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

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

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26 **Summary**

27 The objective was to compare embryo yield and quality in lactating dairy cows superovulated (SO)

28 with varying amounts of gonadotropins and FSH:LH ratios and inseminated with SexedULTRA™

29 sex-sorted semen. The SO treatments (n=77) involved 3 protocols: groups F700 and F1000 were

30 given total doses of 700 and 1000 IU of Folltropin (FSH:LH ratio 49:1), respectively, whereas

31 group F700P300 was given 700 IU of Folltropin + 300 IU of Pluset (FSH:LH ratio 1:1). Cows were

32 artificially inseminated 3 times over a 10-h interval with frozen-thawed SexedULTRA™ sex-sorted

33 semen (total of 10 x 10^6 sex-sorted sperm), starting 18 h after onset of estrus, with embryos/ova

34 recovered 7 d after estrus. Total number of recovered structures and transferable embryos were

35 lower (P<0.05) in F700 (4.7 ± 3.0 and 1.9 ± 1.7, respectively; mean ± SD) compared to F1000 (8.1

36 ± 3.8 and 4.4 ± 2.6) and F700P300 (8.5 ± 6.4 and 4.5 ± 3.3). Percentage of cows ovulating >50% of

37 follicles ≥0.8 cm in diameter was lower (P<0.05) in F700 (35.5%) than in F1000 (82.4%) and

38 F700P300 (73.1%). Percentage of unfertilized oocytes was higher (P<0.05) in F700 (45.0 vs 27.7%

39 for F1000 and 29.0% for F700P300) whereas percentage of morulae was higher (P<0.05) in F1000

40 (19.3 vs 8.7% for F700 and 12.2% for F700P300). Embryo quality was similar among groups

41 (P>0.05). In conclusion, embryo production in lactating dairy cows was improved by increasing

42 total dose of gonadotropins from 700 to 1000 IU, with SexedULTRA™ sex-sorted semen yielding

43 satisfactory fertilization rates and embryo quality.

44

45 **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

72 secretion of LH and an altered (absent, inhibited, premature or late) preovulatory LH surge which

73 reduces ovulation rate, fertilization rates, egg/embryo quality and embryo production (Greve et al.

74 1984, Roberge et al. 1995, Price et al. 1999, Gosselin et al. 2000). In 1 study (Ben Jabara et al.

75 1994), lesser amounts of exogenous LH administered throughout the entire SO protocol resulted in

76 greatest suppression of endogenous LH. However, providing more exogenous LH, only near the end

77 of the SO regimen, increased transferable embryos (Barcelos et al. 2006, Cifuentes et al. 2009).

78 Fertilization rates with sexed semen have always been severely reduced, particularly in

79 superovulated cows versus single-ovulating females or superovulated heifers. Reduced fertilization

80 with sexed semen may be due to low doses of sperm, an abnormal uterine environment or atypical

81 sperm transport in superovulated cows, damage to sperm during sex sorting, or some combination.

82 Superovulated cows inseminated with sex-sorted semen produced a significantly smaller proportion

83 of transferable embryos and significantly larger proportions of unfertilized oocytes and/or

84 degenerate embryos than heifers or cows inseminated with unsorted semen (Peippo et al 2009,

85 Monteiro et al 2016, Mikkola et al 2017). With 4M SexedULTRA™ sex-sorted semen, fertility rates

86 between conventional semen and sex-sorted semen in single-ovulating heifers approached

87 equivalence (Vishwanath and Moreno 2018); however, no data have been published regarding

88 superovulated cattle. Although the economic impact of using sexed semen in an embryo transfer

89 program was deemed profitable only in heifers (Mikkola et al 2017, Hayakawa et al 2009), 4M

90 SexedULTRA™ sex-sorted semen may be appropriate in cows.

91 The objective was to determine whether embryo yield in stressed lactating dairy cows would be

92 altered by changing the FSH:LH ratio and inseminating with SexedULTRA™ sex-sorted semen.

93 Three SO protocols were tested using the 2 products commercially available in Italy: Folltropin,

94 with a high FSH:LH ratio (49:1) (Henderson et al. 1990) and Pluset, with a low FSH:LH ratio (1:1)

95 (Kelly et al. 1995).

96

97 **2. Materials and methods**

98 2.1 Donors

99 The study was conducted on a dairy farm in Emilia Romagna, Italy, with the consent of the owner.

100 No ethical approval was needed for the routine veterinary procedures and drugs used. This herd had

101 300 lactating Holstein cows, housed in a free-stall barn with cooling systems (ventilation and

102 shower system), fed *ad libitum* and with an average daily milk yield of 40 L. The SO protocols were

103 performed during spring or late autumn/winter to avoid the hottest part of the year.

104 Donor cattle (36 primiparous and 41 multiparous cows) had a body condition score between 2.75

105 and 3.5 (scale, 1-5) and had at least 2 physiologic estrous cycles after calving (the SO treatment was

106 initiated 78 ± 15 d after calving). Cows with clinical illness, e.g. mastitis, lameness or

107 gastrointestinal disorders with a considerable reduction in milk production and impaired general

108 health after calving, were not used.

109 2.2 Superovulation protocols

110 Potential donor cows were observed at least twice daily for behavioral signs of estrus and SO was

111 induced by 9 im injections of decreasing dosages of gonadotropins at 12-h intervals over 4.5 d,

112 beginning 9 to 11 d after the onset of standing estrus (Day 0). Concurrent with the seventh and

113 eighth injections of gonadotropins, 150 μg d-cloprostenol (Dalmazin, Fatro, Ozzano dell'Emilia,

114 Italy), a PGF2a analog, was given im.

115 Donors were randomly allocated to receive one of 3 SO protocols: 1) F700 (n=17), a total dose of

116 700 IU of Folltropin (Vetoquinol, Bertinoro, Italy) administered morning and evening as follows:

117 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

118 IU, 5th day 17 IU; 2) F1000 (n=34), a total dose of 1000 IU of Folltropin administered morning and

119 evening as follows: 1st day 170 IU and 155 IU, 2nd day 135 IU and 125 IU, 3rd day 115 IU and 100 IU,

120 4th day 88 IU and 77 IU, 5th day 35 IU; and 3) F700P300 (n=26) cows received 700 IU of Folltropin

121 for the first 5 injections administered as follows: 1st day 170 IU + 155 IU, 2nd day 135 IU + 125 IU,

122 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 $\geq$

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 $\geq$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 $\geq$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

148 Data were analyzed for normality using a Shapiro-Wilk test. Homogeneity of groups for parity, days

149 in milk (DIM), milk production and BCS was evaluated using a one-way ANOVA or a Kruskall-

150 Wallis ANOVA. Statistical differences in total recovery, transferable and non-transferable embryos

151 and mean embryo grade were assessed by one way-ANOVA or Kruskal- Wallis ANOVA, using a

152 Tukey HSD test for *post hoc* comparison or a Wilcoxon-Mann-Whitney test. A Chi-square test was

153 used for analysis of embryo grade, embryo stage, embryo collection yield and ovulation rate. All

154 statistical analyses were performed using IBM SPSS Statistics 23 (IBM Corporation, Milan, Italy).

155 For all analyses, P<0.05 was considered significant.

156

157 **3. Results**

158 Groups were homogeneus for parity, DIM, milk production and BCS (P>0.05). Data regarding

159 embryo collection are summarized in Table 1. Total number of recovered structures and transferable

160 embryos were lower (P<0.05) in F700 versus F1000 and F700P300; however, there was no

161 difference (P>0.05) for mean number of non-transferable structures.

162 Although proportion of collections yielding no transferable embryos was not different (P>0.05)

163 among groups (3/17 – 17.6% F700; 1/34 – 2.9% F1000; 2/26 – 7.7% F700P300)*,* low embryo

164 collections yielding <3 transferable embryos per flushing were higher (P<0.05) in F700 group

165 (11/17 – 64.7%) than in F1000 (9/34 – 26.5%) or F700P300 (7/26 – 26.9%) groups. Percentage of

166 cows ovulating ≥50 % of follicles ≥0.8 cm in diameter present at the first insemination was lower

167 (P<0.05) in F700 (35.5%) than in F1000 (82.4%) and F700P300 (73.1%) groups.

168 Quality grades were similar among groups, with no difference (P<0.05) in mean grade of

169 transferable embryos (Table 2). Unfertilized oocytes were higher (P<0.05) in F700 than in F1000

170 and F700P300 (Table 3). Regarding transferable embryos stages, the only significant difference was

171 percentage of morulae, which was highest in the F1000 group (P<0.05).

172

173 4. **Discussion**

174 In the present study, embryo yield was better in cows given 1000 versus 700 IU of gonadotropins.

175 Group F700 was given 700 IU, the dose recommended by the manufacturer of Folltropin, a drug

176 with a high FSH:LH ratio (49:1) (Henderson et al. 1990), purified to avoid detrimental effects of

177 excessive LH. The other 2 groups received 1000 IU of gonadotropins, using the same drug (1000 IU

178 of Folltropin), without modifying FSH:LH ratio throughout the entire SO treatment, or reducing the

179 FSH:LH ratio in the final part of the SO treatment, switching from Folltropin (700 IU) to Pluset

180 (last 300 IU), a drug with a 1:1 FSH:LH ratio. The rationale for keeping LH low from the beginning

181 to the end of the protocol was based on the premise that exogenous LH is not needed in the SO

182 process, as endogenous LH would support growth of ovarian follicles (Kanitz et al. 2002, Mapletoft

183 et al. 2002). Adding an extra dose of gonadotropin (300 IU) was prompted by reports that high

184 hepatic blood flow and metabolism in dairy cows promoted clearance of steroid hormones

185 (Sangsritavong et al. 2002, Wiltbank et al. 2006), thereby diminishing gonadotropin effect and the

186 SO response. The rationale for an extra dose of gonadotropin with a higher LH content was not only

187 the LH suppression linked to SO treatment, but that LH seems to be required, especially if there is

188 stress and/or negative energy balance, which attenuate or suppress the LH surge (Butler et al. 2003,

189 Matteri et al. 1982). The farm where the study was conducted had greater than average

190 overcrowding and competition, which along with high production and consequent diseases, are

191 important sources of stress for cows, with negative impacts on feed intake, BCS, and reproductive

192 performance.

193 In the F700 group, the number of recovered structures and transferable embryos were the lowest

194 among all 3 protocols. Cows in the F700 group had a good superovulatory response with several

195 follicles reaching pre-ovulatory size by first insemination, although many failed to ovulate, despite

196 displaying estrous behaviors. In F700 group there were very few ovulations and many anovulatory

197 follicles persisting on the ovary 5 h after the last insemination. It is likely that, for the few follicles

198 which reached ovulation, that was a very asynchronous and prolonged process that yielded poor

199 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

226 present study, we inferred that: 1) superior results in both 1000 IU protocols were due to more FSH;

227 2) the amount of LH administered in the F1000 protocol (least amount) was adequate to increase

228 ovulation rate, thus additional LH was not warranted; 3) additional LH in the F700P300 protocol

229 (highest amount) induced more precocious ovulations as compared to F1000 group, as confirmed by

230 a higher percentage of morula-stage embryos in this group.

231 In superovulated cows, SexedULTRA™ sex-sorted semen yielded acceptable results (average of 4.5

232 embryos per flush), which seemed better than most studies involving traditional XY sex-sorted

233 semen in superovulated lactating dairy cows (3.1 embryos Schenk et al 2006; 2.4 embryos

234 Hayawaka et al 2009; 2.1 embryos Peippo et al 2009; and 2.4 embryos Monteiro et al 2016).

235 To our knowledge, only 2 studies with XY sorted semen had better results, with 6.4 (Soares 2011)

236 and 5.4 (Mikkola 2017) embryos per flush. It is noteworthy that cows used in both studies

237 responded successfully to the SO treatment with a standard dose of Folltropin. In Soares' study SO

238 protocol included also P4 and pLH, and the best results were achieved inseminating 18 and 30 h

239 versus 12 and 24 h after pLH (6.4 vs 4.6 embryos; Soares 2011). Comparisons of these studies are

240 challenging and must be done with caution, as there were many differences and many critical points

241 difficult to analyze and consider. It is noteworthy that in the present study, although environmental

242 and management conditions were somewhat stressful, the mean number of DIM was quite low and

243 semen quality was not assessed before insemination, we considered that the SexedULTRA™ sex-

244 sorted semen yielded satisfactory outcomes.

245

246 **5. Conclusions**

247 In conclusion, 700 IU of highly purified gonadotropins provided inadequate stimulation for SO of

248 lactating dairy cows in this study. However, a better SO response was achieved by increasing total

249 gonadotropin dose from 700 to 1000 IU, irrespective of increasing only FSH or both FSH and LH.

250 The use of SexedULTRA™ sex-sorted semen for insemination of superovulated lactating dairy

251 cows was considered satisfactory in terms of proportion of transferable embryos produced.

252

6. Acknowledgements

255

7. Conflict of Interest Statement

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

259
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