Control of ovarian function for in vivo and in vitro embryo production

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Abstract

Knowledge of follicular wave dynamics through use of real-time ultrasonography and development of the means by which follicular wave emergence can be controlled have provided new practical approaches to superstimulation of donors for in vivo and in vitro embryo production. Although some embryo transfer practitioners still initiate superstimulatory treatments during midcycle, elective control of follicular wave emergence and ovulation have greatly impacted on-farm embryo transfer, especially when large groups of donors need to be superstimulated concurrently. A combination of estradiol and progestins has been the most common treatment for synchronization of follicular wave emergence for many years. However, in countries where estradiol cannot be used, practitioners have turned to alternative treatments, e.g. mechanical follicle ablation or gonadotropin releasing hormone administration, for synchronization of follicle wave emergence for superstimulation. In vitro embryo production also depends on synchronization of follicle wave emergence. As *Bos indicus* cattle have high antral follicle populations, large numbers of oocyes can be obtained by ovum pick up without superstimulation. However, in *Bos taurus* donors, synchronization of follicular wave emergence and superstimulation with follicle stimulating hormone is necessary to obtain high numbers of oocytes by ovum pick up and blastocysts following in vitro fertilization. Treatments for synchronization of follicular wave emergence and superstimulation are practical, easy to administer by farm personnel and facilitate application of both in vivo and in vitro embryo technologies in cattle.

Introduction

Objective of ovarian superstimulatory treatments in cattle is to obtain tmaximum number of viable embryos by stimulating growth of antral follicles and ovulation of competent oocytes.[1] Usual regimen for in vivo embryo production has been twice-daily intramuscular (IM) treatments with FSH for 4 or 5 days. Forty eight or 72 hours after initiation of treatment, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is administered to induce luteolysis. Estrus occurs in 36 - 48 hours, with ovulations beginning 24 - 36 hours later.[2,3] Many practitioners prefer decreasing follicle stimulating hormone (FSH) dose schedules, whereas others use constant-dose schedules.[1] Others treat with $PGF_{2\alpha}$ twice on the third day of the treatment protocol, whereas others prefer to treat with $PGF_{2\alpha}$ twice on the fourth day. Furthermore, many do not treat with FSH on the day after administration of $PGF_{2\alpha}$.[1]Although there would appear to be no experimental data supporting 1 approach over others, recent experiments have indicated that follicle maturation and ovulation rate can be improved in at least some donors if FSH treatments are administered over 6 or 7 days.[3,4] Objective of this manuscript is to briefly summarize existing protocols for superstimulating donors for in vivo and in vitro embryo production and to present information on evolution of this technology in South America.

Manipulation of ovarian function for superstimulation

Two very important factors influencing variability in superstimulatory response are intrinsic number of antral follicles in donors and stage of follicular development at initiation of FSH treatments. Response can be predicted by antral follicle counts done with ultrasonography,[5,6] or measurement of circulating concentrations of anti Müllerian hormone (AMH; *Bos Taurus,*[7,8] *Bos indicus*[9]). High antral follicle counts have resulted in more ovulations and more transferable embryos than low antral follicle counts.[10] Similarly, top quartile of circulating AMH values was associated with a greater superovulatory

response than the lowest quartile.[11] Therefore, selection of donors based on antral follicle counts or AMH concentrations may be important for predictable embryo production.

Conventional protocol of initiating ovarian superstimulation during midcycle was based on anecdotal and experimental evidence suggesting a greater superovulatory response when gonadotropin treatments were initiated between days 8 and 12 of the cycle.[1] It is now known that midcycle is the approximate time of emergence of the second follicular wave.[12] However, day of second wave emergence varies between wave types (1 or 2 days later in 2 versus 3 wave cycles). In this regard, Nasser et al.[13] reported that superovulatory response was greater when gonadotropin treatments were initiated at the time of follicle wave emergence; 1 day asynchrony resulted in a reduced ovarian response. The necessity of waiting until midcycle to initiate FSH treatment implies monitoring estrus, the obligatory delay and an inability to group donors. An alternative is to superstimulate donors following synchronization of follicular wave emergence.[14]

Follicle ablation

Transvaginal ultrasound guided follicle ablation followed by FSH treatments 1 or 2 days later is very efficacious,[15,16] but requires specialized skill and equipment and is difficult to apply in the field. However, follicle aspiration for OPU/IVF will synchronize wave emergence and thus, embryos could be produced both in vitro and in vivo in succession, from the same donor.[17]

Estradiol and progesterone

The preferred approach for synchronization of follicular wave emergence prior to superstimulation is administration of 5 mg estradiol 17β plus 50 or 100 mg progesterone and insertion of a progestin device 4 days before initiating FSH treatments. Experimental[18] and commercial[14] results have shown that embryo production following this treatment at unknown stages of the estrous cycle is comparable to that initiated 8 - 12 days after observed estrus. Although estradiol 17β and its esters are available in most countries in South America, this is not the case in many other countries around the world, necessitating the use of alternatives to synchronize follicle wave emergence prior to superstimulation.

Gonadotropin releasing hormone

Attempts to synchronize follicular wave emergence for superstimulation with gonadotropin releasing hormone (GnRH) were initially unsuccessful; however, subsequent field data were more promising. In these cases, GnRH was administered 1.5 - 3.0 days after insertion of a progestin device which may have increased probability of a luteinizing hormone (LH)-responsive follicle. Indeed, Bó et al.[19] reported on the strategic use of $PGF_{2\alpha}$, a progestin device and GnRH to induce ovulation prior to initiating FSH treatments. A persistent follicle was induced by giving $PGF_{2\alpha}$ progestin device insertion; following administration of GnRH 7 days later, ovulation occurred in > 95% of cattle. Superstimulation initiated 36 hours after GnRH (with the progestin device remaining in place) resulted in a superovulatory response that did not differ from controls. More recently, Hinshaw et al.[20] reported no difference in superovulatory response whether GnRH was administered 2 or 7 days after insertion of a progestin device.

Extended superstimulatory treatment protocols

An early study provided rationale for a hypothesis that superstimulatory treatment may recruit follicles into the wave and allow small follicles to attain medium or large diameters.[21] Based on this notion, attempts have been made to increase the superovulatory response by adding equine chorionic gonadotropin (eCG) treatment prior to initiating FSH treatments. Pretreatment with eCG 2 days before the conventional FSH treatment protocol resulted in a numerically greater number of transferable embryos (6.7 ± 1.2 versus 4.9 ± 0.9) in an unselected group of donors[22] and a significantly greater number of transferable embryos in donors defined as poor responders (3.6 ± 0.6 versus 1.0 ± 0.2).[3]

More recently, we evaluated the superovulatory response and embryo recovery in donors treated with either 4 or 7 day FSH superstimulatory treatment protocols.[4] Twenty four beef cows were blocked by numbers of follicles ≤ 5 mm at wave emergence and placed into either a 4 or 7 day FSH treatment protocol utilizing the same total dose of 400 mg FSH (Folltropin-V; Vetoquinol Inc., Canada) administered twice-daily at a constant daily dosage. Mean number of ovulations detected by ultrasonography was greater in the 7 day treatment group (30.9 ± 3.9 versus 18.3 ± 2.9, $p = 0.01$), consistent with a numerically greater number of follicles ≥ 10 mm just prior to ovulation (27.5 ± 4.1 versus 19.5 ± 2.6; $p = 0.11$). Moreover, ovulations occurred more synchronously in the 7 day group (93% of ovulations occurred 12 - 36 hours post LH, compared to 66% in the 4 day group) suggesting that superstimulated follicles were more mature and capable of responding to an LH stimulus. Although total number of ova and embryos, fertilized ova and transferable embryos did not differ statistically, all end points favored the 7-day group. In addition, when data from cows with fertilization failure were removed, number of transferable embryos tended to be higher in the 7 day group (7.6 ± 1.7 versus 4.2 ± 1.5; $p = 0.07$). In another study,[23] a 7 day superstimulation protocol was used to investigate influence of progesterone on follicle growth, ovulation and oocyte competence. Beef cows were superstimulated with 25 mg of FSH twice daily for 4 or 7 days. Again, the superstimultory response (number of large follicles just prior to insemination) was greater ($p < 0.05$) in the 7 day group and numbers of ovulations (15.4 versus 11.6) and embryos (6.7 versus 5.9) were numerically higher in the 7 day group. Duration of treatment appears to be responsible for increases in superstimulatory response, rather than FSH dose. In the 2 studies noted above, number of ovulatory-sized follicles just prior to ovulation was greater following 7 days of superstimulation than 4 days, whether total dose of FSH was greater[23] or the same.[4] In addition, there was no evidence that more follicles were recruited, as total numbers of follicles at the end of FSH treatment was the same as that at the beginning of FSH treatment.

Microarray analysis of large antral follicles

As overcoming follicle selection is a key factor for ovarian superstimulation, exogenous FSH is used to prevent regression of subordinate follicles so that they assume qualities of a dominant follicle. In a recent study of follicles undergoing a 4 day superstimulation protocol, gene expression in granulosa cells was altered compared to a single, naturally occurring dominant follicle.[24,25] Expression of growth-related genes similar to the preLH stage of follicle growth (even though LH had been administered) and those involved in oxidative stress response were upregulated in granulosa cells of follicles undergoing a 4 day FSH superstimulation protocol compared to a preovulatory follicle of an unstimulated follicular wave. Genes related to a disturbance in angiogenesis were also upregulated in superstimulated follicles. We speculate that gene expression during a 7 day superstimulation protocol may be more similar to the naturally occurring single preovulatory follicle.

Acquisition of LH receptors is commonly used as a marker for dominance and a prerequisite for establishment of ovulatory capacity.[26,27] Recently, evaluation of the expression of LHR mRNA in granulosa cells of superstimulated and unstimulated follicles 12 hours after progesterone device removal revealed that expression of LHR was decreased following superstimulation.[28] Consequently, follicles following the conventional 4 day FSH superstimulation protocol seemed to have been delayed in maturational development and response to LH compared to a naturally occurring single preovulatory follicle.[24,25] These findings further support the concept that following a 4 day superstimulation treatment protocol, some follicles do not have sufficient time to mature, upregulate expression of appropriate genes, and acquire capacity to ovulate.

Use of equine chorionic gonadotropin to replace the last 4 follicle stimulating hormone applications

In search of possible improvements to the superstimulatory treatment protocol, Price et al.[29] reported that during superstimulatory treatment, pulses of LH diminish shortly after the first application of FSH and are accentuated during the last applications and the preovulatory period. This occurs due to an increase in estradiol concentrations as a consequence of high steroidogenic activity of superstimulated

cows.[29] However, LH is essential for final growth of superstimulated follicles and for complete maturation of oocytes.[30]

Equine chorionic gonadotropin (eCG) is a complex glycoprotein with FSH and LH activity.[31] In cattle, this gonadotropin has prolonged duration of action, due its sialic acid content (10 - 15%).[32] A remarkable feature of eCG that has been exploited in multiple experimental and commercial contexts is its ability to express FSH activity in nonequid species.[33]

In the early days of bovine embryo transfer, eCG was used to induce superovulation in donor cows.[1] However, its long half-life, which was a feature for induction of superovulation with a single administration, resulted in multiple anovulatory follicles at the time of embryo collection and poor embryo quality (reviewed[1,33]). More recently, the last 2 doses of FSH in a superstimulation protocol have been replaced by different doses of eCG, with the intention of providing more LH support to the growing follicles. Some studies reported beneficial effects of the association of FSH and eCG,[34,35,36,37] whereas other studies did not detect benefits.[38,39]

Although administration of eCG near the end of the FSH treatment protocol did not always improve the superovulatory response, it was certainly not detrimental and raised some interest in using eCG to simplify the superstimulation protocol and decrease costs, as eCG is usually less expensive than pituitary extracts containing FSH. Therefore, we designed a study to evaluate the superovulatory response and embryo production in beef donors using 8 twice-daily injections of FSH or an alternative protocol in which the last 4 FSH treatments were replaced with a single injection of eCG.[40] Twelve (Experiment 1) and 18 (Experiment 2) mature Bonsmara donor cows were superstimulated twice at a 46 day interval in a crossover design. Follicular wave emergence was synchronized by administration of estradiol17β at insertion of a progestin device, with superstimulation initiated 4 d later. Donors in the control group received 8 applications of Folltropin-V IM (total dose: 300 mg) in a twice-daily decreasing dosage schedule over 4 days, whereas donors in the FSH + eCG group received only the first 4 applications of Folltropin-V (total dose: 220 mg) and 48 h after initiating treatment, 800 IU of eCG IM in a single dose. All donors received PGF$_{2\alpha}$ IM in the AM and PM of the same day and intravaginal devices were removed in the AM of the next day. All cows also received GnRH 24 hours later and were inseminated with frozen and thawed semen from 2 bulls, 12 and 24 hours later. Ova and embryos were collected and evaluated according to the IETS standards 7 days after administration of GnRH. In Experiment 2, donors were treated only with FSH + eCG. The total dosage of Folltropin-V was 200 mg and eCG dosage was either 800 or 600 IU. Results of both experiments are presented in Table 1. In Experiment 1, the FSH (control) group produced more ($p < 0.01$) fertilized oocytes, but there were no differences in number of transferable embryos. In Experiment 2, there were no differences between the FSH + 800 eCG and FSH + 600 eCG groups in any parameter evaluated. In conclusion, replacement of the last 4 injections of FSH by a single administration of either 600 IU or 800 IU of eCG decreased the number of treatments required in a superovulation program without negatively affecting production of transferable embryos.

Table 1. Mean ± SEM embryo production in Bonsmara donors treated with FSH or FSH + eCG.[*]

	n	Total ova/embryos	Fertilized ova	Transferable embryos
Experiment 1				
FSH	12	11.7 ± 2.5	10.5 ± 2.3[a]	5.7 ± 1.4
FSH + 800 IU eCG	12	9.6 ± 1.5	6.8 ± 1.0[b]	5.3 ± 1.0
Experiment 2				
FSH + 800 IU eCG	18	6.7 ± 0.7	5.4 ± 0.8	3.6 ± 0.7
FSH + 600 IU eCG	18	6.1 ± 1.1	4.3 ± 1.0	3.7 ± 0.8

[ab]Means without a common superscript are different ($p < 0.05$).
[*]Donors were given 8 intramuscular injections of FSH at 12-hour intervals (FSH group) or the last 4 FSH treatments were replaced by a single intramuscular injection of eCG (FSH + eCG group).

Manipulation of follicular development for in vitro embryo production

In vitro production of embryos, together with obtaining oocytes by ultrasound guided follicular aspiration, known worldwide as ovum pick up (OPU), are reproductive biotechnologies that have advanced greatly in the last 10 years. This technology is highly developed in Brazil, where 57% of in vitro embryos that are transferred in the world are produced.[41] As indicated earlier, *Bos indicus* cattle have more follicles recruited per wave as compared to *Bos taurus* breeds, resulted in recovery of more oocytes after OPU.[42,43,44] Likewise, in field data from our laboratory, Brahman-influenced synthetic breeds produce significantly more viable oocytes and transferable blastocysts than *Bos taurus* breeds (Table 2).[45]

Table 2. Mean ± SEM effects of donor breed on number of viable oocytes aspirated by OPU and embryos produced in vitro without superstimulation.

Breed	n	Viable oocytes	Blastocysts
Dairy *Bos taurus* (Holstein)	620	8.0 ± 0.2^a	1.6 ± 0.1^a
Beef *Bos taurus* (Angus, Hereford, Bonsmara)	229	11.6 ± 0.5^b	3.0 ± 0.2^b
Bos indicus (Brahman)	52	15.8 ± 1.4^c	6.8 ± 0.9^c
Bos indicus x *Bos taurus* (Brangus, Braford)	1045	19.3 ± 0.4^d	5.3 ± 0.2^c

^{ab}Within a column, means without a common superscript are different ($p < 0.01$).

Studies were conducted to evaluate effects of synchronizing follicular wave emergence and superstimulation on number and quality of cumulus-oocyte complexes (COCs) recovered by OPU and in vitro embryo production.[46,47] The most important conclusions of these studies were: 1) Synchronizing follicle wave emergence prior to OPU increased numbers of COCs obtained and blastocysts produced in *Bos taurus,* but not *Bos indicus* cattle; 2) Treatment with estradiol and progesterone and dominant follicle removal (DFR) were equally effective in synchronization of follicular wave emergence for OPU; and 3) Superstimulatory treatment with FSH increased number and quality of oocytes obtained by OPU in *Bos taurus* breeds, but not in *Bos indicus* donors. In an experiment conducted in Brazil with Holstein donors,[48] all cows received a progestin device and 2 mg of estradiol benzoate (Day 0). Cows in the control group received no additional treatments, whereas cows in the FSH group received a total dose of 200 mg of Folltropin-V administered in twice-daily treatments on days 4 and 5. The progestin device was removed and OPU was performed on day 7 (40 hours after last injection of FSH in the treatment group). There were no differences between groups ($p = 0.92$) in numbers of follicles aspirated per OPU session (17.2 ± 1.3 versus 17.1 ± 1.1 in the control and FSH treated cows, respectively); however, FSH-treated cows had a higher blastocyst rate (34.5%, 89/258 versus 19.8%, 55/278, $p < 0.001$) and more transferable embryos per OPU session than those of the control group (3.0 ± 0.5 versus 1.8 ± 0.4, $p = 0.02$). It was concluded that superstimulation of Holstein donors with FSH before OPU increased efficiency of in vitro embryo production by increasing oocyte and embryo quality. In addition, nonlactating donors had a higher percentage of in vitro blastocyst development and produced more embryos per OPU session than lactating cows. In a later study, similar results were obtained when the 4 doses of FSH were replaced by a single injection of 200 mg of Folltropin-V diluted in a 0.5% hyaluronic acid solution (MAP-5, Vetoquinol).[49]

Two other studies were performed in Angus donors. In the first study,[50] administration of FSH resulted in more COCs obtained by OPU. In the second study (Ongarato et al. unpublished), multiparous, nonlactating Angus cows, were randomly allocated to 2 treatment groups and treated twice in a crossover design. Follicular wave emergence was synchronized with estradiol 17β and progesterone, plus a progestin device. Four days later (day 4) donor cows received either 160 mg Folltropin-V diluted in 4 ml of 0.5% hyaluronan by a single IM injection or received no FSH (Control group). COCs were obtained by

OPU 72 hours later (day 7). Results are summarized in Table 3. Number of viable COCs was significantly higher in FSH-treated donors than in controls.

Although administration of FSH prior to OPU has been a common practice for increasing the available follicular population for OPU, most studies have adopted conventional twice daily treatments with FSH, with either positive results in number of oocytes collected and embryos produced[58,49,50,51] or no significant differences in oocyte collection and embryo production in Holstein cows.[52] We understand that in North America, most practitioners superstimulate donors with FSH prior to OPU.

Table 3. Mean ± SEM numbers of total and viable cumulus oocyte complexes (COCs) recovered and number of blastocysts produced following superstimulation of Angus donors.

Group	COCs		Blastocysts
	Total	Viable	Total
Single FSH (n = 9)	21.4 ± 2.4[a]	14.1 ± 1.6[a]	4.2 ± 0.8
Control (n = 9)	15.9 ± 2.7[b]	10.6 ± 2.0[b]	2.7 ± 0.7
p value	0.02	0.02	0.13

[ab]Within a column, means without a common superscript are different (p < 0.05)

Other studies compared effectiveness of multiple vs a single administration of FSH prior to OPU. Chaubal et al.[53] compared administration of 200 mg of FSH diluted in saline, in multiple injections, a single IM injection or 2 subcutaneous injections (120 mg and 80 mg respectively) in Angus cows and reported lower oocyte numbers when FSH was administrated in a single IM injection. Ooe et al.[54] reported that 20 mg FSH in 30% polyvinylpyrrolidone as a single IM treatment prior to OPU in cyclic and pregnant cows resulted in satisfactory follicular responses and oocyte recovery rates. In another study, 100 mg of FSH diluted in saline and administered as a single IM injection in Holstein cows, resulted in a significant increase in number of follicles aspirated, but number of oocytes recovered per animal per session and blastocyst production were unaffected compared to nonFSH treated donors.[55] Finally, Sakaguchi et al.[56] described a different and innovative approach, using a single epidural administration of FSH via caudal vertebrae in Japanese black cows, reporting a higher superstimulatory response and embryo production compared to cows in the control group that received multiple IM injections of FSH for 3 days. Obviously, these results need further investigation.

Application of in vivo and in vitro embryo production in South America
The commercial embryo transfer industry began in North America in the early 1970s and the technology soon spread to South America.[1] Brazil and Argentina have consistently ranked in the top 5 countries outside North America and Europe in production of in vivo-derived (IVD) embryos. Viana[41] reported recently that more than 992,289 IVD and 495,054 in vitro produced (IVP) bovine embryos were generated worldwide in 2017. North America accounted for > 59% (292,755) of IVD embryos, whereas South America only counted for 10% (49,230). Conversely, distribution of IVP embryos were similar in North (475,696; 48%) and South (453,685; 46%) America. This is the first report in which North America produced more IVP than IVD embryos, whereas in South America, number of IVP embryos has exceeded the number of IVD embryos for > 10 years. The use of IVP in Brazil has increased rapidly since 2000, driven primarily by *Bos indicus* breeds which have large numbers of antral follicles from which large numbers of high-quality oocytes can be recovered without superstimulation. Viana et al.[57] reported that embryo transfer accounted for 19.7% of all "Zebu" calves registered in Brazil between 2005 and 2015.

In vitro embryo production in Brazil increased approximately 3.5 times between 2005 and 2016, while numbers of IVD embryos decreased approximately 3 times over the same interval (Viana, personal communication).

Commercial IVP in Brazil has been reported to have gone through 3 phases.[58] The initial phase involved use of proven donors of high genetic merit in both beef and dairy cattle and numbers of IVD and IVP embryos increased similarly. The second phase of growth occurred between 2003 and 2010, driven largely by the need to produce replacement bulls. In 2005, at the peak of this phase, 90.0% of IVP was in beef breeds, with Nelore accounting for 82.7% of all embryos. The third phase has involved use of sex selected sperm and is associated with a shift in IVP from beef breeds to *Bos taurus* dairy breeds. In 2014, IVP in dairy breeds increased by 46.5% (69.0% of total), exceeding that of beef breeds for the first time.

Conclusion

Use of protocols that control follicular development and ovulation facilitate widespread application of assisted reproductive technologies. Current protocols are practical and easy to perform by field staff. In superovulation schemes, estradiol is very effective in synchronizing emergence of a follicular wave, but is not available in many countries. Protocols using dominant follicle removal or GnRH are effective alternatives. Lengthened superstimulation protocols are also an interesting alternative, as they allow the time necessary for all growing follicles to acquire the ability to ovulate. Conversely, 4 day treatment protocols appear to not allow sufficient time for all follicles to acquire this capacity. Regarding in vitro production of embryos, cattle with *Bos indicus* influence adapt very well to this technology as they have a high antral follicle population; consequently, more oocytes are obtained by OPU than in *Bos taurus* breeds. In *Bos taurus* donors, synchronization of follicular wave emergence and use of FSH has resulted in collection of more COCs by OPU and in vitro production of a higher percentage of blastocysts.

Conflict of interest

There are no conflicts of interest to declare

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