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Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows

Ronaldo L A Cerri[1,2], Heloísa M Rutigliano[1], Ricardo C Chebel[1] and José E P Santos[2]

[1]*School of Veterinary Medicine, University of California, Davis, California 95616, USA and* [2]*Department of Animal Sciences, University of Florida, Gainesville, Florida 32611, USA*

Correspondence should be addressed to J E P Santos; Email: jepsantos@ufl.edu

Abstract

Length of dominance of the ovulatory follicle and exposure to oestradiol (OE_2) during proestrus can affect fertility. Lactating cows had their oestrous cycle pre-synchronized and were subjected to one of the four synchronization treatments. Cows in the oestrus detection (OD) treatment received GnRH on day 6 of the oestrous cycle, $PGF_{2\alpha}$ 7 days later, and were inseminated at detected oestrus. The remaining cows were subjected to the Ovsynch (OVS) protocol (day 0 GnRH, day 7 $PGF_{2\alpha}$, day 9 GnRH, and timed artificial insemination (AI) 12 h later) starting on day 3 (OVS3) or day 6 (OVS6 and OVS6E) of the oestrous cycle. Cows in the OVS6E treatment received an injection of 0.5 mg oestradiol cypionate 36 h before AI. Ovaries were examined by ultrasonography and blood was sampled for progesterone and OE_2 concentrations. Uteri were flushed 6 days after AI and recovered embryos–oocytes evaluated. Diameter of the ovulatory follicle at AI differed ($P<0.01$) among treatments, and it was the largest for OVS3 cows, which also had extended ($P<0.01$) length of follicular dominance. During proestrus, OD and OVS6E cows had increased ($P<0.01$) OE_2 concentrations. Fertilization was not altered by treatments, and maximum fertilization was achieved when the number of accessory spermatozoa was >7. Proportions of viable embryos in relation to embryos and embryos–oocytes recovered were smaller for OVS3 cows ($P<0.01$) than the other treatments, and embryos from OVS3 cows also had fewer ($P<0.01$) blastomeres and tended ($P=0.09$) to have a lower proportion of live blastomeres. Extending the period of follicle dominance did not alter fertilization but reduced ($P<0.001$) embryo quality. Embryo quality was compromised even when the dominance of the ovulatory follicle was extended by only 1.5 days.

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Introduction

High-producing lactating dairy cows have greater incidence of two waves of follicle growth during the oestrous cycle compared with growing heifers that are more likely to have three follicular waves (Savio *et al.* 1988). The interval from follicle emergence to oestrus, which is ~3.5 days greater for cows with two follicular waves than for those with three follicular waves (Bleach *et al.* 2004), has been associated with conception rates (Townson *et al.* 2002, Bleach *et al.* 2004). Cows inseminated after spontaneous display of oestrus had a linear reduction in conception rates as the interval from follicular emergence to oestrus increased (Bleach *et al.* 2004). This reduced fertility observed following periods of prolonged follicle dominance is associated with compromised oocyte quality (Revah & Butler 1996) and embryonic development (Ahmad *et al.* 1995), although most of those studies used extremely prolonged periods of dominance that are unlikely to occur spontaneously.

Studies using the Ovsynch (OVS) protocol (Pursley *et al.* 1995) as a platform to synchronize follicle wave emergence, corpus luteum (CL) regression and ovulation have shown that cows that ovulated to the first GnRH injection were more likely to ovulate the pre-ovulatory follicle after the second GnRH injection of the protocol (Vasconcelos *et al.* 1999, Rutigliano *et al.* 2008). Furthermore, ovulation to the first GnRH of the OVS programme increased conception rates (Chebel *et al.* 2006, Rutigliano *et al.* 2008). Therefore, ovulation to the first GnRH injection of the OVS protocol can improve fertility by increasing synchronization of ovulation near artificial insemination (AI), but could also cause the ovulation of a follicle with shorter dominance that might benefit fertilization and embryo quality. Some have suggested that implementation of timed AI programmes have coincided with increased detection of pregnancy losses in dairy cattle (Lucy 2001), although the risk of pregnancy loss seemed to be unaltered by inseminating cows following detection of oestrus or at fixed time (Santos *et al.* 2004). Whether synchronization programmes for AI using timed AI or insemination following detection of a synchronized oestrus affect fertilization and early embryonic development remains unknown.

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Lactating dairy cows have lower concentrations of gonadal steroid hormones compared with non-lactating dairy cows and growing heifers (Sangsritavong *et al.* 2002, Sartori *et al.* 2004). Because hepatic blood flow is correlated positively with feed and energy intakes (Reynolds *et al.* 2003), it has been hypothesized that the greater feed intake in high-producing dairy cows could affect metabolism and concentrations of progesterone and oestradiol (OE_2). In fact, changes in steroid hormone concentrations have been related to acute changes in liver blood flow following feed consumption (Sangsritavong *et al.* 2002). Reductions in peri-ovulatory OE_2 concentrations might compromise spermatozoa transport in the female tract (Hawk 1983) and oviductal oocyte transport (Orihuela & Croxatto 2001). Therefore, it is plausible to speculate that supplementation with OE_2 during a synchronization protocol that might limit follicular OE_2 production could improve fertilization. This may be particularly important in high-producing dairy cows that have inherently low concentrations during proestrus (Wiltbank *et al.* 2006). When lactating dairy cows were supplemented with oestradiol cypionate during proestrus, conception rates increased compared with non-supplemented cows (Cerri *et al.* 2004).

The objectives of the present study were to determine the effects of timing of initiation of the synchronization protocol (day 3 or day 6 of the oestrous cycle), which would alter the length of dominance of the ovulatory follicle, and OE_2 supplementation on OE_2 and progesterone concentrations, ovarian responses to the hormonal treatments, fertilization rate and embryo quality. An additional objective was to determine, whether insemination following a synchronized oestrus or at fixed time altered fertilization and embryo quality.

Results

The mean (±S.D.) and median lactation number did not differ ($P>0.20$) among treatments and were 2.45±1.37 and 2.0 respectively. Similarly, the mean (±S.E.M.) and median body condition score (BCS) on the day of study enrolment was not different ($P>0.50$) among treatments and was respectively 2.80±0.31 and 2.75. The proportion of cows classified as cyclic before the initiation of the synchronization protocols was not ($P=0.87$) different among treatments and averaged 83.9% (Table 1). The number of cows flushed during the study was 313, being 64, 83, 87 and 79 cows in oestrus detection (OD), OVS3, OVS6 and OVS6E respectively. The remaining cows included in the study were not flushed because of lack of oestrus display in the OD group, lack of ovulation to the last GnRH injection of the OVS, or cows that left the study before AI. There were 10 cows with double ovulation following AI and no difference ($P=0.88$) was observed among treatments (OD=3; OVS3=1; OVS6=3 and OVS6E=3). Only five out of the 10 cows with double ovulations yielded two embryos oocytes from the same cow (OD=1; OVS3=1; OVS6=2 and OVS6E=1).

Ovarian structures and responses to the OVS protocol

Diameter of the dominant follicle in OVS3 was reduced ($P<0.001$) at first GnRH injection of the synchronization protocol and differed from OD, OVS6 and OVS6E (Table 1). The proportion of cows that ovulated to the first GnRH injection of the synchronization protocol and started a new follicular wave was decreased ($P<0.001$) in the OVS3 compared with OD, OVS6 and OVS6E. Complete luteolysis was similar ($P=0.70$) among treatments and averaged 95.2%. Treatment affected ($P<0.01$) the diameter of the pre-ovulatory follicle near the time of AI, and OVS3 cows had the largest follicle diameter, followed by OD and then OVS6 and OVS6E. Ovulation to the second GnRH injection of the OVS was similar ($P=0.78$) among OVS3, OVS6 and OVS6E and averaged 87.7%. Although ovulation to the second GnRH was similar among OVS treatments,

Table 1 Effect of treatment on proportion of cyclic cows, and ovarian structures and responses to the synchronization protocols.

	Treatment			
	OD	OVS3	OVS6	OVS6E
Cows (*n*)	118	90	99	89
Cyclic cows (%)	85.5	83.1	86.7	82.0
DF at 1st GnRH (mm±S.E.M.)	14.2±0.4*,†	9.5±0.4‡	15.4±0.4*	15.0±0.4*
Ovulation to 1st GnRH (%)	81.2*	7.1†	88.6*	88.9*
CL regression (%)	94.5	93.2	96.7	96.3
DF near AI (mm±S.E.M.)	19.7±0.3†	20.7±0.4*	18.1±0.4‡	17.7±0.4‡
Ovulation to 2nd GnRH (%)	–	86.7	87.9	88.6
Oestrous detection (%)	58.8	–	–	–
CL on day 6 after AI (mm±S.E.M.)	24.6±0.3*,†	25.3±0.3*	23.7±0.3†,‡	23.1±0.3‡
Dominance length (days±S.E.M.)	7.1±0.2†	8.0±0.2*	5.8±0.2‡	5.7±0.2‡

*,†,‡Superscripts in the same row differ ($P<0.001$). OD, cows inseminated at oestrous detection; OVS3, cows starting the Ovsynch on day 3 of the oestrous cycle; OVS6, cows starting the Ovsynch on day 6 of the oestrous cycle; OVS6E, similar to OVS6 with an additional 0.5 mg of oestradiol cypionate 36 h before AI; DF, dominant follicle diameter; CL, corpus luteum; AI, artificial insemination.

despite major differences in ovulation to the initial GnRH, cows that did not ovulate to the first GnRH had reduced ($P<0.01$) ovulation to the final GnRH of the OVS protocols (77.0 vs 95.8%).

OD for the OD treatment was 58.8% in the 7 days following $PGF_{2\alpha}$, and 96.4% of the cows in oestrus ovulated within 48 h of oestrus. The mean (±S.D.) interval from $PGF_{2\alpha}$ injection to oestrus was 3.7±1.2 days. Distribution of observed oestruses varied from 1.5 to 7 days after the $PGF_{2\alpha}$ injection with 74.3% of the cows displaying oestrus between 2.5 and 4.0 days after the $PGF_{2\alpha}$ injection. The CL diameter 6 days after AI was greater ($P<0.01$) in OVS3 compared with OVS6 and OVS6E. Cows in OD also had greater ($P=0.04$) CL diameter 6 days after AI than OVS6E cows. The length of dominance of the ovulatory follicle differed ($P<0.01$) among treatments, and OVS3 cows had the longest dominance, followed by OD, and then OVS6 and OVS6E, but the latter two treatments did not differ.

Progesterone and OE_2 concentrations

Progesterone concentrations at the first GnRH of the synchronization treatments differed ($P<0.001$) and were lower for OVS3 (0.8±0.1 ng/ml) than the other treatments (Fig. 1). Concentrations were similar ($P>0.46$) for OD, OVS6 and OVS6E (1.7±0.1, 1.6±0.1 and 1.6±0.1 ng/ml respectively). Similarly, concentrations of progesterone differed ($P<0.01$) on the day of $PGF_{2\alpha}$, and cows in OVS3 had lower concentrations (3.1±0.2 ng/ml) than cows in OD, OVS6, OVS6E (3.9±0.2, 4.2±0.2 and 3.8±0.2 ng/ml respectively). Progesterone concentration near AI was similar ($P=0.13$) among treatments and averaged 0.3±0.1 ng/ml. Differences in progesterone concentrations were observed on day 6 after AI, and concentration for cows in OVS6E (1.6±0.1 ng/ml) was lower ($P=0.02$) and tended to be lower ($P=0.09$) than those in OD (2.1±0.1 ng/ml) and OVS3 cows (2.0±0.1 ng/ml) respectively; however, concentration of progesterone on day 6 after AI did not differ between OVS6 (1.90±0.1 ng/ml) and the remaining treatments.

OE_2 concentration at 48 h after the $PGF_{2\alpha}$ injection of the synchronization was greater ($P<0.01$) for OVS6E than all other treatments (Fig. 2). Cows in OVS3 and OVS6 had similar concentrations of OE_2, but they were both greater ($P<0.05$) than OD. At 72 h after the $PGF_{2\alpha}$ injection, OE_2 concentration was greater ($P<0.01$) for OD than all other treatments, and OVS6E had greater ($P=0.05$) concentration than OVS3 and OVS6. Because some OD cows were observed in oestrus after 72 h following $PGF_{2\alpha}$, an additional analysis was performed for concentrations of OE_2 near the time of AI (Fig. 2). Cows in OD and OVS6E had similar ($P=0.14$) OE_2 concentrations, but both were greater ($P<0.01$) than OVS3 and OVS6 treatments.

Embryo–oocyte evaluation

The number of embryos–oocytes recovered relative to the number of CL was not ($P=0.15$) different among treatments and averaged 53.7% (Table 2). Fertilization rate averaged 86.3%, and it did not differ ($P=0.96$) among treatments. The critical number of accessory spermatozoa that resulted in the highest sensitivity and specificity for fertilization was >4 (Fig. 3). Using this cut-off, the sensitivity and specificity were respectively,

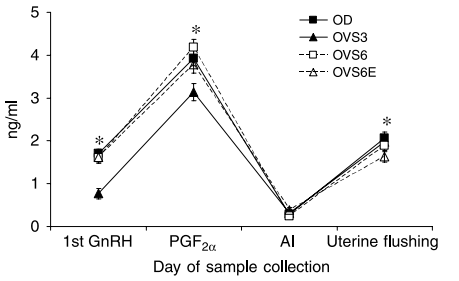

Figure 1 Plasma concentrations of progesterone during the synchronization treatments and on the day of uterine flushing. Within each time point, * denotes difference among treatments ($P<0.05$). The values are mean±S.E.M. concentrations. AI, artificial insemination; OD, cows inseminated at oestrous detection; OVS3, cows starting the Ovsynch on day 3 of the oestrous cycle; OVS6, cows starting the Ovsynch on day 6 of the oestrous cycle; OVS6E, similar to OVS6 with an additional 0.5 mg of oestradiol cypionate 36 h before AI.

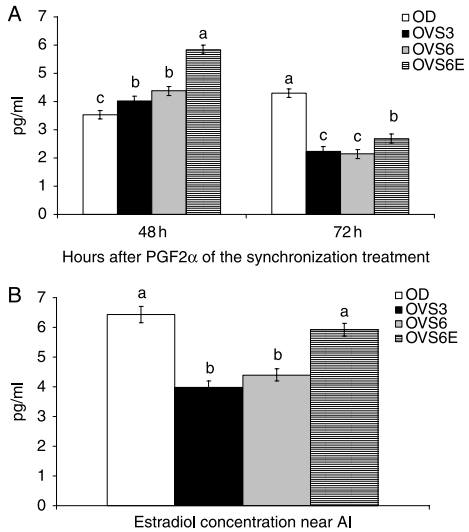

Figure 2 (A) plasma concentrations of oestradiol at 48 and 72 h after $PGF_{2\alpha}$ of the synchronization treatments. [a,b,c]Bars having different letters differ ($P<0.01$). Effect of treatment ($P=0.01$), time ($P=0.001$) and treatment by time interaction ($P=0.01$). (B) plasma concentrations of oestradiol near the time of artificial insemination from all treatments. [a,b]Bars having different letters differ ($P<0.01$). The values are mean±S.E.M. concentrations. OD, cows inseminated at oestrous detection; OVS3, cows starting the Ovsynch on day 3 of the oestrous cycle; OVS6, cows starting the Ovsynch on day 6 of the oestrous cycle; OVS6E, similar to OVS6 with an additional 0.5 mg of oestradiol cypionate 36 h before AI.

Table 2 Effect of treatment on recovery, fertilization and embryo quality parameters of collected embryos–oocytes.

	Treatment			
	OD	OVS3	OVS6	OVS6E
Cows (*n*)	118	90	99	89
Embryos–oocytes (*n*)	41	40	43	44
Recovery (%)	64.1	48.2	49.4	55.7
Embryos–oocytes				
Fertilization (%)	85.4	85.0	86.0	88.6
Embryos grades 1 and 2 (%)	61.0*	40.0†	72.0*	65.9*
Degenerated (%)	21.9‡,§	25.0‡	9.3$^{\S,\|\|}$	11.4$^{\S,\|\|}$
Degenerated-unfertilized (%)	36.6‡,§	40.0‡	23.3§	22.7§
Embryos				
Embryos (*n*)	35	34	37	39
Embryos grades 1 and 2 (%)	71.4*	47.0†	83.7*	74.3*
Degenerated (%)	25.7‡	29.4‡	10.8§	12.8§
Blastomeres				
Mean±S.E.M.	43.5±4.4*	29.6±4.4†	40.9±4.0*	45.2±3.7*
Median (*n*)	49†	32*	42†	45†
Live (%)	91.4†	91.6†	97.9*	98.2*
Accessory spermatozoa				
Mean±S.E.M.	26.2±6.5‡	22.5±6.2‡,§	12.2±6.3§	15.7±6.1‡,§
Median (*n*)	10.5	8.0	7.0	8.5
Embryos–oocytes with ≥1 (%)	95.0*	89.7*	74.4†	95.4*
Embryos–oocytes with >4 (%)	73.0	62.9	57.5	59.5

*,†Superscripts in the same row differ ($P<0.05$). $^{\ddagger,\S,\|\|}$Superscripts in the same row differ ($P<0.10$). OD, cows inseminated at oestrous detection; OVS3, cows starting the Ovsynch on day 3 of the oestrous cycle; OVS6, cows starting the Ovsynch on day 6 of the oestrous cycle; OVS6E, similar to OVS6 with an additional 0.5 mg of oestradiol cypionate 36 h before AI.

71.3 (95% confidence interval (CI)=63.2–78.6) and 91.3% (95% CI=71.9–98.7), and the area under the curve was 0.87 (95% CI=0.81–0.92; $P<0.001$). One-hundred percent fertilization was observed when the number of accessory spermatozoa was >7. Using this cut-off resulted in fertilization rates of 100% (86/86) and 71.3% (57/80) for embryos–oocytes with >7 and ≤7 accessory spermatozoa respectively ($P<0.001$).

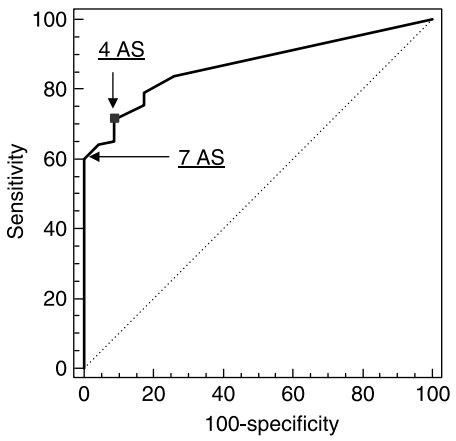

Figure 3 Receiver operating characteristic curve for number of accessory spermatozoa and fertilization rate. The square in the curve illustrates the optimal sensitivity and specificity relationship defined by the analysis; the cut-off number of accessory spermatozoa was 4. The sensitivity and specificity were 71.3 (95% CI=63.2–78.6) and 91.3% (95% CI=71.9–98.7). The area under the curve was 0.87 (95% CI= 0.81 – 0.92; $P<0.001$). For 100% fertilization, the cut-off was >7 accessory spermatozoa. AS, accessory spermatozoa.

Relative to embryos–oocytes, the proportion of embryos graded as 1 and 2 was less ($P<0.02$) for OVS3 than the remaining treatments (Table 2). Similarly, the proportion of degenerated embryos was the greatest for OVS3, but it tended to differ ($P<0.10$) only from OVS6 and OVS6E. When data from both unfertilized and degenerate were evaluated together, cows in OVS3 tended ($P<0.10$) to have a greater proportion of non-viable structures than OVS6 and OVS6E. In relation to embryos, cows in OVS3 had a lower ($P<0.05$) proportion of high-quality embryos than OD, OVS6 and OVS6E. Likewise, cows in OD and OVS3 tended to have ($P<0.10$) greater proportions of degenerated embryos than OVS6 and OVS6E. The mean and median number of blastomeres differed ($P<0.01$) among treatments, and they were both lower for OVS3 than all other treatments. Similarly, cows in OVS3 had a lower ($P<0.05$) proportion of live blastomeres than the remaining treatments. Median number of accessory spermatozoa was similar among treatments, but mean number tended to differ ($P<0.10$) because cows in OD had more accessory spermatozoa than OVS6. In addition, OVS6 had a lower ($P<0.05$) proportion of embryos–oocytes with ≥1 accessory spermatozoon than the remaining treatments. Although, treatment did not influence fertilization despite altering the mean number of accessory speramatozoa and the proportion of embryos–oocytes with ≥1 spermatozoon, it was observed that embryos had increased ($P<0.01$) median number of accessory spermatozoa than oocytes (10.0 vs 1.0). Furthermore, only 65.2% of the oocytes had ≥1

accessory spermatozoon, whereas 92.3% of the embryos had ≥1 accessory spermatozoon ($P<0.01$). Although OVS6 had a reduced proportion of embryos–oocytes with ≥1 accessory spermatozoon compared with the remaining treatments, the same was not observed for the proportion of embryos–oocytes with >4 accessory spermatozoa (Table 2), which was the cut-off for maximum sensitivity and specificity for fertilization.

Because the experimental design aimed to reduce ovulation to the first GnRH in OVS3 and to have maximum ovulation in OD, OVS6 and OVS6E treatments (i.e. OVS3, cows not ovulating; OD, OVS6 and OVS6E, cows ovulating), additional analyses were performed for cows that followed these expected responses for the respective treatments (Table 3). Recovery and fertilization rate were similar among treatments. With respect to embryos–oocytes, the proportion of grades 1 and 2 embryos was lower ($P<0.05$) for OVS3 than the remaining treatments. Degenerated embryos were more prevalent ($P<0.05$) in OD and OVS3 than OVS6, whereas OD tended to have ($P=0.08$) more degenerated embryos than OVS6E. The proportion of non-viable embryos–oocytes tended ($P<0.10$) to be greater for cows inseminated following OD and OVS3 than those in OVS6 and OVS6E. Relative to embryos, the proportion of grades 1 and 2 were lower ($P<0.05$) for OVS3 than the other treatments. Cows in OD and OVS3 had ($P<0.05$) greater proportions of degenerated embryos than those in OVS6; they also tended to have ($P=0.08$) greater proportions of degenerated embryos than OVS6E. The mean and median number of blastomeres were both lower ($P<0.05$) for OVS3 than all other treatments. Similarly, OVS3 had a lower ($P<0.05$) proportion of live blastomeres than the remaining treatments. Cows in OD had a greater ($P=0.03$) mean number of accessory spermatozoa compared with OVS6, but they did not differ from OVS3 and OVS6E. Similarly, the median number of accessory spermatozoa tended ($P<0.10$) to be greater for OD than OVS3 and OVS6. The proportion of embryos–oocytes with >1 accessory spermatozoon was lower ($P<0.05$) for OVS6 than the other treatments.

Although treatments influenced embryo quality and concentrations of progesterone and OE_2, no relationship was observed between proportion of embryos graded as 1 and 2 and OE_2 concentrations at 48 h after $PGF_{2\alpha}$ or near AI. At 48 h after $PGF_{2\alpha}$, concentrations of OE_2 were 4.7 ± 0.2 and 4.7 ± 0.3 pg/ml for cows with and without embryos graded 1 and 2 respectively. At AI, concentrations of OE_2 were 3.6 ± 0.2 and 3.4 ± 0.3 pg/ml for cows with and without embryos graded 1 and 2 respectively. Similarly, progesterone concentrations on day 6 after AI in cows that ovulated within 48 h of AI did not differ between cows with and without embryos graded 1 and 2 and they were 1.8 ± 0.1 and 1.9 ± 0.1 ng/ml respectively.

The distribution of embryos according to grade quality and fertilization shifted with altering the period of follicle dominance (Fig. 4). Follicles with shorter length of dominance yielded a greater proportion of grades 1 and

Table 3 Effect of treatment on recovery, fertilization, and quality parameters of collected embryos–oocytes from cows in the OD, OVS6 and OVS6E treatments that ovulated and cows from the OVS3 treatment that did not ovulate in response to the first GnRH injection of the synchronization protocol.

	Treatment			
	OD	OVS3	OVS6	OVS6E
Cows (*n*)	99	84	90	80
Embryos–oocytes (*n*)	37	36	40	42
Recovery (%)	64.9	46.7	48.2	55.3
Embryos–oocytes				
Fertilization (%)	89.2	83.3	87.5	90.5
Embryos grades 1 and 2 (%)	64.9*	38.9†	77.5*	69.0*
Degenerated (%)	24.2*,‡	22.2*	5.0†	9.5§
Degenerated-unfertilized (%)	35.1‡	38.9‡	17.5§	19.1§
Embryos				
Embryos (*n*)	33	30	35	38
Embryos grades 1 and 2 (%)	72.7*	46.7†	88.6*	76.3*
Degenerated (%)	27.3*	26.7*	5.7†	10.5*,†
Blastomeres				
Mean±S.E.M.	43.8±4.0*	28.0±4.2†	41.3±3.9*	45.7±3.7*
Median (*n*)	49†	32*	43†	45†
Live (%)	91.7†	89.8†	97.9*	98.1*
Accessory spermatozoa				
Mean±S.E.M.	28.4±6.9*	22.8±6.8*,†	10.2±6.7†	15.5±6.4*,†
Median (*n*)	11.0‡	7.0§	6.5§	8.5‡,§
Embryos–oocytes with ≥1 (%)	94.6*	88.6*	72.5†	95.2*
Embryos–oocytes with >4 (%)	71.4	61.8	56.3	56.4

*,†Superscripts in the same row differ ($P<0.05$). ‡,§Superscripts in the same row differ ($P<0.10$). OD, cows inseminated at oestrous detection; OVS3, cows starting the Ovsynch on day 3 of the oestrous cycle; OVS6, cows starting the Ovsynch on day 6 of the oestrous cycle; OVS6E, similar to OVS6 with an additional 0.5 mg of oestradiol cypionate 36 h before AI.

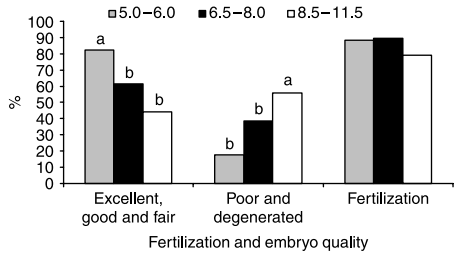

Figure 4 Distribution of frequency of embryos according to grade quality and of oocyte fertilization (IETS 1998) relative to period of dominance of the ovulatory follicle. Fertilization remained unaltered ($P=0.27$), but embryo quality declined ($P<0.001$) with increasing period of dominance. [a,b]Bars having different letters differ ($P<0.01$).

2 embryos, whereas cows with a longer period of dominance had increased proportion of poor quality and degenerated embryos. Fertilization rate remained unaffected by the period of dominance.

Discussion

The present study demonstrated that reproductive programmes that extend the period of follicle dominance influence embryo quality in lactating dairy cows. Follicles from cows in OVS3, which had a longer period of dominance than those from cows in OVS6 and OVS6E, resulted in embryos of reduced quality. Furthermore, embryos from cows in OVS3 had fewer blastomeres and a lower proportion of live blastomeres than embryos from cows in OVS6 and OVS6E. Surprisingly, cows inseminated on oestrus had a greater proportion of non-viable embryos and their embryos had a reduced proportion of live blastomeres compared with cows in OVS6 and OVS6E. Cows in OD had an ovulatory follicle with 1.4 days of dominance longer than those in the OVS6 and OVS6E treatments, suggesting that small increases in the length of follicular dominance compromise embryo quality. Despite treatments, the proportion of viable embryos declined, and that of degenerated embryos and oocytes increased as dominance was extended.

The increased proportion of degenerated embryos with increased period of follicle dominance might explain the reductions in fertility of lactating dairy cows observed when dominance is extended spontaneously (Townson *et al*. 2002, Bleach *et al*. 2004), or of heifers induced to have persistent follicles (Schmitt *et al*. 1996). It is interesting to note that, when cows ovulated to the first GnRH of the synchronization treatments, those in OD had a greater proportion of degenerated embryos and embryos with a smaller proportion of live cells than cows receiving timed AI in OVS6 and OVS6E. Wiltbank *et al*. (2006) suggested that a potential mechanism reducing fertility of high-producing dairy cows is the reduced concentrations of steroids as a result of increased hepatic clearance. The increased hepatic metabolism may require that follicles develop longer to produce sufficient OE_2 to induce oestrus and ovulation, which might compromise fertility. In support, it was observed that cows inseminated in oestrus had longer follicle dominance and increased degenerated embryos in the present study. Therefore, a slight extension in dominance can decrease embryo quality and fertility in cows that come naturally in oestrus. Previously, prolonged follicle dominance was implicated in reduced oocyte and embryo viability (Ahmad *et al*. 1995, Revah & Butler 1996), and reduced pregnancy rate (Schmitt *et al*. 1996); however, in those studies, follicles were induced to become persistent by subluteal concentrations of progesterone. Furthermore, Mihm *et al*. (1999) reported that extending emergence to ovulation to 12 days resulted in oocytes that reached at least metaphase I, thereby characterizing premature resumption of meiosis, which is expected to compromise subsequent embryo development. Although these studies (Ahmad *et al*. 1995, Revah & Butler 1996, Mihm *et al*. 1999) demonstrated deleterious effects of prolonged dominance on the oocyte and embryo, the models used are unlikely to be replicated in cows inseminated following oestrus or when timed AI programmes are properly implemented.

Fertilization was not altered by synchronization protocol or changes in length of dominance of the preovulatory follicle. Consequently, decreased fertility associated with extended dominance of the ovulatory follicle was not caused by alterations in the mechanisms related to oocyte fertilization. Although median number of accessory spermatozoa did not differ among treatments, cows in the OVS6 had embryos–oocytes with reduced number of accessory spermatozoa and proportion of embryos–oocytes with ≥ 1 accessory spermatozoon. It is possible that changes in accessory spermatozoa might be related to decreased OE_2 concentrations near AI in the OVS6 cows. OE_2 is important for uterine motility and spermatozoa transport (Hawk 1983). It was initially hypothesized that increased concentration of OE_2 during proestrus in the OVS protocol would enhance spermatozoa transport and fertilization. Although cows with increased OE_2 concentration during proestrus had a greater number of accessory spermatozoa, particularly in the OD treatment, this improvement did not translate into a better fertilization rate. Embryos had increased numbers of accessory spermatozoa and a greater proportion of them had ≥ 1 accessory spermatozoon than oocytes. The number of accessory spermatozoa with the combination of the highest sensitivity (detection of unfertilized oocytes) and specificity (detection of fertilized oocytes) for fertilization was 4, but the number of accessory spermatozoa resulting in 100% specificity, i.e. 100% fertilization was >7. Previous reports observed a positive correlation between number of accessory spermatozoa and fertilization rate in cows (DeJarnette

et al. 1992, Nadir *et al*. 1993). The cut-off number of seven accessory spermatozoa demonstrates that maximum fertilization in lactating dairy cows can be achieved with a relatively small number of spermatozoa reaching the oocyte.

As expected, ovulation to the first GnRH injection of the synchronization protocols differed among treatments and caused the expected changes in the length of dominance of the ovulatory follicle. Follicles of cows on day 3 of the oestrous cycle, as in the first GnRH of OVS3, probably had not undergone deviation and acquired LH receptors in the granulosa cells (Bao *et al*. 1997), making them incapable to respond to an LH surge and ovulate (Fortune *et al*. 2001). Consequently, fewer OVS3 cows had a new follicular wave recruited, thereby extending follicle growth in ~2.3 days compared with OVS6 and OVS6E cows. Although this resulted in older and larger follicles for OVS3, it did not alter ovulation to the final GnRH in cows receiving timed AI. Others (Vasconcelos *et al*. 1999, Rutigliano *et al*. 2008) observed that cows that did not ovulate to the first GnRH had reduced ovulation to the final GnRH of the OVS protocol. Therefore, decreased fertility observed in cows that do not ovulate to the initial GnRH of the OVS protocol (Chebel *et al*. 2006) may be caused by the reduced synchronization of ovulation when AI is performed, in addition to extension of the period of follicle dominance (Bleach *et al*. 2004).

Progesterone concentration at the first GnRH injection of the synchronization protocols was less for OVS3, which was expected because cows in the OVS3 started the synchronization during metestrus, whereas OD, OVS6 and OVS6E started during early diestrus. Furthermore, progesterone concentration at the $PGF_{2\alpha}$ injection was also reduced in OVS3 cows probably because they were in an earlier stage of the oestrous cycle. Moreover, fewer OVS3 cows responded to the first GnRH and, thus, did not form an accessory CL when $PGF_{2\alpha}$ was injected. In fact, the mean number of CL in the OVS3 treatment was 1.0 as opposed to 1.9 in the other treatments (data not shown). Progesterone concentration was greater in OD and OVS3 treatments compared with OVS6E on day 6 after AI. Both treatments, OD and OVS3, ovulated larger follicles than OVS6 and OVS6E cows and later developed a larger CL that probably had a greater steroidogenic capacity. This agrees with a previous observation showing greater progesterone concentration on day 7 of the oestrous cycle when cows ovulated larger follicles (Vasconcelos *et al*. 2001). OE_2 concentration at 48 h after the $PGF_{2\alpha}$ injection was greater in OVS6E cows likely because of the treatment with oestradiol cypionate 24 h earlier. All treatments, but OD, had a decrease in OE_2 concentration at 72 h after the $PGF_{2\alpha}$ injection. For cows receiving the OVS protocol, the blood sample taken at 72 h after the $PGF_{2\alpha}$ was collected after the final GnRH injection of the protocol that induced an LH surge. Consequently, LH caused down regulation of aromatase mRNA in granulosa cells (Fitzpatrick *et al*. 1997) and cessation of OE_2 synthesis by the pre-ovulatory follicle. On the other hand, most cows in the OD treatment were observed in oestrus 72 h after the $PGF_{2\alpha}$ and, therefore, still had an oestrogen-active pre-ovulatory follicle.

The lack of an effect of increased OE_2 concentration near AI from either an endogenous (OD) or an exogenous (OVS6E) source on fertilization suggests that beneficial effects of OE_2 supplementation on conception rate observed previously (Cerri *et al*. 2004) were probably caused by post-fertilization processes. Mann & Lamming (2000) demonstrated that ovariectomized cows exposed to greater concentrations of exogenous OE_2 during an induced proestrus had reduced concentrations of $PGF_{2\alpha}$ metabolite after an oxytocin challenge in late diestrus of the subsequent simulated oestrous cycle. Attenuating $PGF_{2\alpha}$ release during the period of maternal recognition of pregnancy could improve the embryo survival in cows supplemented with OE_2. Nevertheless, the potential improvement in pregnancy in dairy cows to OE_2 supplementation is probably not mediated by increased fertilization. Furthermore, timed insemination following the OVS protocol does not limit fertilization when compared with cows inseminated at synchronized oestrus.

The deleterious effects on embryo quality observed in OD and OVS3 compared with OVS6 and OVS6E occurred despite similar or even greater progesterone concentrations 6 days after AI. Mann & Lamming (2001) suggested that the rise in progesterone concentration shortly after oestrus was pivotal for embryo development, and Lopes *et al*. (2007) observed a greater likelihood for pregnancy for cows with greater progesterone concentrations after day 5 of the oestrous cycle. However, both studies did not confirm occurrence of ovulation after AI, which would compromise both luteal function and pregnancy (Rutigliano *et al*. 2008). Concentrations of OE_2 during proestrus and progesterone 6 days after AI were similar between cows categorized as having embryos or not as grades 1 and 2. Therefore, the role of OE_2 at proestrus or the progesterone rise during early diestrus that influences pregnancy could be more related to effects on the uterus and not necessarily on early embryo development. Recently, Pereira *et al*. (2009) observed no positive effect of progesterone on embryo development at days 8 and 12 using an *in vitro* culture system with granulosa or oviductal epithelial cells. It is possible that differences in embryo quality relative to ovarian steroid concentrations in the peri-ovulatory period and early diestrus occur only past day 6 of development. Collectively, these data demonstrate that length of dominance of the ovulatory follicle is more important to embryo development until day 6 after insemination than the steroidal endocrine milieu in the peri-ovulatory period.

In conclusion, reducing the period of follicle dominance by optimizing the ovulatory response to the initial GnRH injection of the synchronization protocol improved early embryo development. Reduction in embryo quality was observed even when concurrent extension of follicle dominance was of only 1.5 to 2 days. Furthermore, if embryo quality influences pregnancy in lactating dairy cows, then optimization of fertility should be observed when dominance of the ovulatory follicle is restricted to 5–6 days. It is noteworthy that cows inseminated following a synchronized oestrus had increased proportion of degenerated embryos, which was associated with longer dominance because of the increased interval from CL regression to oestrus and AI. Fertilization and embryo quality on day 6 after AI were not associated with OE_2 concentrations during proestrus, or with progesterone concentrations on day 6 after AI. These data indicate that period of dominance is probably more important for early embryo quality in high producing lactating dairy cows than the endocrine steroidal milieu in which the ovulatory follicle develops. Moreover, these data also indicate that properly implemented timed AI programmes result in similar or better quality embryos than cows inseminated at synchronized oestrus. Therefore, it is plausible that high producing cows with extended interval between follicle deviation to oestrus have reduced fertility because embryo quality is compromised when dominance of the ovulatory follicle is increased by as few as 1.5 days.

Materials and Methods

Animals, housing and diets

The University of California, Davis Institutional Animal Care and Use Committee approved all procedures in this study. Three hundred and ninety six (107 primiparous and 289 multiparous) lactating Holstein cows (*Bos taurus taurus*) from one farm located in the San Joaquim valley of California were enrolled in the present study. The number of lactating cows in the herd during the study was ~900 and the 305 day 3.5% fat-corrected milk rolling herd average was 11 700 kg/cow. Animals were housed in free-stall barns equipped with fans and sprinklers that were automatically turned on when the temperature reached 26.7 °C. All cows were fed with the same diet as a total mixed ration twice daily to meet or exceed the dietary requirements for lactating cows weighing 680 kg, consuming 24 kg of dry matter and producing 45 kg of milk containing 3.5% fat and 3.1% true protein in the first 70 days of lactation (National Research Council 2001).

All cows had their body condition scored at 30±3 and 60±3 days in milk (DIM) according to Ferguson *et al.* (1994), and only cows with 2.50≥BCS≤3.75 at 30±3 DIM were enrolled in the experiment. Cows were classified according to the average BCS measured at 30±3 and 60±3 DIM as having a low BCS if the average was ≤2.75 or moderate BCS if the average was >2.75. Cows diagnosed with any evident health disorder (displacement of the abomasum, lameness, uterine infection and uterine adhesions) were not enrolled in the study.

Treatments and AI

Starting at 30±3 DIM, all cows had their ovulation synchronized with the OVS protocol (Pursley *et al.* 1995) and a controlled internal drug releasing (CIDR; EAZI Breed, Pfizer Animal Health, New York, NY, USA) as follows: day 30, 100 μg of GnRH (gonadorelin diacetate tetrahydrate, Merial Ltd, Iselin, NJ, USA) and a CIDR insert; day 37, an injection of 25 mg of $PGF_{2\alpha}$ (dinoprost tromethamine, Pfizer Animal Health) concurrent with the removal of the CIDR insert; day 39, a second injection of GnRH. The second GnRH injection of the pre-synchronization protocol was considered to be day 0 of the oestrous cycle and of the study. Only cows that had a synchronized ovulation based on disappearance of the dominant follicle within 48 h of the final GnRH of the pre-synchronization were enrolled in the study.

Cows were randomly assigned to one of the four treatments (Fig. 5). OD (n=118) received an injection of 100 μg GnRH on day 6, an injection of 25 mg $PGF_{2\alpha}$ on day 13, and were inseminated after detected in oestrus. Cows in OD were observed for visual signs of oestrus twice daily (am/pm) and AI was performed when cows were first detected in oestrus. The remaining cows were submitted to one of the OVS protocols OVS3 (n=90), OVS6 (n=99) or OVS6E (n=89). In all OVS treatments, cows received AI at fixed time 12 h after the last GnRH injection of the protocols. Cows assigned to the OVS3 treatment started the OVS protocol on day 3 and those assigned to the OVS6 and OVS6E treatment started the OVS protocol on day 6 of the oestrous cycle. Cows in the OVS6E received an additional 0.5 mg injection of oestradiol cypionate (ECP, Pfizer Animal Health) 36 h before AI.

The same person inseminated all cows with semen from a single sire of proven fertility and high expected relative conception rate from field inseminations in lactating cows.

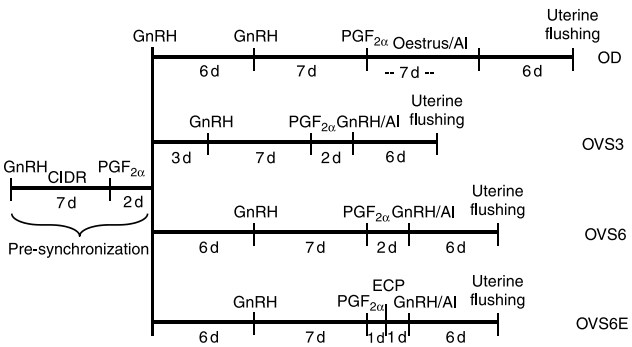

Figure 5 Diagram of the ovulation synchronization protocols used for each treatment. CIDR, controlled internal drug release containing 1.38 g of progesterone; OD, cows inseminated at oestrous detection; ECP, oestradiol cypionate; Oestrus/AI, oestrus observation and artificial insemination; GnRH/AI, GnRH injection and artificial insemination 12 h later; OVS3, cows starting the Ovsynch on day 3 of the oestrous cycle; OVS6, cows starting the Ovsynch on day 6 of the oestrous cycle; OVS6E, similar to OVS6 with an additional 0.5 mg of ECP 36 h before AI.

Blood samples and OE_2 and progesterone analysis

Approximately, 7 ml blood was collected by puncture of the median coccygeal vein or artery utilizing evacuated tubes (Vacutainer systems, Becton & Dickinson, Rutherford, NJ, USA) containing K_2 EDTA. Samples were immediately placed in ice and transported to laboratory within 5 h and centrifuged at 2000 ***g*** for 15 min for separation of plasma. Plasma was harvested and subsequently frozen at −25 °C until later analyses of progesterone and OE_2 concentrations.

Blood samples collected at the GnRH and $PGF_{2\alpha}$ injections of the pre-synchronization protocol (30±3 and 37±3 DIM) were used to determine cyclic status based on progesterone concentration. Samples were collected immediately before CIDR insertion and 30 min after CIDR removal on the days of GnRH and $PGF_{2\alpha}$ injections respectively, to avoid increases in plasma progesterone caused by the supplemental progesterone from the intravaginal insert (Cerri *et al.* 2009). Cows with progesterone concentration ≥1.0 ng/ml in 1 of the two samples were classified as cyclic, whereas those with progesterone concentrations <1.0 ng/ml in both samples were classified as anovular. Blood samples for the analysis of progesterone concentration were also collected concurrent with the hormonal treatments during the synchronization protocols (GnRH, $PGF_{2\alpha}$, and 48 h later) and 6 days after AI when embryo collection was performed. Plasma samples were analyzed in duplicates for progesterone concentration by an ELISA validated by Cerri *et al.* (2004). Each microplate contained the standards 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 ng/ml. The intra- and inter-assay coefficients of variation (CV) were 4.7 and 13.0% respectively. Cows with progesterone concentrations in plasma ≥1.0 ng/ml on the day of $PGF_{2\alpha}$ injection of synchronization treatments and then <1.0 ng/ml 48 h later were classified as experiencing complete luteolysis.

OE_2 concentration was analyzed in plasma samples collected at 48 and 72 h after the $PGF_{2\alpha}$ injection of the synchronization treatments in all cows. An additional blood sample was collected and plasma analyzed for OE_2 at the time of AI in cows in the OD treatment when oestrus was detected after 72 h of the $PGF_{2\alpha}$ injection of the synchronization. Samples were analyzed in duplicates using a validated RIA (Kirby *et al.* 1997). Standards were 0.25, 0.50, 1.0, 2.5, 5.0, 7.5, 10.0 and 20.0 pg/ml. The intra- and inter-assay CV were 10.2 and 13.2% respectively.

Ovarian ultrasonography

Cows had their ovaries examined by ultrasound (Aloka SSD-500, Aloka Co. Ltd, Wallingford, CT, USA) equipped with a 7.5 MHz linear rectal transducer at every injection of the synchronization treatments and again 48 h later. For OD cows, an additional ultrasound examination was performed the day they were observed in oestrus. Ovaries were also examined by ultrasound on the day of embryo collection. Maps of the ovaries were drawn for each individual cow and size and position of follicles ≥5 mm in diameter and CL were recorded. Occurrence of ovulation within 48 h after each GnRH injection was characterized by the disappearance of a previously recorded follicle ≥10 mm in diameter. The length of dominance of the ovulatory follicle was defined by the period between the day of follicular deviation and OD or last GnRH of the OVS protocol (OVS3, OVS6, OVS6E). Follicular deviation was assumed to have occurred 3.5 days after the first GnRH of the synchronization treatment for cows that ovulated to that GnRH (Roche 2004). For cows that failed to ovulate to the first GnRH of the synchronization treatments, deviation was assumed to have occurred 3.5 days after the final GnRH of the pre-synchronization treatment, as all cows enrolled in the study had a synchronized ovulation after the pre-synchronization programme.

Embryo–oocyte collection and evaluation

Cows had their uteri flushed on day 6 after AI by a trans-cervical procedure using a silicon Foley catheter (18 Fr, 30 ml, 56 cm; Minitube of America, Inc., Verona, WI, USA). The balloon of the catheter was placed ∼3 cm passing the external intercornual ligament of the uterine horn ipsilateral to the ovary bearing the CL. Approximately, 300 ml of a flushing solution (ViGro complete flush solution, Bioniche Life Sciences Inc., Belleville, ON, Canada) was used for a single uterine horn. Embryos–oocytes collected were evaluated for fertilization and grade quality (1 = excellent and good; 2 = fair; 3 = poor and 4 = degenerated; IETS 1998). Embryos were stained with 5 μg/ml propidium iodide (Sigma) to determine the number of non-viable blastomeres and then with 5 μg/ml Hoechst 33342 (Molecular Probes Inc., Eugene, OR, USA) to determine the number of accessory spermatozoa using epifluorescence microscopy (365 nm excitation, >400 nm emission). The zona pellucida was then dissolved with a solution of 0.02 N HCl in 0.1% Tween-20 (Sigma). The embryo was again stained with 5 μg/ml Hoechst 33342 and the blastomeres spread in a glass slide and counted using epifluorescence microscopy.

Experimental design and statistical analysis

The experimental design was randomized with incomplete blocks. Lactating Holstein cows were blocked at 30±3 DIM according to parity (primiparous or multiparous) and BCS (low or moderate) and, within each block, randomly assigned to one of the four treatments. More cows were enrolled in the OD treatment because it was expected that OD would be less than 80%.

Dichotomous outcomes were evaluated by logistic regression using the LOGISTIC procedure of SAS (Statistical Analysis Software, SAS Institute Inc., Cary, NC, USA). Count data such as number of accessory spermatozoa and blastomeres were analyzed by the GENMOD procedure of SAS (Statistical Analysis Software, SAS Institute Inc.) using a Poisson distribution. The models included the effects of treatment, parity, cyclic status and average BCS.

Progesterone and OE_2 concentrations were analyzed by ANOVA for repeated measures using the MIXED procedure of SAS (Statistical Analysis Software, SAS Institute Inc.). The covariance structure with the smallest Akaike's information criterion was used for measurements utilized in the MIXED model. The models included the effects of treatment, day of blood collection, interaction between treatment and day of blood collection, parity (primiparous and multiparous) and

average BCS (low and moderate), with cow nested within treatment as the random error. For progesterone, analyses were performed separately for samples collected before and after $PGF_{2\alpha}$ injection during synchronization treatments.

Analysis of dominant follicle diameter was performed by ANOVA with the GLM procedure of SAS (Statistical Analysis Software, SAS Institute Inc.) with a model that included the effects of treatment, parity, cyclic status and average BCS.

Receiver operating characteristic (ROC), analysis using MedCalc version 9.5.1.0 (MedCalc software, Mariakerke, Belgium) was performed to determine the critical number of accessory spermatozoa to optimize fertilization based on sensitivity and specificity. The ROC curve analysis plots the sensitivity against the false positive fraction (1 – specificity) to detect the best combination of sensitivity and specificity for the number of accessory spermatozoa for fertilization, and the point closest to the left upper corner represents the best combination of them.

Differences with $P \leq 0.05$ were considered significant and from $0.05 < P \leq 0.10$ were designated as tendency.

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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