Ovarian Follicle Maturity At Induced Ovulation Influences Fertility In Cattle

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Introduction: Artificial insemination (AI) is the most powerful reproductive technology available to enhance genetic improvement in beef and dairy cattle. Tremendous increases in milk production have been achieved in the dairy industry over the past 4 decades as a result of incorporation of this technology. In beef production, the increasing emphasis on value – based marketing, and producing beef for targeted, specialized markets will drive the increased incorporation of this technology into this industry.

Rationale: Fixed time AI, where all cows are bred at a similar time regardless of spontaneous estrus, has become increasingly important in order to decrease the time required and problems associated with estrous detection and the process of AI. Most fixed time AI protocols in the US utilize an injection of gonadotropin releasing hormone (GnRH) at the time of, or 8 – 16 hours before AI to ensure that ovulation occurs in a timely manner relative to AI. Another method to induce a synchronized LH surge, and hence ovulation, is through the use of estradiol, given 24-36 hours before AI. For synchronized ovulation to be effective, it is necessary to coordinate a wave of follicular development so that the ovulatory follicle is of adequate size and maturity to respond to the induced LH surge. The Ovsynch (Pursley et al., 1998) and CO-Synch (Geary et al., 1998) systems of estrous control use an injection of GnRH to initiate a new wave of follicular development and GnRH is again administered 9 days later to ovulate the newly formed dominant follicle. Geary et al., (2000) reported, however, that follicular wave control occurred in only 66% of the animals treated in this type of system. Estradiol, given in conjunction with progesterone, is another method that can be used to initiate a new wave of follicular development (Day and Burke, 2001). When estradiol is used for this purpose, a new wave is initiated in nearly 100% of females (Burke et al., 2001) however, the timing of emergence varies with reproductive status of the animal, dose of estradiol and stage of the estrous cycle (Burke et al., 2002; Day and Burke, 2001; Bogacz 1999, 2000). With these variations in the precision of control of follicle waves with either GnRH or estradiol, it is accepted that follicles of varying sizes and maturity will be present at the time of the LH surge that is induced with GnRH or estradiol to synchronize ovulation for AI. It has been reported that estradiol will induce ovulation of most dominant follicles that are 8-10 mm in diameter in beef heifers (Burke et al., 2001) and GnRH will induce ovulation of most follicles that average 11.5 mm in diameter in lactating dairy cows (Vasconcelos et al., 2001). In anestrous postpartum beef cows, estradiol ovulated only 44% of follicles that averaged 9 mm in diameter (Burke et al., 2001). In these same studies, it was reported that in lactating dairy cows, and in anestrous beef cows, the CL resulting from ovulation of an immature follicle was smaller in diameter and produced less progesterone. Cows in the study by Vasconselos et al., (2001) were inseminated at the induced ovulation, and a reduction in conception rate was detected in the treatment that ovulated smaller follicles. However, ovarian follicle aspiration was performed 6 days before AI in those ovulating smaller follicles, whereas this procedure was not performed in the control group that ovulated larger follicles, potentially confounding this finding.

With the increasing use of timed AI programs, the impact of variation in stage of follicular development and thus maturity of the preovulatory follicle at the time of synchronized ovulation is of increasing interest. The precision, or lack thereof, of the current methods available to coordinate follicular development in cattle means that follicles of varying maturity will be induced to ovulate when timed AI/synchronized ovulation programs are used. Based upon previous work from our laboratory and that of others, it is obvious that follicles across a wide range of sizes will ovulate in response to GnRH or estradiol, and will subsequently form a CL. In most instances, the CL resulting from ovulation of an immature dominant follicle was of smaller size, and produced lesser concentrations of progesterone in circulation. Furthermore, data from one study suggested that ovulation of immature follicles may influence conception rate at the induced ovulation. Therefore, we initiated a line of investigation to directly address the influence follicle size and/or maturity at the time of ovulation on fertility and subsequent CL function. An animal model that provided precise control of follicular development was used to achieve ovulation of dominant follicles at specific diameters. This model was used in the first 2 experiments with the objective to determine the impact of ovulation of small (immature) follicles vs. large (mature) follicles on conception rate to a timed AI and to verify the influence of ovulatory follicle size on luteal progesterone production.

Experiment 1: The specific aim of this experiment was to compare conception rate and luteal function between animals induced to ovulate an immature follicle with GnRH, to females that spontaneously ovulated in response to an endogenous LH surge. Estrus was synchronized in 56 Angus crossbred cows. At an average of 6.4 $\pm$ 0.1 days following the synchronized estrus, all ovarian follicles > 4 mm in diameter were aspirated with a 17-gauge needle utilizing a transvaginal probe in order to synchronize a new wave of follicular development (D 0 of the experiment). Trans-rectal ultrasonography was performed daily to monitor follicular growth, beginning on D -2 and continuing until ovulation occurred. Two injections of $PGF_{2\alpha}$ were administered 12 hours apart on D 1.5 and 2 after aspiration to induce luteolysis. Animals were alternatively allotted to treatments as the largest, newly emerged follicle reached 10 mm. Treatments consisted of administering 100 μg GnRH when the largest follicle was 10 mm in diameter (GnRH-10) or providing no further treatment and permitting the female to exhibit spontaneous estrus and ovulation (SPON). AI was performed 12 hours after the GnRH injection (GnRH-10 treatment) or the onset of estrus as detected with Heatwatch™ (SPON treatment). Blood samples for determination of progesterone concentrations were collected beginning two days after AI and every other day thereafter until pregnancy diagnosis on D 30. Ovulation of a smaller follicle at an earlier time following aspiration was induced in the GnRH-10 as compared to the SPON treatment (Table 1). Conception rate to the prematurely induced ovulation in the GnRH-10 treatment was reduced by 24.1% relative to a spontaneous ovulation. Concentrations of progesterone were lower in the GnRH-10 than SPON treatment on most days monitored during the mid – luteal phase. On d 12 (Table 1) of the estrous cycle, concentrations differed by .8 ng/ml, representing a 13% reduction in progesterone concentrations from that of the SPON treatment.

Table 1. Ovulatory characteristics, luteal progesterone and pregnancy rate (Exp. 1).

Variable	GnRH-10	SPON
Interval to estrus (D)	na	6.2 ± 0.2
Incidence of Ovulation (%)	29/29 (100)	24/27 (88.9)
D of Experiment at Ovulation	6.8 ± 0.1*	7.7 ± 0.1
DF1 Diameter at Ovulation (mm)	10.7 ± 0.1*	12.0 ± 0.3
P4 - 12 d post-ovulation (ng/ml)	5.4 *	6.2
Pregnant 30 d after AI (%)	22/29 (75.9)*	24/24 (100)

* GnRH-10 vs SPON, $P < 0.05$

The animal model used in this experiment resulted in a high incidence of double ovulations (GnRH 41 %, SPON 54%). Although this was an interesting finding, it was difficult to ascertain how this unusual ovulatory response may have affected the outcome of this experiment. For example, although a reduction in conception rate was detected between treatments, conception rates in both treatments in this experiment were higher than expected, and may have been influenced by the greater ovulation rate.

Regardless, these findings clearly indicated that conception rate and luteal function are reduced in animals induced to ovulate an immature follicle, relative to a spontaneous ovulation. While we hypothesized that this difference was due to follicle diameter, an alternative interpretation would be that the difference in fertility was a result of the contrasting proestrus environments created by induction of the LH surge with GnRH as compared to a spontaneously generated LH surge. Therefore a subsequent experiment was designed to compare conception rate and luteal function between animals induced to ovulate an immature or mature dominant follicle with GnRH.

Experiment 2: The specific objective of this experiment was to compare conception rate and luteal function between females induced to ovulate either large, mature follicles, or small immature follicles with GnRH. Estrus was synchronized in postpartum beef cows (n = 101) and aspiration of ovarian follicles and administration of $PGF_{2\alpha}$ was performed as described in Experiment 1 with one key modification. Rather than administering the $PGF_{2\alpha}$ on D 1.5 and 2 as in Experiment 1(coincident with emergence of the new wave of follicles), it was given as a single dose at the time the newly emerged dominant follicle attained a diameter of 8 mm. Most cows would have received $PGF_{2\alpha}$ on D 4, or 2 days later than in Experiment 1. We speculated that the high incidence of double ovulations observed in Experiment 1 may have been the result of the synchronous timing of new wave emergence and luteal regression. Furthermore, if we permitted the dominant follicle to attain a diameter usually associated with follicle deviation, perhaps the high incidence of double ovulations could be avoided. Trans-rectal ultrasonography was performed to monitor follicular growth following aspiration and as the largest follicle reached a diameter of 10 mm, animals were alternatively assigned to receive 100 μg of GnRH at

that time (GnRH-10) or when this follicle was 13 mm in diameter (GnRH-13). AI was performed 12 hours after the administration of GnRH in both treatments. Blood samples were collected during the mid-luteal phase to monitor circulating concentrations of progesterone.

Table 2. Ovulatory characteristics, luteal progesterone and pregnancy rate (Exp. 2).

Variable	GnRH-10	GnRH-13
Incidence of Ovulation (%)	45/47(95.7)	54/54 (100)
D of Experiment at Ovulation	7.1 $\pm$ 0.1*	8.3 $\pm$ 0.2
DF1 Diameter at Ovulation (mm)	11.1 $\pm$ 0.2*	13.6 $\pm$ 0.2
P4 - 12 d post-ovulation (ng/ml)	4.9 *	5.5
Pregnant 30 d after AI (%)	2/45 (4.4)*	31/54 (57.4)

* GnRH-10 vs GnRH-13, $P < 0.05$

The incidence of double ovulations was18 % and 12 % in the GnRH-10 and GnRH-13 treatments, respectively and did not differ. Thus, postponing the injection of PGF until follicle diameter was 8 mm appeared to reduce the incidence of multiple ovulations that were evident in Experiment 1. The approach taken in this study resulted in GnRH-induced ovulation of follicles that differed in diameter by 2.5 mm (Table 2). Results provided compelling evidence that fertility can be severely compromised if follicles are induced to ovulate prematurely with GnRH. Pregnancy rate in the GnRH-13 treatment was within the typical range for a timed AI protocol in cows of this type, suggesting that the model used did not in itself have major detrimental effects on conception rate. As in Experiment 1, concentrations of progesterone were lower in cows that were induced to ovulate a smaller follicle. On d 12 (Table 2) of the estrous cycle, concentrations were .6 ng/ml less in the GnRH-10 than GnRH-13 treatment, representing an 11% reduction in progesterone concentrations in circulation.

In Experiments 1 and 2, data were compiled on 157 animals to indicate that cattle induced to ovulate a small or immature follicle with GnRH will have lower fertility at this ovulation. These initial experiments do not address the mechanisms responsible for the reduction in pregnancy rates or whether the failure of animals ovulating small follicles to be pregnant 30 days later is due to defects in fertilization, embryonic development, early embryonic mortality, or some combination of these types of these factors. A challenge is to isolate the potential multiple sources for pregnancy failure when immature follicles are induced to ovulate. A systematic approach involving a series of specifically focused investigations will be required. One such experiment has been completed to identify the mechanisms responsible for the reduction in fertility when cattle are induced to ovulate immature follicles. Results of this study are reported below (Experiment 3).

Experiment 3: The objective of this experiment was to determine the influence of ovulation of immature follicles on the pregnancy rate of heifers used as recipients for embryo transfer. Yearling beef heifers (n=24) were subjected to similar treatments as described for Experiment 2. Ovarian follicle aspirations were performed to synchronize a follicular wave (D 0) and animals were induced to ovulate either a small dominant follicle (10 mm) or a large dominant follicle (13 mm) with GnRH as previously described. These heifers were not inseminated at the induced ovulation. Embryos were implanted on day 7 following induction of the LH surge. Embryo's used for implantation were stratified across treatments based on source, breed and grade. Blood samples were collected during the mid-luteal phase for analysis of circulating concentrations of progesterone. Trans-rectal ultrasonography was performed on day 30 to assess pregnancy rates.

Table 3. Ovulatory characteristics, luteal progesterone and pregnancy rate (Exp. 3).

Variable	GnRH-10	GnRH-13
Incidence of Ovulation (%)	12/12 (100)	12/12 (100)
D of Experiment at Ovulation	6.8 $\pm$ 0.1*	7.8 $\pm$ 0.2
DF1 Diameter at Ovulation (mm)	11.1 $\pm$ 0.2*	13.7 $\pm$ 0.2
P4 - 12 d post-ovulation (ng/ml)	3.4 *	5.6
Pregnant 30 d after AI (%)	1/12 (8.3)*	8/12 (66.7)

* GnRH-10 vs GnRH-13, $P < 0.05$

Similar to Experiment 2, the design of this experiment resulted in GnRH-induced ovulation of follicles that differed in diameter by 2.6 mm (Table 3). Although only 12 heifers were in each treatment, the magnitude of the difference in pregnancy rate indicates that the capacity of heifers to maintain a pregnancy established by embryo transfer is severely compromised if follicles are induced to ovulate prematurely with GnRH. In this case, pregnancy failure was not due to an impairment of fertilization or early embryo development. Pregnancy rate in the GnRH-13 treatment was within the upper range for embryo transfer, again confirming that the model used did not in itself have major detrimental effects on pregnancy rate. Concentrations of progesterone were lower in heifers that were induced to ovulate a smaller follicle, and the magnitude of this difference was more pronounced than the previous 2 experiments (39.3% reduction).

Discussion: Some of the potential sources of inadequacy to establish and support pregnancy following induced ovulation of immature follicles include the ovulation of incompetent oocytes, an inadequately prepared uterine environment and/or subsequent development of a subfunctional CL.

Results from Experiment 3 imply that the quality or maturity of the oocyte released from immature follicles may not be a primary factor in the reduction of fertility that was observed in Experiments 1 and 2 when females were inseminated at the induced ovulation. The use of

embryo transfer removed the direct influence of the competence of the oocyte for determination of whether pregnancy would be established. Similar reductions in pregnancy rates were observed between Experiments 2 and 3, suggesting a mechanism other than, or in addition to, oocyte incompetence is of primary importance. Results of Experiment 3 clearly indicate that the uterine environment into which the embryo was placed was inadequate to sustain development of the embryo and lead to establishment of pregnancy. Whether this inadequacy is a direct function of the levels of progesterone being produced by the CL or caused by some other alteration in the hormonal milieu to which the uterus is exposed as a result of premature ovulation is unknown. Premature induction of ovulation would by necessity disrupt the normal sequence of the preovulatory changes in estradiol and the gonadotropins. It is possible that this interruption alters the preparation of the uterus to adequately support a pregnancy. For example, the lack of estradiol exposure of the uterus during proestrus could conceivably cause a decrease in functional progesterone receptors on the endometrium. When this effect is combined with a decrease in progesterone concentrations, perhaps the uterus is not able to attain the appropriate status required to support a pregnancy.

A consistent finding in all experiments was that concentrations of progesterone were reduced following ovulation of immature follicles. The magnitude of the reduction was 13, 11 and 39% in Experiments 1, 2 and 3, respectively. It is unknown at present whether this variable reduction in progesterone is sufficient to be responsible for the profound differences in fertility detected. It is interesting that in Experiment 1, although the reduction in progesterone concentrations in the GnRH-10 treatment relative to the SPON treatment was numerically greater than between the GnRH-10 and GnRH-13 treatments in Experiment 2, pregnancy rate was much higher in the GnRH-10 treatment in the first study (75.9 vs. 4.4%). The model used in these two experiments differed slightly. In Experiment 1, follicles emerged coincidentally with luteal regression whereas in Experiment 2, luteal regression was not initiated until the dominant follicle was at least 8 mm in diameter. Thus, in the first study, follicles were presumably exposed to the increased level of gonadotropic stimulus characteristic of proestrus from the time of emergence to ovulation. In the second study, proestrus was initiated at a more advanced stage of follicular development. Perhaps, at a given diameter, follicles in the first study were more developmentally advanced due to constant gonadotropic stimulus from the time of emergence. That this may be the case is inferred by comparing the diameter of the ovulatory follicle between the SPON treatment in Experiment 1, and the GnRH-13 treatment in Experiment 2. Cows in the first study showed estrus and ovulated a follicle that averaged 12 mm in diameter. In the second study, diameter of the ovulatory follicle was 13.6 mm at ovulation, and cows in this group had not exhibited estrus at the time of GnRH administration. From collective consideration of these data, it was hypothesized that the proestrous environment to which a follicle has been exposed may be of at least equal importance to actual diameter, in determining the competency of the follicle to lead to a pregnancy. An experiment is in progress to test this hypothesis.

In conclusion, the results from these experiments clearly show that fertility is compromised when animals are induced to ovulate a dominant follicle that has not reached full maturity. Whether there is a specific size or maturity status of the dominant follicle at ovulation that is required to prevent this decrease in fertility has not been established. In addition, the mechanisms mentioned previously such as oocyte quality, uterine environment and CL function that may be responsible for this reduction in fertility have not been confirmed. It is likely that there is a

cumulative effect of several mechanisms that contribute to the reduction in fertility observed in these experiments. Obviously, further research needs to be performed to substantiate these ideas and to identify the mechanism(s) that are affected as a result of ovulating a premature dominant follicle.

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