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Innovative strategies for superovulation in cattle

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Abstract

Although superstimulatory protocols in cattle are usually initiated during mid-cycle, the elective control follicular wave emergence and ovulation have had a great impact on the application of on-farm embryo transfer. However, the most commonly used approach for the synchronization of follicular wave emergence involves the use of estradiol which cannot be used in many parts of the world. Therefore, the need for alternative treatments has driven recent research. An approach that has shown promise is to initiate FSH treatments at the time of the emergence of the first follicular wave following GnRH-induced ovulation. Alternatively, it has been shown that it may be possible to ignore follicular wave status, and by extending the treatment protocol induce smaller follicles to grow and reach maturity and superovulate. Finally, the short half-life of pituitary FSH necessitates twice daily treatments which are time-consuming, stressful and subject to error. Recent treatment protocols have permitted superstimulation with a single or alternatively, two FSH treatments, reducing the need for animal handling during FSH treatments.

Keywords: eCG, estradiol, follicle wave, FSH, GnRH.

Introduction

Although research efforts in recent years have resulted in little or no increase in the number of transferable embryos following superovulation, protocols that control emergence of the follicular wave (Bó *et al.*, 1995; 2002) and the timing of ovulation (Baruselli *et al.*, 2006; Bó *et al.*, 2006) have allowed the treatment of groups of donors, regardless of the stage of the estrous cycle and permitted fixed-time AI in donors, without the need to detect estrus. However, the most commonly used treatment for synchronization of follicular wave emergence for superovulation involves estradiol-17β or one of its esters, which cannot be used in many countries because of concerns about the effects of steroid hormones in the food chain (Lane *et al.*, 2008). The purpose of this paper is to review new developments in superovulation of beef cattle utilizing readily available pharmaceutical products.

Traditionally, gonadotropin treatments have been initiated during the mid-luteal phase, approximately 8 to 12 days after estrus (reviewed in Bó *et al.*, 1995, 2002; Mapletoft *et al.*, 2002), around the time of emergence of the second follicular wave (Ginther *et al.*, 1989). However, a greater superovulatory response occurred when treatments were initiated on the day of follicular wave emergence, as apposed to 1 day before, or 1 or 2 days after wave emergence (Nasser *et al.*, 1993). Therefore, conventional treatment protocols have two drawbacks: 1) the requirement to have trained personnel dedicated to the detection of estrus, and 2) the necessity to have all donors in estrus at the same time in order to initiate treatments in groups of animals. We have recently summarized current superovulation protocols for cattle (Bó *et al.*, 2010; Mapletoft and Bó, 2012).

Manipulation of the follicular wave for superstimulation

The ability to electively induce follicular wave emergence permits initiation of superstimulation without regard to the stage of the estrous cycle and eliminates the need for estrus detection or waiting 8 to 12 days to initiate gonadotropin treatments (Mapletoft *et al.*, 2009). In the 1990's, we reported on the use of progestins and estradiol to induce synchronous emergence of a new follicular wave (Bó *et al.*, 1995). This approach to superovulation in the cow has been reviewed extensively (Bó *et al.*, 2002; Mapletoft *et al.*, 2002; 2007). It has been used by practitioners around the world and has recently been incorporated into protocols that permit fixed-time AI of donors (Baruselli *et al.*, 2006; Bó *et al.*, 2006).

Estradiol and progesterone

The most common hormonal treatment to synchronize the emergence of a new follicular wave for superstimulation has involved the administration of 5 or 2.5 mg estradiol-17β or 2 mg estradiol benzoate, and 100 or 50 mg progesterone by intramuscular injection at the time of insertion of an intravaginal progestin device (reviewed in Bó *et al.*, 2002, 2006; Mapletoft *et al.*, 2002). The estradiol treatment causes a suppression of FSH release and follicle atresia. Once the estradiol is metabolized, FSH surges and a new follicular wave emerges, on average 4 days after treatment (Bó *et al.*, 1995). Superstimulatory FSH treatments are then

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initiated at that time, and continued for 4 days i.e., eight intramuscular injections. Prostaglandin (PGF) is normally administered with the fifth and sixth injections FSH and the progestin device is removed with the sixth injection. Estrus normally occurs approximately 48 h after the first PGF injection; inseminations are done 12 and 24 h after the onset of estrus.

Fixed-time AI of donors

Barros and Nogueira (2005) have developed what they refer to as the P-36 protocol in which the progestin device is left in place for up to 36 h after PGF administration (the reason the protocol is called P-36) and ovulation is induced by the administration of exogenous pLH (or GnRH) 12 h after withdrawal of the progestin device (i.e., 48 h after PGF administration). Since ovulation occurs between 24 and 36 h after pLH administration (Nogueira and Barros, 2003), fixed-time AI was scheduled 12 and 24 h after pLH, avoiding the necessity of estrus detection.

In a series of experiments in which the timings of ovulations were monitored ultrasonically, Bó *et al.* (2006) showed how synchronization of follicular wave emergence with estradiol could be incorporated into a protocol for fixed-time AI in donors without the need for estrus detection and without compromising results. Basically, FSH treatments were initiated on day 4 (day 0 was the time of estradiol and progestin treatment), PGF was administered on day 6 and progestin devices were removed in the morning of day 7 (preventing early ovulations and allowing late developing follicles to “catch-up”), followed by induction of ovulation with GnRH or pLH 24 h later. Fixed-time AI was done 12 and 24 h later. From a practical perspective, fixed-time AI of donors has been shown to be ideal for busy embryo transfer practitioners (Larkin *et al*., 2006).

Studies in high-producing dairy cattle in Brazil have indicated that it is preferable to allow an additional 12 h before removing the progestin device (i.e., day 7 PM) followed by GnRH 24 h later (i.e., day 8 PM) with fixed-time AI 12 and 24 h later (Bó *et al*., 2006). In *Bos indicus* breeds, it was found that it was preferable to remove the progestin device on day 7 PM, followed by GnRH 12 h later (i.e., day 8 AM; Baruselli *et al*., 2006).

Alternative approaches for follicle wave synchronization and superstimulation

Recently, the use of estradiol has been restricted in countries such as USA, New Zealand and the European Union. This restriction created the need to develop treatments that do not involve the use of estradiol esters (Mapletoft *et al*., 2009). However, the inaccessibility of effective synchronization tools leaves many embryo transfer practitioners with a serious dilemma.

Follicle ablation

An alternative to estradiol is to eliminate the suppressive effect of the dominant follicle by ultrasound-guided follicle ablation and initiate superstimulatory treatments 1 or 2 days later (Bungartz and Niemann, 1994; Bergfelt *et al*., 1997). Initial studies involved the ablation of all follicles ≥ 5 mm (Bergfelt *et al*., 1997), but we subsequently showed that it was only necessary to ablate the two largest follicles (Baracaldo *et al*., 2000) to ensure that the dominant follicle was removed. Superstimulatory treatments may then be initiated 1 to 2 days later, at the time of emergence of a new follicular wave. Although this treatment has been shown to be highly effective (reviewed in Bó *et al*., 2006), the disadvantage is that it requires ultrasound equipment and trained personnel which is only appropriate for embryo production centers, where all the donors are maintained at the same location; it is very difficult to apply in the field.

GnRH or pLH

GnRH has been reported to induce ovulation or luteinization of the largest follicle at the time of treatment (Macmillan and Thatcher, 1991), with emergence of a new follicular wave approximately 2 days later. However, it has also been shown that follicular wave emergence occurs only when treatment resulted in ovulation (Martinez *et al*., 1999), and ovulation rates after GnRH treatment at random stages of the estrous cycle have been reported to range from 44.3% (Colazo *et al*., 2009) to 85% (Pursley *et al*., 1995). Therefore, the interval from GnRH treatment to wave emergence may not be as consistent as is required for superstimulation. Indeed, Deyo *et al*. (2001) reported unsatisfactory embryo production following synchronization of follicular wave emergence for superstimulation with GnRH or pLH. However, recent results from commercial embryo transfer practitioners (Hinshaw, 2009; AABP; personal communication; Steel and Hasler, 2009) and a research report involving 411 dairy donor cows (Wock *et al*., 2008) have revealed much more promising results, with no difference in embryo production when compared to the use of an estradiol-based protocol. Basically, a progestin device is inserted at random stages of the estrous cycle and GnRH is administered 2 or 3 days later with superstimulation treatments beginning 1.5 to 2.5 days later. Although controlled and appropriately designed experimental studies must be designed to confirm these promising results, it is noteworthy that most of these protocols involve the insertion of a progestin device 2 or 3 days before GnRH is administered which may ensure the presence of an LH-responsive follicle.

Superstimulation at emergence of the first wave after GnRH-induced ovulation

The first follicular wave emerges consistently on the day of ovulation (the day after the onset of estrus) in cattle (Ginther *et al*., 1989). Nasser *et al*. (1993) showed that superstimulation can be initiated at the time of emergence of the first follicular wave, and first wave follicles have been shown to be as responsive to superstimulation as second wave follicles (Adams *et al*., 1994). However, a progestin device must accompany gonadotropin treatments initiated at the time of emergence of the first follicular wave to ensure high oocyte/embryo quality (Nasser *et al*., 2011).

An alternative approach for the synchronization of ovulation for superstimulation is to combine the use of GnRH and a progestin device as reported recently (reviewed by Bó *et al*., 2008; Carballo Guerrero *et al*., 2009). In this protocol, a persistent follicle was induced by administration of PGF at the time of insertion of a progestin device (Small *et al*., 2009); then 7 days later, GnRH very effectively induced ovulation of the persistent follicle. The most user-friendly and efficacious protocol consisted of insertion of a progestin device and the administration of PGF on random days of the estrous cycle (day 0). Progestin devices are not removed and in fact stayed in place until the end of the superstimulation treatment protocol. GnRH or pLH was given on day 6.5 and FSH treatments were initiated 36 h later (i.e., day 8) at the expected time of ovulation (and wave emergence). By adding a second GnRH injection 24 h after progestin device removal (at expected onset of estrus), it was possible to do fixed-time AI with this protocol. Collectively, data suggest that superstimulation protocols involving the first follicular wave after a GnRH-synchronized ovulation can be used at a self-appointed time without estrus detection in groups of donors and with no decrease in embryo production.

Subordinate follicle breakthrough

During a normal follicular wave, subordinate follicles regress because of decreasing concentrations of FSH, caused by the secretion of estradiol and inhibin by the cohort, and especially of the dominant follicle (Adams *et al*., 1992, 1993). Small follicles require FSH to continue their growth, and evidence suggests that follicles as small as 1 mm in diameter will commence growth under the influence of FSH (reviewed by Adams *et al*., 2008). We hypothesized that it would be possible for exogenous FSH to cause these follicles to grow to a diameter of 3 or 4 mm at which time the regular 4- or 5-day superstimulatory treatment protocol could be initiated. Assuming a growth rate of 1 to 2 mm per day, this should take 2 to 3 days. Thus, these follicles could be recruited by adding 2 to 3 days to the superstimulation treatment protocol. The presence of a dominant follicle may not have any effect on superovulatory response under these circumstances because the exogenous FSH replaces that being depressed by the estradiol and inhibin. Indeed, Bó *et al*. (2008) successfully superstimulated donors at random stages of the estrous cycle, without regard to the presence of a dominant follicle, using this approach.

Alternatively, the 2 days of FSH pretreatment might be replaced with an injection on 500 IU of equine chorionic gonadotropin (eCG) 2 days before initiating FSH treatments. Indeed, Bó *et al*. (2008) have shown that pretreatment of poor responding donors with 400 IU of eCG 2 days before follicular wave emergence followed by FSH treatments 2 days later (Caccia *et al*., 2000) resulted in an improved superovulatory response over that achieved previously without the use of eCG. Although not studied critically, it was hypothesized that the eCG recruited additional follicles into the wave.

More recently, we investigated the effect of lengthening the superstimulatory treatment protocol from the traditional 4 days to 7 days in order to recruit more follicles into the wave (García Guerra *et al*., 2012). Lengthening the FSH treatment protocol to 7 days, without increasing the total amount of FSH administered, increased the percentage of follicles that ovulated, the number of ovulations and the synchrony of ovulations, and tended to increase the mean numbers of total ova/embryos, fertilized ova, and transferable embryos. In other words, the lengthened superstimulatory treatment protocol resulted in more follicles reaching an ovulatory size and acquiring the capacity to ovulate with an increased number of ovulations, and with no decrease in oocyte/embryo quality. It was concluded that prolonged FSH treatment protocols may be an effective strategy to recruit small follicles into the follicular cohort available for superstimulation, while providing the additional time needed for these follicles to reach an ovulatory size and acquire the capacity to ovulate. In addition, these results suggest that traditional 4-day superstimulatory treatment protocols may not provide adequate time for all follicles within the cohort to acquire the capacity to ovulate. This requires further study.

Reducing the number of FSH treatments in a superstimulation protocol

Because the half-life of pituitary FSH has been shown to be 5 h in the cow (Laster, 1972), traditional superstimulatory treatment protocols consist of twice daily injections of pituitary FSH over 4 or 5 days (Mapletoft and Bó, 2012). This requires frequent attention by farm-personnel and increases the possibility of failures due to non-compliance. In addition, twice daily treatments may cause undue stress in donor cows with a subsequent decreased superovulatory response, and/or altered preovulatory LH surge (Stoebel and Moberg, 1982). Thus, simplified protocols may be expected to reduce donor handling and improve response, particularly in less tractable animals.

More than 15 years ago, we reported that a single subcutaneous administration of FSH in beef cows in high body condition (>3 out of 5) resulted in a superovulatory response equivalent to the traditional twice daily treatment protocol over 4 days (Bó *et al.*, 1994). However, the results were not repeatable in Holstein cows, which had less adipose tissue (Hockley *et al.*, 1992). In a subsequent study in Holstein cows, the single injection was split into two, with 75% of the FSH dose administered subcutaneously on the first day of treatment and the remaining 25% administered 48 h later, when PGF is normally administered (Lovie *et al.*, 1994). Although superovulatory response was improved, it was still numerically less than the twice daily injection protocol.

An alternative to induce a consistent superovulatory response with a single injection of FSH would be to combine the pituitary extract with agents that cause the hormone to be released slowly over several days. These agents are commonly referred to as polymers, are biodegradable and non-reactive in the tissue, facilitating use in animals (Sutherland, 1991). We have recently completed a series of experiments in which FSH diluted in a 2% hyaluronan solution was administered as a single intramuscular injection, to avoid the effects of body condition. Overall, the single injection protocol resulted in a similar number of ova/embryos as the traditional twice-daily FSH protocol (Tríbulo *et al.*, 2011). However, 2% hyluronan was viscous and difficult to mix with FSH, especially in the field. We speculated that although more dilute preparations of hyaluronan were less efficacious as a single injection, their use could be improved by splitting them into two injections 48 h apart, as we had shown previously with subcutaneous injections of FSH. The split intramuscular treatment protocol consisted of diluting the FSH lyophilized powder with 10 ml of the reduced concentration hyaluronan solution and the administration of two-thirds of the total dosage of FSH on the first day, followed by a second injection with the remaining one-third of the total dosage of FSH 48 h later, when PGF is normally administered (Tríbulo *et al.*, 2012). Overall, the numbers of transferable embryos did not differ among treatment groups (Control: 4.0 ± 0.8; 1% hylauronan: 5.0 ± 0.9; 0.5% hyaluronan: 6.1 ± 1.3). Data were interpreted to suggest that splitting the FSH dose in either reduced concentration of hyaluronan into two intramuscular injections 48 h apart would result in a superovulatory response comparable to the traditional twice-daily intramuscular injection protocol in beef cattle. Furthermore, the less concentrated solutions of hyaluronan were not difficult to mix with FSH, even under field conditions.

Barros *et al.* (2008) conducted an experiment in which Nelore cows were superstimulated with Folltropin-V over 3 days; the two FSH injections on day 4 were replaced by two injections of 200 IU of eCG. Donors in the control group were superstimulated with the conventional treatment of eight twice-daily decreasing doses of FSH over 4 days. Treatment with eCG significantly increased the number of ova/embryos and numerically increased the number of transferable embryos. Reano *et al.* (2009) examined the use of this protocol in Brangus cows and heifers and found that it resulted in an increased number of transferable embryos. It is tempting to speculate that a single injection of eCG might prove to be useful in a split-dose superstimulation scheme. Obviously, more research is required.

Summary and conclusions

The use of protocols that control follicular development and ovulation has the advantage of being able to apply assisted reproductive technologies without the need for detecting estrus. These treatments have been shown to be practical and easy to perform by the farm staff. Estradiol is very efficacious in synchronizing follicle wave emergence for superovulation schemes, but is not available in many countries. Although the administration of GnRH to synchronize follicular wave emergence yields variable results, presynchronization with a progestin-releasing device has been shown to improve the response to GnRH allowing for superstimulation during the first follicular wave after ovulation, with results that did not differ from the use of estradiol. Lengthened superstimulation treatment protocols would appear to result in the recruitment of additional follicles into the wave and allow for the time needed for these follicles to acquire the capacity to ovulate. On the other hand, 4-day treatment protocols may not provide sufficient time for all superstimulated follicles to acquire the capacity to ovulate. Finally, the use of a hyaluronan-based, slow-release formulation has shown that it is possible to induce a consistent superovulatory response in beef cattle following two intramuscular injections of FSH, without adversely affecting the number of transferable embryos.

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