FSH withdrawal improves developmental competence of oocytes in the bovine model

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Abstract

Combinations of genetic, environmental, and management factors are suspected to explain the loss in fertility observed for over 20 years in dairy cows. In some cases, IVF is used. When compared with *in vivo* embryo production, IVF resulted in low success rates until the FSH coasting process (FSH starvation after superstimulation) was introduced in 2002. Increased competence associated with FSH withdrawal of aspirated oocyte for *in vitro* maturation and IVF has not been optimized nor explained yet. The goal here was to determine and characterize the optimal oocyte competence acquisition window during the coasting period by determining blastocyst rates and follicular cohort development. Commercial milking cycling cows ($n=6$) were stimulated with 3 days of FSH (6×40 mg NIH Folltropin-V given at 12 h intervals) followed by a coasting period of 20, 44, 68, or 92 h. Each animal was exposed to the four conditions and served as its own control. At the scheduled time, transvaginal aspirations of immature oocytes were performed followed by IVF of half the oocytes. The outcomes were as follows: i) FSH coasting was optimal at a defined period: between 44 and 68 h of coasting; ii) The best estimated coasting duration was $\sim 54 \pm 7$ h; iii) Under these conditions, the best statistical blastocyst rate estimation was $\sim 70\%$; iv) Between 44 and 68 h of coasting, follicle size group proportions were similar; v) Follicle diameter was not linearly associated with competence. In conclusion, coasting duration is critical to harvest the oocytes at the right moment of follicular differentiation.

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Introduction

Dairy cow fertility has been decreasing over the last 20 years (Sakaguchi 2011). The combination of high milk production, genetic, environmental, and managerial factors is likely to explain this decrease in fertility. A direct consequence of this decreased fertility is the increase in the use of IVF in cattle, especially when repeated inseminations are not successful. Up to now, the number of embryos obtained has been relatively low using transvaginal aspiration in stimulated animals when compared with IVF (Walsh *et al.* 2011). Compared to humans, IVF is normally done with *in vitro*-matured rather than *in vivo*-matured oocytes of cattle. The reason for this is not very clear in the literature but our own experience has been lower fertilization rates with *in vivo*-matured oocyte and the difficulties of working with animals very close to ovulation. *In vitro* maturation (IVM) was developed in cows in the mid-1980s (Sirard *et al.* 1988) with the use of slaughterhouse ovaries from unstimulated cows. Despite hundreds of technical papers on the improvement of culture conditions since

then, the average developmental rate to blastocyst has not changed significantly (Sirard *et al.* 2006). This situation has changed with the introduction of follicle-stimulating hormone (FSH) coasting – FSH withdrawal – introduced a few years ago (Blondin *et al.* 2002). It is known that maximal oocyte competence acquisition occurs in large animals, including cows, between the FSH surge and the pre-ovulation luteinizing hormone (LH) surge (Sirard *et al.* 2006).

The initial coasting time was chosen based on previous studies indicating that, following one bolus injection of FSH, the optimal blastocyst rate was obtained when oocytes were recovered 48 h later instead of 24 or 72 h (Blondin *et al.* 1997). Therefore, the initial clinical trial was set for a coasting of 33 vs 48 h following the last FSH injection and both periods resulted in a significant increase in competence compared with slaughterhouse controls.

Assuming that a period between FSH and LH surge is favorable for oocyte competence, the optimal competence acquisition window calls for a better

Table 1 Potential blastocyst yield per cow.

Coasting period	V1	V2	V3	V4	V5	V6	Total
20 h ^{b,c}	3/7 (43%)	6/9 (67%)	8/10 (80%)	1/3 (33%)	4/7 (57%)	1/11 (9%)	23/47 (49.0%)
44 h ^{a,c}	3/5 (60%)	10/10 (100%)	12/18 (67%)	4/6 (67%)	2/4 (50%)	5/8 (63%)	36/51 (71.0%)
68 h ^a	6/7 (86%)	7/9 (78%)	7/7 (100%)	4/8 (50%)	3/6 (50%)	2/9 (22%)	29/46 (63.0%)
92 h ^b	3/4 (75%)	7/8 (88%)	11/17 (65%)	1/6 (17%)	2/7 (29%)	2/9 (22%)	26/51 (51.0%)
Total	15/23 (65%)	30/36 (83%)	38/52 (73%)	10/23 (44%)	11/24 (46%)	10/37 (27%)	113/195 (58.5%)

Data are expressed as follows: number of blastocyst/half of COCs recovered (blastocyst rate). Coasting periods are followed by letters, common letters do not differ significantly ($P>0.05$). COC, cumulus-oocyte complex.

characterization of FSH coasting conditions by defining the conditions prior to and following this change, in other words the borders of the window of competence. We hypothesize that oocyte competence is initially reduced by the growing status of the follicle under FSH influence and then also if FSH withdrawal is done for too long, resulting in atresia in a non-ovulatory context.

To resolve this very important question, we have designed an experiment where each animal ($n=6$) was exposed to 1, 2, 3, or 4 days of coasting periods to characterize the oocyte developmental competence based on the ability to reach Day 8 blastocyst stage. Our results indicate that the timing was crucial for competence and that most animals had a common window of competence. The longer coasting was not beneficial to oocytes despite the increase in follicular size even 4 days after FSH withdrawal.

Results

Developmental competence according to the coasting period

The impact of coasting on blastocyst rate is described in Table 1 (absolute data) and Fig. 1. In this figure, each cow is presented individually to illustrate the variations among animals in relation to blastocyst developmental rates. The fact that each animal represents its own control adds to comparison values and supports the observation of the progressive effect of FSH withdrawal.

To better describe the overall effect of FSH, blastocyst rates were analyzed in box plots (Fig. 2). When oocytes were collected 20 h after the last FSH injection, blastocyst rates varied from 9 to 80%. They varied from 50 to 100% at 44 h, from 22 to 100% at 68 h, and finally from 22 to 88% at 92 h. At 20, 44, 68, and 92 h, the median and mean values were 50 and 49%, 65 and 71%, 64 and 61%, and 47 and 51% respectively.

Considering the coasting period as a mathematical categorical variable, the probability of blastocyst production was estimated to be 48.9, 70.6, 63, and 46% for the four coasting periods (20, 44, 68, and 92 h) respectively. There were no differences between 20 and 92 h and between 44 and 68 h. There was a tendency for a higher blastocyst rate at 44 h than at 20 h ($P=0.063$). There were significantly more blastocysts at 44 h than at 92 h ($P=0.0199$) as well as

more blastocysts at 68 h than at 20 h ($P=0.0129$) or 92 h ($P=0.0004$). Considering the coasting period as a mathematical continuum variable, a quadratic regression was calculated converging with the blastocyst data (Fig. 3). The equation of the blastocyst probability model is as follows: $\text{Log}(P/1-P) = -1.2353 + 0.0757 \text{ coasting} - 0.0007 \text{ coasting} \times \text{coasting}$.

Using this equation, the ideal FSH coasting to maximize blastocyst rate (69%) is 54.07 ± 7.71 h.

Follicular dynamics according to the coasting period

In our analysis of the follicular dynamics in this experiment, the follicles were classified into three groups (5–6, 7–10, and above 10 mm respectively). For the 5–6 mm follicles, there were significantly ($P<0.0001$) more follicles in this group at the earliest time (20 h) of coasting compared with 44, 68, or 92 h (Fig. 4 and 7). There were fewer 7–10 mm follicles at 20 h of coasting than at 44 h ($P=0.0047$) and 68 h ($P=0.0243$). Also, there were fewer 7–10 mm follicles at 92 h than at 44 h ($P=0.0017$) and 68 h ($P=0.0097$). In the last group of follicles (>10 mm), there was no statistical difference between 44 and 68 h. Finally, there were statistically more >10 mm follicles at 92 h than at 20 h ($P<0.0001$), 44 h ($P=0.0052$), and 68 h ($P=0.0290$).

The other meaningful observation when the follicles were analyzed was that the overall number did not change after FSH withdrawal and remained at around 20 follicles per animal (over 5 mm; Fig. 5). Figure 5 also illustrates the shift from the 7–10 mm to the longer than

Figure 1 Blastocyst rate per cow and coasting period. Each color/symbol represents one cow. Blastocyst rate is expressed as percentage.

Figure 2 Blastocyst rate dispersion per coasting period. For each coasting period, from the bottom: minimal value, first quartile, median, third quartile, maximal value. Red dotted line=mean.

10 mm at the last coasting time (92 h), indicating that the follicles were still growing. This increase in follicular size occurred at the same time as the observation of lower blastocyst rates from 68 to 92 h.

This change in follicular size prompted us to analyze the possible relationship between the number/proportion of follicles 7–10 mm in size and blastocyst rate. The first obvious observation was that the blastocyst curve mimics the 7–10 mm curve as shown in Fig. 6B, while evolution of the proportion of larger follicles did not reflect the developmental outcome of enclosed oocytes. Considering the best blastocyst rate for each cow, the proportion of 7–10 mm follicles was statistically positively correlated with blastocyst rate ($R^2=0.844$, $P=0.0096$).

Discussion

The main weaknesses of assisted reproduction technologies when applied to large mammalian species are a low success rate attributed to low oocyte competence and a high degree of variability among individuals that could be associated with genetic and environmental factors. Using animals housed in the exact same conditions and applying the same exact treatments to each animal in a randomized scheme represent an important effort to minimize the impact of individuals and/or the environment on the ovarian response. In addition, for more relevance, the chosen animals were not heifers but lactating commercial cows to better reflect the population normally presenting fertility issues and thus requiring infertility treatments.

In terms of blastocyst rate and follicle grouping, data dispersion was similar for all coasting periods (Fig. 1). This reflects the observed diversity in superovulation responses (Kafi & McGowan 1997, Mapletoft *et al.* 2002). In this group of six animals, when considering

data dispersion, blastocyst rate, and follicle groups, 44 h of coasting was clearly the less variable condition tested. This result illustrates for the first time that some synchrony is possible throughout a cohort of follicles in mono-ovulating species.

Although follicles are usually and basically reported by their sizes, it has been a challenge to link follicle size and competence in several species. In cows, a higher number of blastocysts are obtained from follicles >6 mm than from 2–6 mm follicles (Lonergan *et al.* 1994), and from 2–8 mm follicles than from 1–2 mm ones (Pavlok *et al.* 1993). Individually cultured cumulus–oocyte complexes (COCs) originating from 3–5 mm and >5 mm follicles are linked to higher blastocyst rates (Blondin & Sirard 1995). Nevertheless, in general, a linear positive correlation between follicular size and competence is observed, at least until the 8 mm follicle stage in cows. Surprisingly, this study indicated that the best blastocyst rate (44–68 h) was not related to the largest cohort of follicles (92 h), i.e. the follicle cohort including the higher number of >10 mm follicles. Statistically, the presumed largest follicles are still growing from 68 to 92 h and the rate of blastocyst is lower at such time. Therefore, we can assume that, for unknown reasons, the correlation between size and competence is not positive in the largest follicles. Consistent with our hypothesis, Hagemann *et al.* (1999) did not obtain a better developmental rate with follicles >13 mm compared with that of 9–12 mm follicles.

The fact that growing follicles may contain compromised oocytes has been observed before. Two other different experimental conditions support such an observation. The first one was extending exposure to progesterone (P_4) supplementation, thereby preventing

Figure 3 Mathematical model of blastocyst probability. Coasting was considered as a continual variable and embryo as a binary variable, blastocyst at day 8 or not, in a GEE model. The equation of blastocyst probability modelization was $\text{Log}(P/1-P) = -1.2353 + 0.0757 \text{ coasting} - 0.0007 \text{ coasting} \times \text{coasting}$. With this consideration, the coasting duration maximizing blastocyst rate (69%) was 54.07 ± 7.71 h. GEE, generalised estimating equation.

Figure 4 Follicle size groups and coasting period. The proportion of each follicle size group is associated to a color and a motif. Data with a common superscript do not differ significantly ($P > 0.050$).

ovulation. It was associated with continuous follicle growth and reduced fertility (Sirois & Fortune 1990, Bridges & Fortune 2003). A second study used gonadotropin-releasing hormone inhibition to obtain similar results. Increased atresia rate associated with decreased blastocyst rate was noted (Oussaid *et al.* 2000, van de Leemput *et al.* 2001). Although previous studies are consistent with our data supporting a link between largest follicles and a reduced blastocyst rate, it is not possible to rule out that decreased competence may be caused by smaller follicles as the oocytes were not evaluated according to follicular dimension (7–10 mm).

Follicles collected in an active growth phase have a low developmental competence (Blondin *et al.* 1996). Here, oocytes from follicles following 20 h of coasting displayed a lower blastocyst rate than those from the plateau (68 h). Accordingly, oocytes obtained at 20 h of coasting are likely to remain under FSH influence and may not have completed competence differentiation. From 20 to 92 h, there was no new follicle recruitment. This can logically be explained by the inhibition of larger follicles and the LH level, illustrating the functional repression of a dominant cohort (Ginther *et al.* 2003). Here, we observed that the number of follicles was constant throughout coasting, although there were more large follicles at 92 h, consistent with the results of

Goodhand *et al.* (1999). Therefore, we estimate that our coasting period mimics single follicle dominance and prevents the recruitment of new follicles. It is important to remember that FSH reduction during 4–5 days is physiological in normal cycling cows (Cooke *et al.* 1997) and women (Dighe *et al.* 2005, Stricker *et al.* 2006). During coasting, all follicles longer than 7–8 mm have a pseudo-dominant status, with LH receptors (Ginther 2000), and have undergone differentiation under basal LH as in normal cycles. However, since our protocols are initiated when P_4 is high, the amplitude of LH pulses does not increase, and these follicles will eventually regress.

Using IVM of immature bovine oocytes, the average reported blastocyst rate has not often exceeded 30% over the last 25 years (Sirard *et al.* 1988, Rizos *et al.* 2002). Here, we define coasting limitations while at the same time highlighting coasting benefits. Our results indicate an oocyte competence window between 44 and 68 h of coasting with the best results for a coasting period of 54 ± 7 h. According to our mathematical model, an optimal statistical blastocyst rate (70%) can be reached with these conditions. Considering blastocyst rate and data dispersion on the one hand and coasting timing as a categorical mathematical variable on the other, the best period revealed here was 44 h with a mean outcome of 71% blastocysts, which is relevant in the superstimulation IVM–IVF context. This rate is higher in comparison to *in vivo*-produced and fertilized oocytes in an ovary stimulation context (60%; Sirard & Lambert 1985) and is close to the success rate of oocytes produced by natural cycle (80%; Merton *et al.* 2003).

We have observed 2 years of industrial application of such protocol with a rise of around two embryos obtained by ovum pick-up (OPU) and a pregnancy rate of 53% (39/74) and 55% (439/801) for 43 and 54 h of coasting respectively (P Blondin, J Belanger, C Vigneault, 2011 personal communication).

In our experiment, half of the oocytes, randomly chosen, were frozen for subsequent RNA analysis, which limits the absolute number of embryos produced in this study. The theoretical blastocyst outcome (that would

Figure 5 Follicles, COCs, and theoretical blastocyst absolute data per coasting period. COC, cumulus-oocyte complex.

Figure 6 Follicle size group proportion dispersion per coasting period. For each coasting period, from the bottom: minimal value, first quartile, median, third quartile, maximal value.

apply the same development rate to all oocytes obtained) – the embryo amount per OPU – is 13.3 at 44 h of coasting, which is higher than the best estimation for OPU-multiple observation and embryo transfer (MOET), 5–8 h (Merton *et al.* 2003). This difference could be partly explained by the fact that in our study, the cows used were relatively young and fertile. These results also indicate that, if not in the optimal period, coasting may lead to reduced developmental capacity. These observations call for further analysis to identify the related causes of such oocyte changes.

In summary, our results provide new information about the importance of FSH decrease in oocyte competence acquisition. First, we showed that the precise coasting duration defined here was a key element to obtain the best success rates. Secondly, decreased oocyte quality occurred in a continuous

follicular growth context, reinforcing previous observations showing the negative impact of artificially extended follicle life. Therefore, we have demonstrated that the optimal period between FSH surge and transvaginal aspiration is 54 ± 7 h and that a well-defined competence window is crucial to obtain optimal oocyte quality in ovarian stimulated milking cows.

Materials and Methods

Chemicals

All reagents and media supplements used in these experiments were of tissue culture grade and were obtained from Sigma-Aldrich Co. unless otherwise indicated.

Ovarian stimulation treatment and oocyte recovery from superovulated animals

Each animal (six commercial milking cycling *Bos taurus* Holstein cows, 4 years old on average, 3.04 ± 0.2 years old on average at calving) was exposed in a randomized scheme to the four conditions with at least one complete regular sexual cycle between two treatments and served as its own control. Each animal was treated during the luteal phase to prevent spontaneous ovulation. Hormonal synchronization was not performed; FSH application and OPU were done using natural diestrus. We performed P_4 serum assays showing no significant differences between coasting durations. The P_4 level allowed to determine that there was a corpus luteum. We performed estradiol (E_2) serum assays; there was no significant difference between treatments in term of E_2/P_4 . We could indirectly confirm that LH level was basal. The mean duration between calving and OPUs is 271.8 ± 28.9 days. There is no correlation between the order of OPUs and efficiency; there are no significant differences between cows in terms of duration between two OPUs. There are no correlations between blastocyst rate and duration between calving and OPUs, or between coasting duration and duration between calving and OPUs, or between the efficiency to collect COCs and the duration between calving and OPUs. The efficiency to collect COCs (76% on average) is not significantly different from the beginning to the end of the protocol. There is no correlation between coasting duration and efficiency. There is no significant difference between cows in term of duration between calving and OPUs. The mean milk production was 8630.17 ± 771.1 l. The energy balance of the cows was positive. The cows were part of the same herd.

The dominant follicle was aspirated 36 h before administration of hormones. Cows were stimulated for 3 days with FSH (6×40 mg NIH Folltropin-V, Bioniche Animal Health, Belleville, ON, Canada), followed by a coasting (no FSH) period of four different durations (20, 44, 68, and 92 h). Using transvaginal ultrasonography, follicular diameters were measured and COCs were collected by transvaginal puncture (PTV), under epidural, with an 18G needle and COOK aspiration unit (COOK Medical, Bloomington, IN, USA). COCs and granulosa cells were collected in warm HEPES-buffered Tyrode's medium (TLH) containing Hepalean

		5–6 mm				7–10 mm				>10 mm			
		20 h	44 h	68 h	92 h	20 h	44 h	68 h	92 h	20 h	44 h	68 h	92 h
5–6 mm	20 h		*** <0.0001	*** <0.0001	*** <0.0001	NS				*** <0.0009			
	44 h			NS	NS		*** <0.0001				*** <0.0001		
	68 h				NS			*** <0.0001				*** <0.0001	
	92 h								*** <0.0003				*** <0.0001
7–10 mm	20 h						** <0.0047	* <0.0243	NS	** <0.0023			
	44 h							NS	** <0.0017		*** <0.0001		
	68 h								** <0.0097			** <0.0065	
	92 h												** <0.0399
>10 mm	20 h										NS	* <0.0137	*** <0.0001
	44 h											NS	*** <0.0052
	68 h												* <0.0290
	92 h												

Figure 7 *P* values associated with follicle size group and coasting period in cows. NS, non-significant. **P* value <0.05, ***P* value <0.01, ****P* value <0.001.

(10 UI/ml) and transferred to laboratory for IVM (half of the COCs) or treated (oocytes, cumulus, and granulosa cells) for further studies.

In vitro maturation

The COCs were placed in HEPES-buffered TLH solution (supplemented with 10% bovine serum, 0.2 mM pyruvate, and 50 µg/ml gentamicin) and washed three times to remove follicular fluid. Healthy COCs were placed in droplets of maturation medium under embryo-tested mineral oil (#8410; Sigma-Aldrich). Maturation medium was composed of TCM199 (Gibco 11150059, Invitrogen Life Technologies), 10% fetal bovine serum (FBS), 0.2 mM pyruvate, 50 µg/ml gentamicin, 5 µg/ml FSH and 0.5 µg/ml LH, and 1 µg/ml E₂. Maturation droplets were incubated for 24 h at 38.5 °C with 5% CO₂ in maximal humidity.

IVF

After 24 h of IVM, COCs were collected and washed twice in TLH medium before being transferred in groups of 5 into 48 µl droplets under mineral oil. The droplets consisted of modified Tyrode's lactate (TL) medium supplemented with 0.6% (w/v) free fatty acid BSA, 0.2 mM pyruvic acid, 2 µg/ml heparin, and 50 µg/ml gentamicin. Oocytes were transferred 15 min prior to semen addition and 2 µl penicillamine, hypotaurine, epinephrine (PHE) (1 mM hypotaurine, 2 mM penicillamine, and 250 mM epinephrine) were added to each droplet to stimulate sperm motility. The same semen was used for each IVF (Centre d'Insémination Artificielle du Québec, St-Hyacinthe, QC, Canada). The spermatozoa previously stored in liquid nitrogen were thawed for 1 min in 35 °C water, added to a discontinuous Percoll gradient (45 over 90% Percoll (Sigma-Aldrich)), and centrifuged at 700 *g* for 30 min at 26 °C. The supernatant containing the cryoprotectant and the dead spermatozoa was discarded, and the pellet was then

resuspended in 1 ml of modified TL and centrifuged at 250 *g* for 5 min at 26 °C. The resuspended spermatozoa were counted on a hemocytometer and diluted with IVF medium to obtain a final concentration of 1×10^6 cells/ml. Finally, 2 µl sperm suspension were added to the droplets containing the matured COCs. The fertilization medium was incubated at 38.5 °C for 15–18 h in a humidified atmosphere of 95% air and 5% CO₂.

In vitro culture

For culture, embryos were placed in groups of 10 in 10 µl droplets of modified synthetic oviduct fluid (mSOF) with non-essential amino acids, 3 µM EDTA, and 0.4% fatty acid-free BSA (ICP bio, Auckland, New Zealand) under embryo-tested mineral oil (#8410, Sigma-Aldrich). The embryo culture dishes were incubated at 38.5 °C with 6.5% CO₂, 5% O₂, and 88.5% N₂ in 100% humidity. Embryos were transferred in new 10 µl droplets of mSOF containing non-essential and essential amino acids 72 h post-fertilization and again 120 h post-fertilization in 20 µl droplets of mSOF containing non-essential and essential amino acids to prevent toxicity due to ammonium concentration and nutrient depletion caused, respectively, by amino acid degradation and embryo metabolism. Blastocyst development was monitored at days 7 and 8 post-fertilization.

Statistical analysis

Statistical analyses were performed using SAS Software (SAS Institute, Cary, NC, USA). The dichotomic variable blastocyst stage followed a binomial distribution and was used in the generalized estimating equation (GEE) model. The variable coasting period was first considered as a continuous variable. This permitted the regression model of the blastocyst stage data to be determined. The coasting period resulting in the maximal blastocyst probability was determined with the Delta method. Secondly, the coasting period was considered as a categorical variable and the different treatments were compared (χ^2).

Follicle data followed a Poisson distribution and were used in a log linear model to compare each coasting period within each follicle group and each follicle group within each coasting period (χ^2).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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