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Effect of preovulatory follicle maturity on pregnancy establishment in cattle: the role of oocyte competence and the maternal environment

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Abstract

Reproductive technologies to synchronize estrus and ovulation in cattle have enhanced the ability to practically utilize artificial insemination to increase both genetic merit and reproductive management of beef and dairy herds. The ability to successfully synchronize a follicular wave and ovulation, in heifers and cows, has improved substantially in recent years. Consequently, pregnancy rates to a single fixed-time artificial insemination (FTAI) can approximate that of insemination following spontaneous estrus. Despite these advances, a subset of heifers and cows often has a physiologically immature dominant follicle at the time of GnRH-induced ovulation. These animals will exhibit reduced pregnancy rates and decreased embryonic survival if a pregnancy happens to become established. The physiological mechanisms underlying the preceding decreased fertility have been a focus of our laboratories and may include an effect of the follicular microenvironment on both oocyte competence and the maternal environment. Oocytes must have adequate opportunity to complete cytoplasmic and molecular maturation during the final stages of oocyte maturation that occur within the preovulatory follicle. Follicular status, during the proestrus period, must be such that adequate circulating concentrations of estradiol are present before FTAI to increase oviductal transport of gametes and enhance both the luteinizing capacity of granulosa cells and progesterone receptor population in the post-ovulatory uterus. Following ovulation, the follicle's transformation to a functional corpus luteum to secrete adequate amounts of progesterone is essential for the establishment of pregnancy. The physiological status of the preovulatory follicle, prior to FTAI, greatly affects the concepts discussed above and has an important impact on pregnancy establishment and maintenance in cattle.

Keywords: bovine, follicle, oocyte, pregnancy, synchronization of ovulation.

Introduction

Synchronization of estrus/ovulation and artificial insemination (AI) are powerful techniques for both genetic improvement and reproductive management in beef cattle (Seidel, 1995). However, the time and labor associated with the detection of estrus has been a deterrent to the adoption of AI in beef herds. Therefore, significant effort has been directed toward development of fixed-time AI (FTAI) protocols that allow heifers and cows to be inseminated at a predetermined time and achieve pregnancy rates that are similar to those following the detection of estrus and AI. Furthermore, FTAI protocols increase the proportion of heifers and cows that conceive early in the breeding season, which has important benefits for reproductive management and beef production. Significant progress has been made toward developing FTAI protocols that precisely control the time of ovulation. Consequently, increased effort has been directed toward understanding the ovarian, uterine, and embryonic mechanisms controlling the establishment and maintenance of pregnancy (see reviews by Pohler *et al.*, 2012; Bridges *et al.*, 2013; Geary *et al.*, 2013), with the purpose of developing strategies for increasing the pregnancy rate to a single insemination. The purpose of this paper is to review the effect of ovulatory follicle size, at the time of FTAI, on pregnancy rates and late embryonic/fetal survival, to discuss why physiologically immature follicles may be present at FTAI, and to discuss mechanisms by which the physiological maturity of a dominant follicle may affect the establishment and maintenance of pregnancy in beef cattle.

Overview of synchronization of ovulation

Ovarian mechanisms controlling the expression of estrus, ovulation of a competent oocyte, and establishment of an oviductal/uterine environment conducive to embryonic development is likely optimized when a female expresses estrus and ovulates spontaneously. However, when the preceding events are artificially manipulated with FTAI protocols, pregnancy rates can be reduced. Cattle have recurrent follicular waves, beginning prior to puberty and continuing until late gestation, and the development of FTAI protocols require both synchronization of follicular waves and the induction of luteolysis. Consequently, FTAI protocols for cattle frequently involve the following physiological sequence: 1) Turnover of a dominant follicle to initiate a new follicular wave. This is accomplished by administration of exogenous gonadotropin releasing hormone (GnRH; e.g. USA) or estradiol in the presence of progesterone (e.g. Brazil) to induce ovulation or dominant follicle turnover, respectively (see reviews by Bó *et al.*, 1995; Diskin *et al.*, 2002), 2) Induction of luteolysis, five to seven days later, by administration of prostaglandin F2α (PGF), and 3) Administration of

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estradiol or GnRH to induce ovulation following insemination. Essentially all FTAI protocols in the USA are variations of the preceding GnRH-PGF-GnRH injection sequence with some differences in timing of insemination and many protocols include a progestin between the first GnRH and PGF injections to better control estrus expression. For FTAI protocols, the timing of insemination is scheduled to result in an overlap between the period of oocyte viability following ovulation and availability of capacitated sperm in the ampulla of the oviduct. However, at the time of FTAI, there is a mixed population of heifers or cows that have or have not expressed estrus. Animals that have not expressed estrus by the time of FTAI require an injection of GnRH or estradiol to induce a preovulatory gonadotropin surge and ovulation so that all animals can be inseminated at the same time. Females that exhibit estrus prior to or at the time of FTAI normally have a spontaneous gonadotropin surge and experience higher pregnancy rates compared to those that fail to exhibit estrus (Perry *et al.*, 2005; Larson *et al.*, 2006). Therefore, a challenge with FTAI is to manipulate the estrous cycle or the induction of ovulation such that the follicular microenvironment is optimal for acquisition of oocyte competence and programming the maternal environment for the establishment and maintenance of pregnancy.

Effect of ovulatory follicle size on pregnancy in beef heifers and cows

In *Bos taurus* and *Bos indicus* cattle, antral follicles acquire the ability to ovulate in response to an endogenous or exogenous preovulatory gonadotropin surge at 7 or 10 mm in diameter, respectively, which is associated with the time of follicular divergence between the newly selected dominant follicle and subordinant follicles (Sartori *et al.*, 2001; Gimenes *et al.*, 2008). This time frame corresponds to acquisition of LH receptors in bovine granulosa cells by the selected follicle (see review by Lucy, 2007). However, a larger dose of LH was required to induce ovulation in a 10 mm follicle versus larger sized follicles (Sartori *et al.*, 2001), suggesting a difference in the physiological maturity of small versus large dominant follicles.

When ovulation is induced, the size or physiological maturity of the preovulatory follicle influenced pregnancy rate and late embryonic survival in beef and dairy cattle (Lamb *et al.*, 2001; Vasconcelos *et al.*, 2001; Perry *et al.*, 2005, 2007; Waldmann *et al.*, 2006; Dias *et al.*, 2009; Meneghetti *et al.*, 2009; Sá Filho *et al.*, 2009). In a study from our laboratory, postpartum beef cows induced to ovulate small dominant follicles (less than 11.3 mm in diameter) experienced lower pregnancy rates and higher incidences of late embryonic mortality than did those induced to ovulate large (greater than 11.3 mm in diameter) dominant follicles. Interestingly, ovulatory follicle size did not affect pregnancy establishment or maintenance when animals exhibited estrus and underwent spontaneous ovulation (Perry *et al.*, 2005). This led to the hypothesis that the physiological maturity, rather than the diameter, of a preovulatory follicle affects the establishment and maintenance of pregnancy (Perry *et al.*, 2005; Atkins *et al.*, 2013).

Why do heifers and cows have small dominant follicles at fixed-time insemination?

Our laboratories have utilized the CO-Synch FTAI protocol (GnRH-1 seven days before PGF, and GnRH-2 at FTAI 48 h after PGF; Geary *et al.*, 1998) to examine the effect of ovulatory follicle size on pregnancy establishment in beef heifers and postpartum cows (Perry *et al.*, 2005, 2007; Atkins *et al.*, 2013).Although this protocol has been modified for current use in the industry, we have continued to use it since it results in significant variation in dominant follicle size at GnRH-2. Approximately 40 to 50% of heifers (Atkins *et al.*, 2008) and 66% of postpartum beef cows (Geary *et al.*, 2000) have a dominant follicle capable of responding to GnRH-1. It is logical that small dominant follicles present at the time of GnRH-2 (FTAI) could result from failure to ovulate a dominant follicle and initiate a new follicular wave following GnRH-1 administration. Consequently, at GnRH-2 there will be heifers and cows that have and do not have a synchronized follicular wave. We hypothesized that cows that do not have a synchronized wave at GnRH-2 may have a small dominant follicle at GnRH-2. Alternatively, a slower growth rate of the dominant follicle could result in a small dominant follicle at GnRH-2. To test the preceding hypothesis we administered GnRH-1 to beef heifers, cycling postpartum cows, and anestrous postpartum cows at times when they would or would not have a follicle capable of ovulating to the induced gonadotropin surge (Atkins *et al.*, 2008, 2010a, b). Administration of GnRH-1 occurred on days 2, 5, 10, 15 and 18 or 2, 5, 9, 13, and 18 after estrus (day 0) in cycling heifers and postpartum cows, respectively. In beef heifers, day of the cycle at GnRH-1, but not ovulatory response to GnRH-1 had an effect on dominant follicle size at GnRH-2. Heifers receiving GnRH-1 in the latter part of the cycle (i.e. days 15 and 18) had a greater incidence of spontaneous luteolysis before PGF administration and earlier onset of estrus regardless of the presence of an accessory corpus luteum after GnRH-1, which resulted in smaller follicles at GnRH-2. Consequently, a strategy to reduce the presence of small, physiologically immature follicles at GnRH-2 in heifers may be to pre-synchronize their follicular development, such that follicles are in an earlier stage of the estrous cycle (≤day 10) at GnRH-1. In cycling cows, the day of the cycle at GnRH-1 did not affect dominant follicle size or the proportion of cows ovulating at GnRH-1. However, in both the cycling and anestrous groups, cows that ovulated in response to GnRH-1 had a larger follicle at GnRH-2 than cows that did not ovulate. In summary, induction of ovulation at GnRH-1 increased preovulatory follicle size at GnRH-2 in postpartum cows but not heifers.

Follicular determinants of pregnancy establishment in beef cattle

The decrease in pregnancy rate and late embryonic/fetal survival (days 28 to 70 post breeding) following GnRH-induced ovulation of physiologically immature follicles is likely due to a combination of decreased oocyte competence and (or) an inadequate preparation of the maternal environment for pregnancy establishment. Atkins *et al.* (2013) performed a reciprocal embryo transfer experiment to distinguish between effects of the follicular microenvironment on oocyte competence *vs*. the maternal environment. Single GnRH-induced ovulations were synchronized in recipient and donor postpartum beef cows. Animals were classified into large (≥12.5 mm) and small follicle (<12.5 mm) groups at GnRH-induced ovulation, and none of the animals were detected in estrus. Donor animals were inseminated, and embryos or unfertilized oocytes were recovered seven days later. Viable embryos from donors with small or large follicles were transferred into recipients with small or large follicles to differentiate between effects of the follicular microenvironment on oocyte competence and(or) the uterine environment. Evidence of inadequate oocyte competence and a compromised uterine environment in females induced to ovulate a small compared to a large ovulatory follicle was reported and is discussed in more detail below.

Oocyte determinants of fertility

Oocyte competence is defined as the oocyte's ability to resume meiosis, cleave after fertilization, develop to the blastocyst stage, and bring to term a successful pregnancy (Sirard *et al.*, 2006). Developmental competence is acquired throughout oocyte and follicular growth as the oocyte progresses through meiotic, cytoplasmic, and molecular maturation. During the period of oocyte growth, the bovine oocyte increases in size from an intra-zonal diameter of less than 30 µm in primordial follicles to greater than 120 µm in tertiary follicles (Hyttel *et al.*, 1997). Bovine oocyte competence has been examined by evaluating fertilization rate, cleavage rate, proportion of embryos that reach the blastocyst stage, as well as embryo quality (Otoi *et al.*, 1997; Hendricksen *et al.*, 2000; Atkins *et al.*, 2013) with increased oocyte competence observed in oocytes of larger size (Otoi *et al.*, 1997) and originating from larger follicles (Arlotto *et al.*, 1996; Hendricksen *et al.*, 2000; Atkins *et al.*, 2013).

Acquisition of oocyte competence can be divided into three major events: 1) Acquisition of the ability to undergo meiotic maturation, 2) Acquisition of cytoplasmic maturation, and 3) Accumulation and storage of mRNA transcripts and proteins (i.e. molecular maturation). In fetal life, DNA synthesis doubles the chromatin content in the oocyte. The chromatin enters the diplotene stage of meiosis I and is arrested in a state of intermediate chromatin condensation, which allows for transcription of mRNA that can be stored within the oocyte for weeks due to polyadenylation of the 3' untranslated region (Sirard, 2001). Oocytes remain in diplotene arrest until they are either removed from their surrounding follicular cells or exposed to the preovulatory gonadotropin surge. As the oocyte gains meiotic competence, it acquires the ability to be released from meiotic diplotene arrest, fully condense its chromatin, expel a polar body, and progress to metaphase II (MII). It is commonly accepted that actively growing oocytes are meiotically incompetent, and acquisition of meiotic competence is a progression that takes place as the oocyte grows (Sirard, 2001). At an intrazonal diameter of 100 µm, the bovine oocyte acquires the ability to resume meiosis, but full meiotic competence to reach MII is not acquired until the oocyte reaches a diameter of 110 µm, which is normally contained in a 3 mm bovine follicle (Hyttel *et al.*, 1997).

While oocytes from bovine follicles greater than 3 mm may be competent to resume meiosis, they must progress through cytoplasmic maturation or oocyte capacitation to attain full developmental competence. Early changes in the oocyte's ultrastructure occurred at the secondary stage of follicular development as the zona pellucida and cortical granules were synthesized (Sirard, 2001). However, few changes in oocyte ultrastructure were observed from this point until the follicle reached a size of 8 to 9 mm (Hendrickson *et al.*, 2000). As the follicle progressed to ovulatory size, morphological changes in the mitochondria, ribosomes, endoplasmic reticulum, Golgi complex, and cortical granules occurred as the oocyte transitioned from the germinal vesicle (GV) to MII stage (reviewed by Ferreira *et al.*, 2009). The preceding reorganization of organelles is presumably regulated by cytoskeletal microfilaments and microtubules and is essential to oocyte viability (e.g. providing ATP to the nucleus for meiotic maturation and fertilization, proper translation of proteins, and the production of a calcium gradient and cortical granule release to block polyspermy; reviewed by Ferreira *et al.*, 2009).

In cattle, transcripts produced and stored by the oocyte are essential for subsequent oocyte maturation and early embryonic development up to activation of the embryonic genome (reviewed by Sirard *et al.*, 2006). Molecular maturation refers to the transcription of the mRNA blueprint (i.e. transcriptome) as well as storage of transcripts through the incorporation and extension of a 3' poly(A) tail (Brevini-Gandolfi *et al.*, 1999). Maternal mRNAs are rapidly transcribed and stored beginning at the secondary follicle stage (Fair *et al.*, 1997) and throughout the period of rapid oocyte growth up to the 3 mm follicular size (Fair *et al.*, 1995). Past this point, transcriptional activity continued, at a lower rate, until condensation of the chromosomes following germinal vesicle breakdown (GVBD; Fair *et al.*, 1995; Mourot *et al.*, 2006; Mamo *et al.*, 2011).

Molecular maturation of the bovine oocyte is also influenced by the surrounding follicular cells where the innermost layer of cumulus cells, the corona radiata, possesses cellular projections (i.e. transzonal projections) that penetrate the zona pellucida and directly contact the oolemma (Macaulay *et al.*, 2014). Although it is well known that small molecules (e.g. cAMP) can be delivered from cumulus cells to the oocyte, via transzonal processes, transport of mRNA to the oocyte has recently been reported and transported transcripts were observed to increase as the oocyte progressed from metaphase I (MI) to MII and to be associated with polyribosomes (Macaulay *et al.*, 2014, 2016). Transport of mRNAs is reportedly terminated upon exposure to the gonadotropin surge and subsequent breakdown of transzonal projections (Macaulay *et al.*, 2014).

Induced ovulation of small preovulatory follicles, in cows that have not expressed estrus, may negatively impact acquisition of oocyte competence. While meiotic competence is mostly complete by the time a bovine follicle reaches 3 mm, inadequate cytoplasmic and(or) molecular maturation could compromise oocyte competence in small preovulatory follicles at GnRH-induced ovulation. An inadequate transcriptome may be observed in oocytes from small preovulatory follicles, which are induced to ovulate prematurely, since transcription ends at GVBD and does not resume until activation of the embryonic genome. Analysis of the transcriptome of bovine oocytes from dominant follicles of postpartum beef cows that differed in size (smaller than 11.7 mm versus larger than 12.5 mm) or physiological status (estrous expression versus no estrous expression) revealed a list of differentially abundant transcripts that could regulate pathways associated with acquisition of oocyte competence (Dickinson, 2016).

Endocrine requirements for the establishment of pregnancy

Protocols for precisely synchronizing ovulation in beef and dairy cows have been developed and are widely employed by the industry (Binelli *et al.*, 2014; Bó and Baruselli, 2014; Colazo and Mapletoft, 2014). The next challenge in protocol development is to further increase the pregnancy rate following FTAI. Accomplishing this goal will require an increased understanding of the endocrine and physiological mechanisms controlling acquisition of oocyte competence, ovulation, fertilization, gamete transport, early embryonic development, maternal recognition of pregnancy, and placentation. Binelli *et al.* (2014) identified three biological principles of FTAI protocols that govern pregnancy success: 1) Regulation of circulating concentrations of progesterone to increase oocyte competence and efficacy of PGF-induced luteolysis prior to FTAI, 2) Adequate estradiol priming during proestrus, and 3) Adequate progesterone priming during the early luteal phase. In postpartum beef cows, GnRH-induced ovulation of small dominant follicles resulted in decreased circulating concentrations of estradiol at FTAI and decreased postovulatory concentrations of progesterone (Perry *et al.*, 2005; Busch *et al.*, 2008; Atkins *et al.*, 2010a, b, 2013). These concepts are discussed in more detail below.

Role of proestrus and preovulatory estradiol

Proestrus includes the period from luteolysis to the onset of estrus and is characterized by increased pulsatile secretion of LH, increased circulating concentrations of estradiol, estrogenic changes in the reproductive tract (e.g. cervix, uterus, and oviduct), and preovulatory follicular growth and maturation. Pregnancy rates following FTAI were positively associated with length of proestrus in beef (Mussard *et al.*, 2007; Bridges *et al.*, 2008, 2010; Geary *et al.*, 2013) and dairy (Santos *et al.*, 2010) cattle. Ovulation synchronization protocols that increase length of proestrus influence the follicular and uterine steroid environment by increasing serum concentrations of estradiol at estrus and progesterone during the subsequent luteal phase. Increased serum concentrations of estradiol at FTAI were associated with increased pregnancy rates (Jinks *et al.*, 2013). Therefore, the effects of increased proestrus on pregnancy rates were more likely an effect of increased estradiol rather than a function of follicular age (Bridges *et al.*, 2008).

Increased pregnancy rates associated with increased circulating estradiol at FTAI may be due to a direct effect of estradiol on the cumulus-oocyte complex, oviduct and uterine environment, and(or) an indirect effect on gamete transport. The bovine oocyte and surrounding cumulus cells contain estradiol receptor mRNA (Driancourt *et al.*, 1998; Beker-van Woudenberg *et al.*, 2004) and oocytes from preovulatory bovine follicles that had increased intrafollicular concentrations of estradiol were more likely to develop into blastocysts (Mermillod *et al.*, 1999). However, addition of estradiol to in vitro maturation media had either no effect or a negative effect on nuclear maturation of bovine oocytes (Beker-van Woudenburg *et al.*, 2004, 2006). Interestingly, treatment of beef cows with estradiol cypionate, during the preovulatory period, increased pregnancy rates in cows following GnRH-induced ovulation of small, but not large ovulatory follicles (Jinks *et al.*, 2013). Circulating concentrations of estradiol may affect the establishment and maintenance of pregnancy in a manner that is independent of oocyte competence. For example, increased follicular secretion of estradiol may increase pregnancy rates through modulating uterine pH (Perry and Perry, 2008a, b), by altering sperm transport and longevity (Allison and Robinson, 1972; Hawk, 1983), by inducing oviductal secretions (e.g. oviductal glycoprotein; reviewed by Buhi, 2002), by modulating progesterone action via induction of progesterone receptors in the uterus (Stone *et al.*, 1978; Zelinski *et al.*, 1982; Ing and Tornesi, 1997), and(or) by increasing luteal progesterone secretion. Madsen *et al.* (2015) demonstrated the necessity of preovulatory estradiol on embryo survival and placental attachment in beef cows using an ovariectomized cow model. In regards to the latter effect of estradiol, Atkins *et al.* (2013) reported that circulating concentration of estradiol at FTAI (day 0) was positively associated with serum concentrations of progesterone on day 7 and independent of ovulatory follicle size. The ability of luteinized human granulosa cells to secrete progesterone increased when the cells were collected from follicles having increased follicular fluid concentrations of estradiol (McNatty, 1979). In addition, ewes treated with an aromatase inhibitor prior to induced ovulation had a delayed rise in serum progesterone (Benoit *et al.*, 1992). Consequently, estradiol may have a role in preparing follicular cells to luteinize.

Role of postovulatory progesterone

The preovulatory gonadotropin surge induces luteinization and transformation of the ovulatory follicle into a corpus luteum, which serves as the primary source of progesterone during the establishment and maintenance of pregnancy in cattle (Smith *et al.*, 1994). Luteal development is a continuation of follicular maturation; consequently, an inadequate follicular microenvironment (e.g. decreased gonadotropin stimulation and[or] estradiol production) may impair subsequent luteal function (Garverick and Smith, 1986). In beef heifers and postpartum beef cows, GnRH-induced ovulation of small dominant follicles was associated with decreased postovulatory concentrations of progesterone (Perry *et al.*, 2005; Atkins *et al.*, 2008, 2010a, b) and decreased pregnancy rates in postpartum beef cows (Atkins *et al.*, 2013). Potential mechanisms by which decreased circulating concentrations of progesterone, during the early luteal phase, might result in decreased pregnancy rates are discussed below.

In ruminants, the early conceptus relies on progesterone-stimulated production of growth factors and uterine secretions collectively known as histotroph for nourishment (Geisert *et al.*, 1992; Spencer and Bazer, 2002). Ovarian steroids can have an indirect effect on uterine function through estradiol induction of uterine progesterone receptors (Zelinski *et al.*, 1982; Ing and Tornesi, 1997) and progesterone effects on histotroph production (Garrett *et al.*, 1988). Alternatively, progesterone may also have a direct effect since the bovine embryo possesses progesterone receptor mRNA (Clemente *et al.*, 2009) and may respond directly to progesterone supplementation in culture (inconsistencies reviewed by Lonergan, 2009).

Beginning on day 9 after GnRH-induced ovulation and FTAI, circulating concentrations of progesterone were greater in pregnant versus nonpregnant postpartum beef cows (Perry *et al.*, 2005). A delayed rise in circulating progesterone may compromise pregnancy establishment due to decreased embryonic size and production of interferon-tau (IFN-tau). Production of IFN-τ from the trophoblast on approximately days 14 to 20 is an essential signaling mechanism for maternal recognition of pregnancy and IFN-tau has been shown to reduce pulsatile uterine PGF secretion by blocking expression of endometrial oxytocin receptors (reviewed by Spencer *et al.*, 2007). A delayed rise in progesterone, following ovulation, was associated with lower rates of bovine embryonic development and reduced IFN-tau production by day 16 embryos (Mann and Lamming, 2001). In summary, an adequate increase in the postovulation concentration of progesterone is necessary for pregnancy establishment and maintenance in cattle.

Conclusion

Ovulation of a competent oocyte, as well as adequate preovulatory secretion of estradiol and postovulatory secretion of progesterone are essential for the establishment and maintenance of pregnancy. When ovulation was induced with GnRH in postpartum cows not detected in estrus, positive associations among ovulatory follicle size, circulating concentrations of preovulatory estradiol, fertilization rates, embryo quality, circulating concentrations of progesterone during the postovulatory period, and pregnancy rate have been reported (Atkins *et al.*, 2013). In the preceding study, preovulatory estradiol at GnRH-induced ovulation and postovulatory progesterone seven days later were the two most important factors affecting pregnancy establishment. Continued research on FTAI protocols in modern beef and dairy production systems should focus on strategies to increase preovulatory estradiol, postovulatory progesterone, and oocyte competence to increase pregnancy rates to a single insemination.

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