



## Control of ovarian function for assisted reproductive technologies in cattle

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### Abstract

Although the discovery that follicles in bovine ovaries grow in a wave-like fashion has presented challenges for estrus synchronization and superovulation, recent protocols, designed to control follicular function have permitted fixed-time AI or fixed-time embryo transfer with high pregnancy rates, and the initiation of superstimulatory treatments at a self-appointed time, all without the necessity of estrus detection. The key is the synchronization of follicle wave emergence. More recent studies have revealed that it is not only possible to synchronize the timing of ovulation for fixed-time AI in single ovulating animals, but also in superstimulated donors. Ultrasound-guided follicle ablation is very efficacious in synchronizing follicle wave emergence but is difficult to apply in the field. Similarly, the induction of ovulation with GnRH or LH will effectively synchronize follicle wave emergence, but ovulation occurs in only 60 to 70% of cases. The administration of estradiol benzoate in progestin-treated cattle effectively synchronizes follicle wave emergence for both superovulation and estrus synchronization, but estradiol benzoate is not available in many countries. The challenge now is to use knowledge of follicle wave dynamics to design alternatives.

**Keywords:** artificial insemination, cattle, embryo transfer, follicle wave emergence, synchronization.

### Introduction

Our expanding knowledge of ovarian function during the bovine estrous cycle has given us new approaches for the precise synchronization and control of ovulation. Recent protocols, designed to control both luteal and follicular function, permit fixed-time AI with high pregnancy rates, and the initiation of superstimulatory treatments at a self-appointed time, and provide opportunities to do fixed-time AI in donors and fixed-time embryo transfer in recipients. The intention of the following review is to discuss how these events impact the application of assisted reproductive technologies in cattle.

### The estrous cycle

Ovarian follicles in cattle grow in waves. A

follicular wave has been described as the synchronous emergence of a group of antral follicles 4 to 5 mm in diameter; one follicle is selected to become dominant while the remaining (subordinates) become atretic (Ginther *et al.*, 1989a; Adams, 1998). Estrous cycles in cattle are composed of primarily either two or three follicular waves (Ginther *et al.*, 1989b); however, 4-wave cycles have been observed occasionally in *Bos indicus* cattle (Rhodes *et al.*, 1995; Bó *et al.*, 2003). In Both 2- and 3-wave cycles, emergence of the first follicular wave has been shown to occur on the day of ovulation which has been designated as Day 0 (as apposed to the day of estrus when simple estrus detection is done). However, more recent studies suggest that follicular wave emergence may occur as much as 2 days earlier when follicles are 1 to 2 mm in diameter (Jaiswal *et al.*, 2004). Based on the emergence of follicles 4 to 5 mm in diameter, the second wave emerges on Days 9 or 10 in 2-wave cycles, and on Days 8 or 9 in 3-wave cycles, with the third wave emerging on Days 15 or 16. Duration of the estrous cycle (interovulatory interval) is approximately 20 and 23 days in 2- and 3-wave cycles, respectively. The dominant follicle present at the time of luteolysis becomes the ovulatory follicle (Kastelic and Ginther, 1991), and emergence of the next follicular wave is delayed until the ensuing ovulation. Follicular waves have also been reported in heifers before puberty (Evans *et al.*, 1994) and postpartum cows before the first ovulation (Savio *et al.*, 1990).

Recruitment of follicular waves and selection of the dominant follicle are based on differential responsiveness to FSH and LH (Adams *et al.*, 1992a, b, 1993; Ginther *et al.*, 1996). Surges in plasma FSH concentrations are followed in 1 to 2 days by emergence of a new follicular wave, while FSH is subsequently suppressed by products of the growing follicles (e.g. estradiol and inhibin). In each wave, the dominant follicle acquires LH receptors and continues to grow while subordinates (that continue to depend on FSH) undergo atresia (Ginther *et al.*, 2001). Suppression of LH, as a consequence of progesterone secretion by the corpus luteum (CL), causes the dominant follicle eventually to cease its metabolic functions and regress; this leads to a new FSH surge and emergence of a new follicular wave (Adams *et al.*, 1992b). Luteal regression allows LH pulse frequency to increase, and the dominant follicle present at that time increases its growth; elevated estradiol concentrations result in positive feedback on

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the hypothalamo-pituitary axis, an LH surge, and ovulation.

### **Synchronizing estrus for artificial insemination and embryo transfer**

#### *Prostaglandin F<sub>2α</sub> (PGF)*

Although PGF is the most commonly used treatment for synchronizing estrus in cattle (Seguin, 1987; Odde, 1990; Larson and Ball, 1992), it has some important limitations. Cattle must be cycling and in an appropriate stage of the estrous cycle for luteal regression to occur. When luteolysis is induced by PGF treatment, the onset of estrus is distributed over a 6-day period (Seguin, 1987); this variation is due to ovarian follicular status at the time of treatment (Kastelic *et al.*, 1990). In a two-dose PGF synchronization scheme, an interval of 10 or 11 days between doses has been used; theoretically, all cattle should have a PGF-responsive CL at the second treatment. However, a higher conception rate has been reported with a 14-day interval (Folman *et al.*, 1990) because a growing dominant follicle is more likely to be present at the time of the second PGF. Most other methods of estrus synchronization require the use of PGF in the protocol.

Acceptable pregnancy rates in embryo transfer are partially dependent upon the onset of estrus in the recipient being within 24 hours of synchrony with that of the embryo donor (Hasler *et al.*, 1987). Recipients can be selected by detection of natural estrus in untreated animals or following estrus synchronization. Regardless of the method of synchronization used, timing and critical attention to estrus detection are important. Recipients synchronized with PGF must be treated 12 to 24 hours before donors because PGF-induced estrus will occur in recipients in 60 to 72 hours and in superstimulated donors in 36 to 48 hours (Mapletoft, 2006). Pregnancy rates do not seem to differ in recipients with natural or PGF-induced estrus; in fact, pregnancy rates were higher in PGF-synchronized recipients in at least one study (Hasler *et al.*, 1987).

#### *Progesterone/progestins*

Progesterone alters ovarian function in cattle by suppressing estrus and preventing ovulation (Christian and Casida, 1948). Progesterone also suppresses LH release (Savio *et al.*, 1993), which in turn suppresses growth of the dominant follicle in a dose-dependent fashion (Adams *et al.*, 1992a). It is noteworthy that progesterone does not suppress FSH secretion (Adams *et al.*, 1992a); therefore, follicular waves continue to emerge in the presence of a functional CL. Although progestins given for intervals exceeding the lifespan of a CL (i.e., >14 days) result in synchronous estrus upon withdrawal, fertility at the ensuing estrus is low. The types and doses of progestins

used to control the estrous cycle in cattle are generally less efficacious than endogenous progesterone (from a CL) for suppressing LH; they result in high LH pulse frequency, development of "persistent" follicles (Savio *et al.*, 1993) which contain aged oocytes, and poor fertility (Revah and Butler, 1996).

Progesterone-releasing intravaginal devices are now commonly used to synchronize estrus in cattle (Mapletoft *et al.*, 2003). The vaginal insert is normally removed after 7 or 8 days and PGF is given at that time or 24 hours earlier and estrus detection begins 48 hours after progestin removal. Because of the short treatment period (7 or 8 days), the incidence of persistent follicles is reduced and fertility following AI is normal. Progestin devices are well suited for estrus synchronization of recipients, and for various approaches used to synchronize follicular development and ovulation.

As protocols designed for estrus synchronization depend on estrus detection for AI or embryo transfer, results are often disappointing. Estrus detection is time consuming, inaccurate and inefficient with estrus detection efficiencies of 40% or less in most modern dairy herds (Washburn *et al.*, 2002). Although acceptable conception rates are often reported, pregnancy rates are low as a consequence of poor estrus detection. Similarly, estrus detection efficiency has an adverse effect on the application of bovine embryo transfer. For example, when two injections of PGF are administered 11 to 14 days apart (reviewed in Bó *et al.*, 2004) to synchronize recipients, about 80% should show signs of estrus within 5 days of treatment, if all are cycling. However, due to the inefficiency of estrus detection, about 50% of the treated recipients will have a CL and receive an embryo 7 days after estrus (Bó *et al.*, 2002). This situation may be even worse if the recipients are *Bos indicus* or *Bos indicus* crosses under pasture conditions. In one study, an overall pregnancy rate of 13% was observed, due largely to the low number of recipients seen in estrus (863/1554, 55.5%) and/or with a CL at the time of embryo transfer (449/1554, 28.9%; Bó *et al.*, 2004). The alternative to increase efficiency and pregnancy rates is to eliminate the need for estrus detection by applying protocols for fixed-time AI or embryo transfer.

### **Manipulation of ovarian function for fixed-time AI or embryo transfer**

#### *Follicular ablation*

Treatments that eliminate the dominant follicle will result in emergence of a new follicular wave. In that regard, transvaginal ultrasound-guided ablation of the dominant follicle hastens the emergence of the next follicular wave by removing the suppressive effects of follicle products on FSH (Bergfelt *et al.*, 1994). Although follicular ablation in combination with PGF is



very efficacious in synchronizing estrus and ovulation, it is not practical for widespread use in the field.

### *Estradiol and progesterone*

Several years ago, Wiltbank *et al.* (1961) showed that estradiol causes uterine-induced luteolysis. Soon thereafter, estradiol was given at the start of a short-term (9 to 11 days) progestin treatment protocol to eliminate the need for PGF, and following progestin removal a synchronous, fertile estrus occurred (Wiltbank *et al.*, 1965). However, the effects of estradiol on ovarian follicular growth were elucidated more than 25 years later (Bó *et al.*, 1991). In a series of studies, it was shown that estradiol treatment suppressed antral follicle growth, and suppression was more profound when estradiol was given after insertion of a progestin insert (Bó *et al.*, 1993, 1994, 1995a, b). The mechanism of estrogen-induced suppression of follicular growth appears to be systemic, and involves suppression of FSH (Bó *et al.*, 1993). Once the estradiol was metabolized, there was an FSH surge, and a new follicular wave emerged. The administration of 5 or 2.5 mg estradiol-17 $\beta$  (E-17 $\beta$ ) (reviewed in Bó *et al.*, 2002) or 2 mg of estradiol benzoate (Caccia and Bó, 1998) or estradiol valerate (Colazo *et al.*, 2005a) in progestin-treated cattle at random stages of the cycle was followed by the emergence of a new follicular wave approximately 4 days later, with little variability.

In estrus synchronization protocols, estradiol is normally injected (with or without progesterone) at the time of insertion of a progestin device (Martinez *et al.*, 2000, 2005; Mapletoft *et al.*, 2003) which is removed 7 or 8 days later, at the time of administration of PGF. A lower dose of estradiol is normally given 24 hours after progestin removal to induce a synchronous LH surge (approximately 16 to 18 hours after treatment) and ovulation approximately 24 to 32 hours later (Mapletoft *et al.*, 2003; Martinez *et al.*, 2005, 2007). This has permitted fixed-time AI (FTAI) with very high pregnancy rates. Pregnancy rates following FTAI have been shown to be improved in suckled beef cows and suckled *Bos indicus* cows and heifers when 400 IU of eCG was administered at the time of progestin removal (reviewed in Baruselli *et al.*, 2004; Bó *et al.*, 2005). The beneficial effect of eCG treatment would seem to be through stimulation of dominant follicle growth and maturation, resulting in increased progesterone production by the subsequent CL (Baruselli *et al.*, 2004).

Compared to *Bos taurus* cattle, *Bos indicus* breeds have several differences in reproductive physiology. Follicular diameters at deviation and at the time that ovulatory capability (Sartori *et al.*, 2001) is acquired are smaller (Sartorelli *et al.*, 2005; Gimenes *et al.*, 2008), and *Bos indicus* breeds have a shorter duration of estrus, often expressed during the night (Bó *et al.*, 2003). Although *Bos indicus* cattle respond to

estradiol and progesterone with synchronous emergence of a new follicular wave (Sá Filho *et al.*, 2005, 2006), they tend to be more sensitive to steroid hormones than *Bos taurus* cattle. These differences must be considered when designing assisted reproductive programs for *Bos indicus* cattle.

Estradiol and progesterone treatments have been used increasingly for fixed-time embryo transfer (FTET; Bó *et al.*, 2002, 2005, 2007). In general, treatments are very similar to those used for FTAI, except the PGF treatment may be given earlier. Therefore, recipients receive a progestin device and an injection of 2 mg EB on Day 0, PGF on Day 5 (1 day after wave emergence), progestin devices are removed on Day 8 and 1 mg EB is given on Day 9 (Day 10 is considered the day of estrus) and embryos are transferred on Day 17 in all recipients with a CL. Treatment with PGF on Day 5 has resulted in a larger diameter of dominant follicle, higher progesterone concentrations at the time of FTET, a larger proportion of recipients selected for transfer and higher overall pregnancy rates.

The effects of 400 IU eCG on Day 5 of the FTET treatment protocol on pregnancy rates has also been investigated mainly in *Bos taurus* x *Bos indicus* crossbred recipients (Bó *et al.*, 2002, 2005, 2007; Nasser *et al.*, 2004). Although eCG treatment did not result in more recipients selected for transfer, it did result in increased CL diameters and improved conception rates. Furthermore, plasma progesterone concentrations in recipients treated with eCG were significantly higher than in those not treated with eCG, regardless of the number of CL at the time of embryo transfer (Bó *et al.*, 2004). An interesting observation in these studies was that pregnancy rates did not differ whether recipients were seen in estrus or not (Bó *et al.*, 2007).

### *Gonadotropin releasing hormone (GnRH)*

In cattle with a growing dominant follicle (at least 10 mm in diameter), treatment with GnRH induces ovulation (Macmillan and Thatcher, 1991), with emergence of a new follicular wave approximately 2 days after treatment (Twagiramungu *et al.*, 1995), but only when ovulation occurred (Martinez *et al.*, 1999). An ovulation synchronization scheme utilizing GnRH for fixed-time AI (Ovsynch) in lactating dairy cattle was developed by Pursley *et al.* (1995). The first injection of GnRH is followed 7 days later with an injection of PGF, followed in 48 hours by a second injection of GnRH; fixed-time AI is performed 0 to 24 (optimally 16 to 18) hours later. The Ovsynch protocol has been much more efficacious in lactating dairy cows than in heifers, and has been used successfully for several years (Seguin, 1997). Although the cause for the discrepancy between cows and heifers is not known, ovulation following the first injection of GnRH



occurred in 85% of cows but only 54% of heifers (Pursley *et al.*, 1995). In addition, 19% of heifers were in estrus before the injection of PGF, dramatically reducing fertility following FTAI (Wiltbank, 1997).

An alternative is to ensure that a viable growing dominant follicle is present at the time of GnRH treatment. Stage of development of the dominant follicle (Martinez *et al.*, 1999), or stage of the estrous cycle (Vasconcelos *et al.*, 1999) at the time that GnRH is administered has been shown to affect results. If GnRH is administered when the dominant follicle is immature or post-mature, ovulation may not occur and a new follicular wave will not emerge (Martinez *et al.*, 1999). It has been suggested that cattle will respond most consistently to GnRH administered between Days 5 and 12 of the estrous cycle; this can be accomplished by using a PGF presynchronization treatment, with the last PGF given 12 to 14 days before the first injection of GnRH (Moreira *et al.*, 2001). We have also increased the numbers of beef cattle responding to the first GnRH by presynchronization with a progestin device for periods ranging from 5 to 14 days (Colazo *et al.*, 2005b, 2006).

We have investigated the use of GnRH-based FTAI protocols in beef cattle; GnRH caused ovulation of the dominant follicle in only 56% of heifers and therefore it did not consistently induce the emergence of a new follicular wave (Martinez *et al.*, 1999). Lactating beef cows appeared to be more similar to heifers than lactating dairy cows, with seldom more than 60% ovulating following administration of GnRH at random stages of the estrous cycle (Colazo *et al.*, 2007). We also showed that circulating concentrations of progesterone affect LH release following the administration of GnRH in beef cattle (Colazo *et al.*, 2008). The presence of a progestin insert between the first injection of GnRH and the injection of PGF 7 days later overcame the problem of low pregnancy rates in beef heifers, essentially doubling pregnancy rates in two different studies (Martinez *et al.*, 2002). Although GnRH-based protocols have been used successfully in suckled beef cows, the addition of a progestin insert has also been beneficial, especially if cows are early postpartum or in low body condition. The addition of eCG at the time of progestin removal was also shown to improve pregnancy rates in first-calf, suckled beef cows (Colazo *et al.*, 2005b, 2006).

GnRH-based protocols have also been used to synchronize ovulation in recipients (reviewed in Bó *et al.*, 2005, 2007). In two studies involving *Bos indicus* x *Bos taurus* crossbred heifers (Baruselli *et al.*, 2000; Zanenga *et al.*, 2000), the overall pregnancy rate was higher in recipients treated with the GnRH-based protocol than those treated with PGF, because more recipients received embryos. The inclusion of a progestin device to a GnRH-based protocol has also been shown to result in higher pregnancy rates. In one study involving the transfer of frozen embryos, Beal

(1999) observed a conception rate in Heat-Watch-detected controls (62%) that was significantly higher than in nondetected recipients synchronized with an Ovsynch protocol (48%) or numerically higher than those synchronized with an Ovsynch protocol that included a progestin device between the first injection of GnRH and PGF treatment 7 days later (54%). However, more pregnancies were produced with the GnRH-based protocols because more recipients received embryos i.e., recipients were used regardless of whether they were seen in estrus. In a field trial involving 1637 recipients treated with the GnRH protocol plus a progestin device and embryo transfer without estrus detection, overall pregnancy rate was 59.9% (Beal and Hinshaw; personal communication). A recent experiment examined the effect of the addition of eCG to a GnRH-based treatment protocol in *Bos indicus* x *Bos taurus* recipients that received embryos at a fixed-time (Mayor *et al.*, 2008). Recipients received a progestin device and GnRH on Day 0, 400 IU eCG on Day 3 (1 day after expected time of follicle wave emergence), PGF at progestin removal on Day 7, a second GnRH on Day 9 and FTET on Day 16. The number of recipients selected/treated was higher than in the control group, which were treated similarly but without eCG on Day 3 (70.0%, 28/40 vs 47.5%, 19/40), and conception rates and pregnancy rates were higher (16/26, 61.5% and 16/40, 40%) than in the control group (9/19, 47% and 9/40, 22.5%).

In summary, results of these studies indicate that acceptable pregnancy rates can be achieved following FTAI or FTET in animals that have received treatments that synchronize follicle wave emergence and ovulation, without the necessity of estrus detection. Furthermore, the administration of eCG at the expected time of follicle wave emergence in recipients, or at the time of progestin removal in certain classes of animals to be inseminated, was shown to improve pregnancy rates.

#### **Manipulation of ovarian function for superstimulation**

The conventional protocol of initiating ovarian superstimulation during mid-cycle was originally based on anecdotal and experimental information which suggested a greater superovulatory response when gonadotropin treatments were initiated 8 to 12 days after estrus (Lindsell *et al.*, 1986; Bó *et al.*, 1995b; Mapletoft, 2002). However, these early studies did not utilize ultrasonography to evaluate ovarian status when superstimulation treatments were initiated. It is now known that 8 to 12 days after estrus (Days 7 to 11 after ovulation) would be the approximate time of emergence of the second follicular wave (Ginther *et al.*, 1989b). However, the day of emergence of the second follicular wave varies within wave type and is 1 or 2 days later in 2-wave cycles than in 3-wave cycles. In this regard, it



has been shown that superovulatory response was higher when gonadotropin treatments were initiated at the time of wave emergence; 1 day asynchrony reduced the response (Nasser *et al.*, 1993). However, the necessity of waiting until mid-cycle to initiate superstimulatory treatments implies monitoring estrus and an obligatory delay. An alternative approach is to initiate gonadotropin treatments subsequent to the synchronization of follicular wave emergence.

#### *Follicular ablation*

A simple approach to the synchronization of follicle wave emergence involves transvaginal ultrasound-guided follicular ablation of all follicles  $\geq 5$  mm, followed by FSH treatments 1 day later (Bergfelt *et al.*, 1994, 1997). However, the timing of estrus was more synchronous when a progestin implant was inserted for the period of superstimulation and two injections of PGF were administered on the day of progestin removal. Combined over two experiments, there was no difference in the superovulatory response between ablated and non-ablated groups (Bergfelt *et al.*, 1997). In another study, ablation of the two largest follicles at random stages of the cycle was as efficacious in synchronizing follicular wave emergence for superstimulation as ablating all follicles  $\geq 5$  mm (Baracaldo *et al.*, 2000). In addition, ablation of the dominant follicle during mid-diestrus (Bungartz and Niemann, 1994; Kim *et al.*, 2001), followed by gonadotropin treatments 2 days later resulted in a higher superovulatory response than when the dominant follicle was not ablated. In a retrospective study of lactating dairy cows (Shaw and Good, 2000), follicle ablation resulted in a significantly higher number of ova/embryos, but a comparable number of transferable embryos as superstimulation 7 to 13 days after estrus. Although highly efficacious, follicle ablation requires specialized skill and equipment and tends to be difficult to apply on a widespread basis in the field.

#### *Estradiol and progesterone*

The preferred approach for synchronization of follicular wave emergence prior to superstimulation in the field is an injection of 5 mg E-17 $\beta$  plus 100 mg progesterone at progestin device insertion, with gonadotropin treatments beginning 4 days later (Bó *et al.*, 1996). Experimental (Bó *et al.*, 1996) and commercial (Meyer *et al.*, 2000; Bó *et al.*, 2002) embryo transfer results have shown that the superovulatory response and embryo production following this treatment at unknown stages of the estrous cycle was comparable to that of donors superstimulated 8 to 12 days after observed estrus. By synchronizing follicle wave emergence, the full extent of the estrous cycle was available for superstimulation and the need to detect estrus or ovulation, and waiting 8

to 12 days to initiate gonadotropin treatments was eliminated. At the same time, numbers of transferable embryos were not compromised.

Traditionally, donor cows have been subjected to embryo collection at approximately 60-day intervals, however, the elective synchronization of wave emergence permits successful superstimulation every 25 to 35 days, without regard to expression of estrus (Mapletoft *et al.*, 2002). Once multiple CL regress and cows ovulate, normal follicular wave patterns are established and superstimulation can be rescheduled. Briefly, cows receive a progestin insert at random stages of the estrous cycle and an injection of estradiol plus progesterone; 4 days later gonadotropin treatments are initiated. Progestin inserts are removed 12 hours after administration of PGF and cows are inseminated 12 and 24 hours after estrus; 7 days later ova/embryos are collected and cows receive PGF (often repeated in 4 to 5 days). The protocol is repeated 10 to 15 days later without regard to the stage of the estrous cycle.

Unfortunately, E-17 $\beta$  is often not commercially available. Therefore, the use of estrogen esters (i.e., estradiol benzoate or valerate) has been investigated. Treatment with 2.5 mg estradiol benzoate (EB) and 50 mg progesterone at the time of progestin insertion, resulted in synchronous emergence of a new follicular wave in 3 to 4 days (Caccia and Bó, 1998), and superovulatory responses comparable to those initiated 4 days after treatment with 5 mg or 2.5 mg E-17 $\beta$  plus 50 mg progesterone (Caccia *et al.*, 2002) or those initiated 8 to 12 days after estrus (Bó *et al.*, 1996). On the other hand, 5 mg estradiol valerate (EV) and 3 mg norgestomet resulted in less synchronous follicular wave emergence and a lower superovulatory response; however, a dose of 1.0 or 2.0 mg EV resulted in follicular wave emergence in 3 to 4 days, with little variability (Colazo *et al.*, 2005a).

#### *Gonadotropin releasing hormone (GnRH)*

Attempts to synchronize follicular wave emergence for superstimulation with GnRH initially had limited success (Kohram *et al.*, 1998). The use of GnRH or pLH treatments to synchronize follicular wave emergence for superstimulation resulted in reduced numbers of embryos in three successive experiments (Deyo *et al.*, 2001). Recently, Wock *et al.* (2008) reported more promising results with the use of GnRH in dairy cattle. GnRH-treated animals received a CIDR on random days of the estrous cycle (Day 0), GnRH on Day 3 and superstimulation was initiated on Day 5. Results revealed no significant differences in the total number of ova/embryos ( $9.8 \pm 0.6$  vs  $9.7 \pm 0.6$ ), and grades 1 and 2 embryos ( $4.7 \pm 0.4$  vs  $4.5 \pm 0.4$ ) between GnRH- and estradiol-treated groups ( $n = 411$ ). Results from two commercial embryo transfer practitioners (Steel and Hinshaw; personal communication) have also indicated a similar number of transferable embryos in

GnRH- and estradiol-treated donors. Controlled and appropriately designed experiments need to be done to confirm these promising results.

#### *Superstimulation during the first follicle wave*

Another alternative is to initiate gonadotropin treatments at the time of emergence of the first follicular wave. The first follicular wave emerges consistently on the day of ovulation (or the day after the onset of estrus) in cattle (Ginther *et al.*, 1989a). Nasser *et al.* (1993) have shown that superstimulation can be initiated successfully at the time of emergence of the first follicular wave, and Adams *et al.* (1994) showed that the superovulatory response did not differ whether gonadotropin treatments were initiated at the time of the emergence of the first or second follicular wave.

To avoid the need to detect estrus or ovulation, Nasser *et al.* (2003) induced synchronous ovulation in *Bos indicus* donor cows with an 8-day, EB-progestin protocol and the administration of pLH 24 hours after progestin removal to induce ovulation approximately 24 hours later. Superstimulatory treatments were initiated at the expected time of emergence of the first follicular wave (i.e., ovulation); donors received or did not receive a new progestin device during superstimulation. There was no difference in the number of transferable embryos in progestin-treated cows whether FSH treatments were initiated at the time of emergence of the first follicular wave ( $8.0 \pm 1.8$ ) or 4 days after the injection of 2.5 mg EB and 50 mg progesterone (Control Group;  $6.6 \pm 2.0$ ), but both were greater than when treatments were initiated at the time of emergence of the first follicular wave without the use of a progestin device ( $0.2 \pm 0.2$ ;  $P < 0.05$ ).

It may also be possible to synchronize ovulation prior to superstimulation by inducing ovulation of a persistent follicle with GnRH or pLH. It has been shown previously that it is possible to induce a persistent follicle with a device for 7 to 10 days and PGF at the time of insertion to regress the CL (Colazo *et al.*, 2006). Administration of GnRH at the time of progestin removal resulted in ovulation and follicular wave emergence 1 to 2 days later. A similar approach has recently been applied in a series of experiments to a superstimulation treatment protocol (Carballo Guerrero *et al.*, 2008). Basically, a growing dominant follicle was induced by the strategic use of PGF and a progestin device, and GnRH or pLH was used to induce ovulation, at which point gonadotropin treatments were initiated. The most user-friendly and efficacious protocol consisted of insertion of a progestin device and the administration of PGF on random days of the cycle (Day 0). PGF is administered 5 days later, but progestin devices are not removed and in fact stay in place until the end of the gonadotropin treatment protocol. GnRH is given 36 hours after PGF (Day 6.5) and gonadotropin treatments are initiated 36 hours later (i.e., Day 8) with

twice daily decreasing doses of FSH over 4 d (Days 8 to 11). PGF is administered on Day 10, the progestin is removed on Day 11, and pLH or GnRH is administered on Day 12 with FTAI 12 and 24 later. Preliminary data suggest that protocols involving the first follicular wave after ovulation can be used successfully to superstimulate groups of donors at a self-appointed time without estrus detection and with no decrease in embryo production.

#### **Fixed-time AI in superstimulated donors**

The timing of estrus, the endogenous LH surge and ovulation are especially variable among superstimulated cattle (Callesen *et al.*, 1987; D'Occhio *et al.*, 1997), and a significant inverse relationship has been reported between ovulation rate and the interval from administration of PGF to the LH surge (Greve *et al.*, 1983). On average, donors have been observed to ovulate between 60 and 108 hours after the first PGF treatment, but donors with more than four CL ovulated significantly earlier ( $79.6 \pm 1.8$  hours) than those with less than four CL ( $90.2 \pm 3.7$  hours; Bó *et al.*, 2006). Variability in superovulatory response makes estrus detection critically important to ensure that AI is conducted at the most appropriate time to maximize fertilization rate and embryo quality.

In efforts to synchronize ovulation times in superstimulated cattle, the main strategy has been to postpone the LH surge in relation to PGF treatment, allowing more follicles to develop and acquire the capacity to ovulate. However, delaying the LH surge in superstimulated cattle with GnRH antagonists (Rieger *et al.*, 1990) or agonists (D'Occhio *et al.*, 1997) followed by induction of ovulation with pLH has had variable results. Other studies have involved postponing the removal of progestin inserts (Vos *et al.*, 1994; Barros and Nogueira, 2001) with the administration of GnRH at the time of progestin removal to induce ovulation.

Recent studies, mainly in Brazil, have been directed toward the development of a superstimulation protocol that allows for fixed-time AI in *Bos indicus* cattle. Gonadotropin treatments were initiated on Day 4 after the administration of the EB plus progestin treatment (Barros and Nogueira, 2001; Baruselli *et al.*, 2003), and PGF was given in the AM and PM of Day 6 with removal of progestin devices at varying times thereafter, but before induction of ovulation with pLH 48 hours after the first PGF (i.e., the AM of Day 8). All donors were inseminated 12 and 24 hours after pLH treatment.

Based on these results, a series of experiments were designed to evaluate the effect of the time of removal of a progestin insert and GnRH or pLH treatment on the distribution of ovulations and embryo production in superstimulated Angus and Brangus donors (Chesta *et al.*, 2006, 2007). Donors were treated with progestin devices and EB plus progesterone on



Day 0, FSH from Day 4 through Day 7 and PGF in the AM and PM of Day 6. Progestin devices were removed at various times from the PM of Day 6 to the PM of Day 7 and GnRH was administered at various times after removal of progestin devices and FTAI was done 12 and 24 hours later. Although there was no effect of treatments on the number of ovulations, delaying the removal of the progestin devices from the PM of Day 6 to the AM or PM of Day 7 had the largest effect in preventing early ovulations. A 24-hour interval from progestin removal to GnRH or pLH was preferable for Angus donors, whereas either a 12- or 24-hour interval was acceptable in Brangus donors. In a more recent study, cows had progestin inserts removed in the AM of Day 7 and pLH was administered in the AM of Day 8; the number of transferable embryos did not differ between those that were inseminated 12 and 24 hours after the onset of estrus (detected by Heat-Watch) and those that were fixed-time inseminated 12 and 24 hours after the administration of pLH (Larkin *et al.*, 2006). In high-producing Holstein cows in Brazil, delaying the time of GnRH or pLH treatment to the PM of Day 8 with FTAI 12 and 24 hours later was optimal, probably because of the additional time required for superstimulated follicles to acquire the capacity to ovulate (Bó *et al.*, 2006).

In *Bos indicus* cattle, the optimal protocol involves the administration of PGF on Day 6 with the removal of the progestin insert 36 hours later (PM of Day 7) and the administration of GnRH or pLH 12 hours later i.e., Day 8 AM, 48 hours after PGF, with FTAI 12 and 24 hours later (Baruselli *et al.*, 2006). In 136 superstimulations of Nelore cattle using this protocol, the number of ova/embryos and transferable embryos and pregnancy rates following nonsurgical transfer of fresh embryos were  $13.3 \pm 0.8$ ,  $9.4 \pm 0.6$  and 43.5% (528/1213), respectively (Baruselli *et al.*, 2006) which is comparable to other reports where donors were inseminated 12 and 24 hours after onset of behavioral estrus (Barros and Nogueira, 2001; Nogueira *et al.*, 2002; Nogueira and Barros, 2003). It was also possible to use a single FTAI 16 hours after pLH treatment without compromising ova/embryo production (Baruselli *et al.*, 2006).

In summary, exogenous control of follicle wave emergence has been shown to offer the advantage of initiating superstimulatory treatments at a time that is optimal for follicle recruitment, regardless of the stage of the estrous cycle. Treatments are practical, easy to follow and, more importantly, eliminate the need for detecting estrus or ovulation and waiting 8 to 12 days to initiate FSH treatments. Furthermore, the utilization of these protocols permit repeated superstimulations, with embryo collections at 25 to 35 day intervals. More recent studies have revealed that it is possible to synchronize the timing of ovulation, permitting FTAI in superstimulated donors. Although there are similarities in the protocols used in *Bos taurus* and *Bos indicus*

breeds, *Bos taurus* donors tend to require slightly longer for superstimulated follicles to acquire the capacity to ovulate.

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