In vitro and *in vivo* embryo production in cattle superstimulated with FSH for 7 days

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Abstract

Over the past two decades, research efforts have resulted in superstimulation protocols that are user-friendly, but embryo production has increased only marginally. Studies to-date have not adequately answered the question of whether superstimulatory protocols can be used to overcome the follicle wave pattern, increase the number of follicles that enter the wave, or rescue a greater number of small follicles within the wave. Studies which appear to facilitate greater utilization of follicles within the wave are described in this review. The number of large follicles at the time of first AI tended to be greater, and more ovulations and CL occurred with lengthened protocol (7-day) than with the convention 4-day FSH treatment. In addition, there was greater synchrony of ovulations and the mean numbers of total ova/embryos, fertilized ova, transferable or freezable embryos were numerically higher in the 7-day group. When used in an *in vitro* fertilization model, FSH treatment for 7 days resulted in a greater number of follicles for aspiration, a greater proportion of expanded cumulus-oocyte-complexes, and more transferable embryos after *in vitro* culture. Daily ultrasonography revealed a reduction in the number of small (1-2 mm) antral follicles from the beginning to the end of the superstimulatory treatment that was associated with a progressive shift of follicles to the next size category in both 4-day and 7-day groups. The number of follicles ≤ 5 mm decreased during superstimulation suggesting that there was no continuous recruitment of small follicles, and the number of follicles ≥ 1 mm at the end of superstimulation did not differ from the number of follicles ≥ 1 mm at the beginning of superstimulation. However, the total number of follicles ≥ 3 mm at the end of superstimulation, was greater than the number of follicles ≥ 3 mm at the beginning of superstimulation due to growth of the 1-2 mm population into larger size categories during treatment. Results support the hypothesis that both 4-day and 7-day superstimulatory treatment protocols result in rescue of small antral follicles present at the time of wave emergence. However, the lengthened superstimulatory treatment protocol resulted in more follicles acquiring the capacity to ovulate with an increased number of ovulations, and a tendency for increased embryo production without a decrease in oocyte/embryo competence.

Introduction

Improvements in our understanding of ovarian function, and gonadotropin preparations has led to important changes in strategies used for superovulation in cattle. Advances in superstimulatory treatment protocols have included the ability to initiate treatments at a self-appointed time (Bó *et al*., 1995; Mapletoft and Bó, 2012), the use of fixed timed AI (Bó *et al*., 2006), and a reduction in the number of FSH treatments (Bó *et al*., 1994; Tríbulo *et al*., 2012; Carvalho *et al*., 2014), all of which have made protocols easier to implement. More recently, it has been revealed that the superovulatory response is related to the number of follicles present at the time of wave emergence and that this number is inherent and repeatable within individuals (Singh *et al*., 2004; Ireland *et al*., 2007).

The number of transferable embryos is defined ultimately by the superovulatory response, fertilization rate and subsequent embryo development. The objective of this manuscript is to review recent thoughts on follicular growth and ovulatory response during superstimulation, and new information on how the duration of the superstimulatory treatment protocol might optimize donor response.

Follicle recruitment and initiation of FSH treatments

During emergence of follicular waves in cattle, a variable number of 3-5 mm follicles appear over a period of 2 to 4 days (Ginther *et al*., 1989; Lucy *et al*., 1992; Adams *et al*., 2008), as a result of a surge in circulating FSH 1 to 2 days earlier (Adams *et al*., 1992). Improvements in the resolution of ultrasound equipment revealed that small antral follicles (1-3 mm) also develop in a wave-like pattern in response to increases in endogenous FSH (Jaiswal *et al*., 2004). The number of 1-3 mm follicles was maximal 1 to 2 days before the previously defined day of wave emergence and was temporally associated with the earliest rise in the pre-wave surge of FSH. In addition, the future dominant follicle was first detected at a diameter of 1 mm approximately 66 h before the previously defined day of wave emergence (based on detection of the future dominant follicle at a diameter of 4 mm), and coincident

with the beginning of the FSH surge. This discovery of an extension of the wave pattern to include follicles at a smaller developmental stage has important implications for ovarian superstimulation.

The presence of a functional dominant follicle was found to suppress the superovulatory response (Guilbault *et al*., 1991; Bungartz and Niemann, 1994), and it has been shown that superstimulatory treatments must be initiated at the time of wave emergence to optimize response (Nasser *et al*., 1993; Adams, 1994). These findings led to the development of methods to synchronize wave emergence before ovarian superstimulation by treatment with a combination of estradiol and progesterone (Bo *et al*., 1995) or by transvaginal ultrasound-guided follicle ablation (Bergfelt *et al*., 1994). In another study, the superovulatory response and the number of freezable embryos were greater when FSH treatment was initiated 2 days rather than 1 day after ovum pick-up (OPU), a procedure that mimics the effect of follicular ablation (Surjus *et al*., 2014). As all follicles ≥3 mm were aspirated during oocyte collection, the additional day may have provided time for smaller follicles in the wave to reach an ovulatory stage during superstimulation treatment.

In contrast, preliminary data from others suggest that superstimulation initiated in the presence of a functional dominant follicle resulted in the emergence of a new follicular wave, and that the presence of a progestin implant for 4.5 days during the period of FSH treatment allowed sufficient additional time for the FSH-induced wave of follicles to reach the ovulatory pool, regardless of the fate of the dominant follicle (Bednar and Pursley, 2000). Similarly, in a review of alternative approaches to setting up donors for superstimulation (Bó *et al*., 2008), preliminary data were presented on the effects of a 6-day superstimulation protocol initiated without regard to follicular wave status. Ovarian response, and the number of ova/embryos and transferable embryos did not differ between the extended protocol without synchronization of wave emergence and a conventional 4-day protocol with synchronization. However, studies to-date have not directly addressed the question of whether superstimulatory protocols can be used to overcome the wave pattern (allow superstimulation regardless of wave status), increase the number of follicles that enter the wave, or rescue small follicles within the wave.

Extended superstimulatory treatment protocols

Based on the notion that exogenous gonadotropins can overcome the wave pattern and result in "subordinate follicle break-through", attempts have been made to increase the superovulatory response by adding eCG treatment prior to initiating FSH treatments. Pre-treatment with eCG 2 days before the conventional FSH treatment protocol resulted in a numerically greater number of ovulations (16.1 ± 1.5 *vs*. 12.4 ± 1.4) and transferable embryos (8.1 ± 1.6 *vs*. 6.5 ± 1.3) in an unselected group of donors (Caccia *et al*., 1999), and a significantly greater number of transferable embryos in donors that were defined as poor responders (3.6 ± 0.6 *vs*. 1.0 ± 0.2; Bó *et al*., 2008).

More recently, we evaluated the superovulatory response and embryo recovery in donors treated with either a 4-day or a 7-day FSH superstimulatory treatment protocol (García Guerra *et al*., 2012). Twenty-four beef cows were blocked by number of follicles ≤5 mm at the time of wave emergence and placed into either a 4-day or 7-day FSH protocol utilizing the same total dose of 400 mg FSH (Folltropin-V; Vetoquinol Inc/Bioniche Animal Health; Fig. 1).The mean number of ovulations detected by ultrasonography was greater in the 7-day treatment group (30.9 ± 3.9 *vs*. 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 *vs*. 19.5 ± 2.6; P = 0.11; Table 1). Moreover, ovulations occurred more synchronously in the 7-day group (93% of ovulations occurred 12 to 36 h post-LH as compared to 66% in the 4-day group) suggesting that the superstimulated follicles were more mature and capable of responding to an LH stimulus. Although the total number of ova/embryos, fertilized ova and transferable embryos did not differ statistically, all end-points favoured the 7-day group. In addition, when data from cows with fertilization failure were removed, the number of transferable embryos tended to be higher in the 7-day group (7.6 ± 1.7 *vs*. 4.2 ± 1.5; P = 0.07).

In another study (Dias *et al*., 2013a), a 7-day superstimulation protocol was used to investigate the effects of the length of the follicle growth phase under the influence of progesterone on follicle growth, ovulation and oocyte competence. Beef cows were superstimulated with 25 mg twice-daily of FSH 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 the numbers of ovulations (15.4 *vs*. 11.6) and embryos (6.7 *vs*. 5.9) were numerically higher in the 7-day group.

The duration of treatment appears to be responsible for the increase in the superstimulatory response rather than the FSH dose. In the two studies referred to above, the number of ovulatory-sized follicles just prior to ovulation was greater following 7 days of superstimulation than 4 days, whether the total dose of FSH was greater (Dias *et al*., 2013a) or the same (García Guerra *et al*., 2012). However, further study is needed to determine the optimal dose of FSH when an extended superstimulatory treatment is used.

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Table 1. Superovulatory response (mean ± SEM) and ova/embryo recovery in beef cows given a conventional 4-day *vs.* a 7-day superstimulatory treatment protocol.

End point	Treatment group		P-value
	4-day	7-day	
Number of cows	12	12	
Corpora lutea	20.8 ± 2.2	27.2 ± 2.1	0.04
Total ova/embryos recovered	11.3 ± 2.0	13.8 ± 2.3	0.44
Fertilized ova	5.6 ± 1.5	8.0 ± 2.0	0.24
Percentage of fertilized ova	59.0 ± 10.7	54.2 ± 9.5	0.58
Degenerate embryos	1.4 ± 0.7	1.7 ± 0.6	0.73
Transferable embryos (Grades 1-3)	4.2 ± 1.5	6.3 ± 1.6	0.25
Percentage of transferable embryos	44.4 ± 11.8	44.1 ± 8.4	0.69

Adapted from García Guerra *et al.*, 2012.
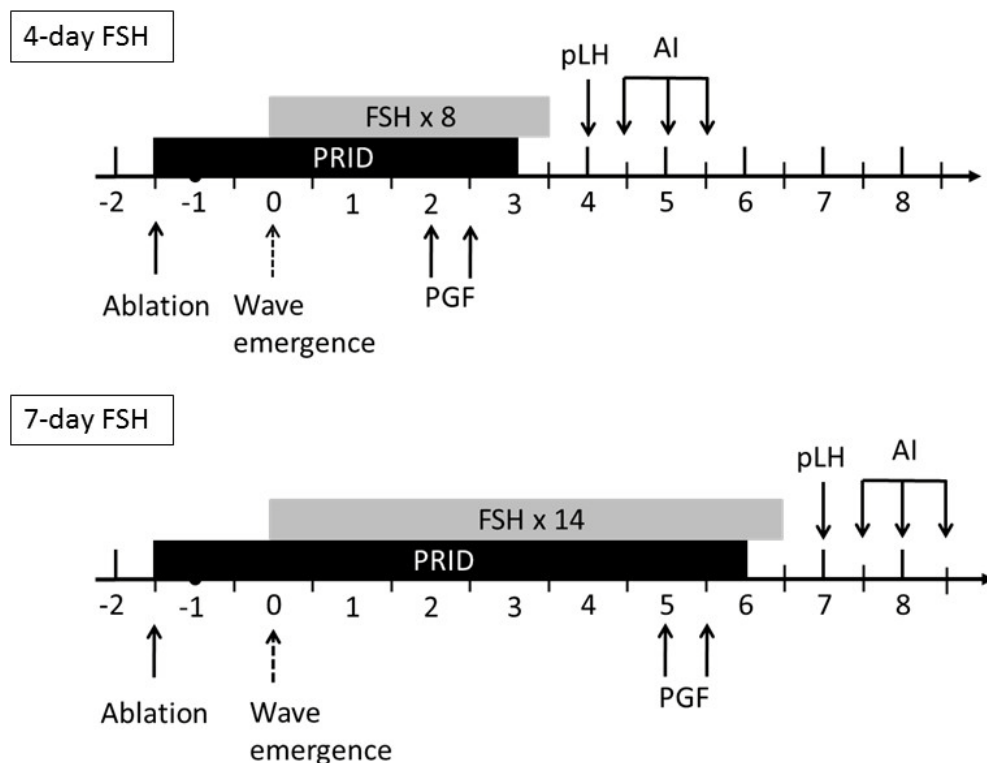
Figure 1. Experimental design used to compare the superovulatory response between a conventional 4-day *vs.* a 7-day superstimulatory protocol in cattle. From García Guerra *et al.* (2012).

Follicle development during superstimulatory treatments

An earlier study provided rationale for a hypothesis that superstimulatory treatment does not recruit more follicles into the wave, but rather permits more small follicles of a wave to attain medium and large diameters (Adams *et al.*, 1993). This hypothesis was tested in a recent study designed to compare the effects of a conventional (4-day) *vs.* a lengthened (7-day) superstimulation protocol on follicle dynamics (Garcia Guerra *et al.*, 2015). The number of follicles ≥1 mm at the end of superstimulation did not differ from the number of follicles ≥1 mm at the beginning of superstimulation, but the number of small (1-5 mm) antral follicles decreased dramatically during superstimulation. In addition, there was a negative correlation between the number of follicles ≤5 mm and those >5 mm. There was also a greater number of follicles ≥3 mm at the end of superstimulatory treatment

than at the start which was attributed to the rescue of the 1-2 mm population and their growth into larger categories during treatment. These results indicate that superstimulatory treatment rescued small antral follicles within the wave from atresia rather than recruiting more follicles into the wave (Fig. 2).

Although small follicles in the wave appeared to have been rescued in both groups, significant differences were detected in the developmental pattern of the growing cohort (Fig. 2). When the 7-day and the 4-day treatment protocols were compared, the decrease in the number of small follicles and the increase in the number of large follicles tended to occur at a relatively lower accumulated dose of FSH, and the mean growth rate of follicles between initiation of treatment and ovulation was lower (1.3 ± 0.1 *vs.* 1.9 ± 0.1 mm/day) in the 7-day group (Garcia Guerra *et al.*, 2015). Because follicles in 7-day protocol grew at a slower rate, and appeared to exist at an ovulatory size for a longer period of time, they may have had more time to acquire the capacity to ovulate, as indicated by the greater number and synchrony of ovulations than in the 4-day protocol (Garcia Guerra *et al.*, 2012).
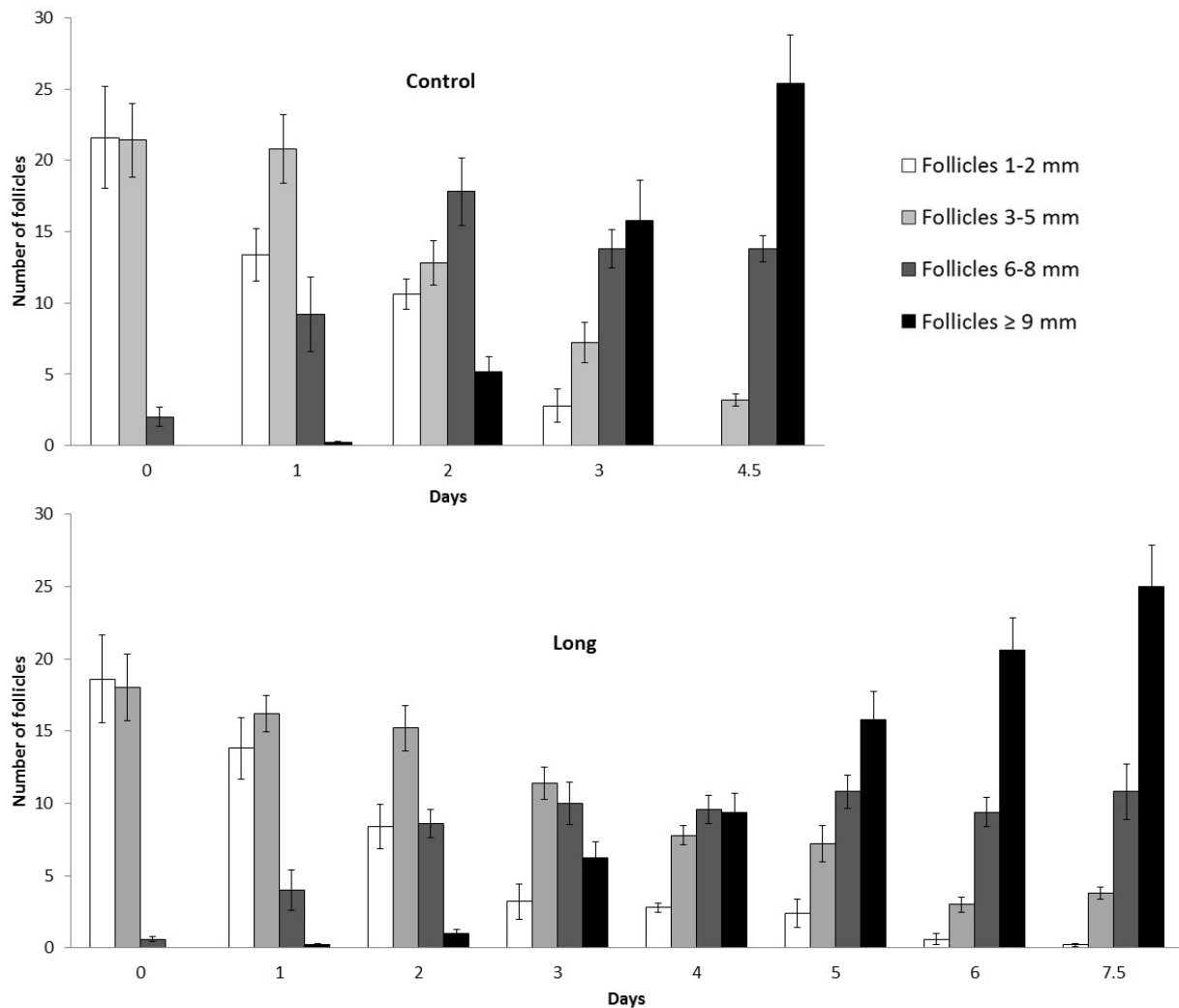

Figure 2. Mean ($\pm$ SEM) number of follicles in different size categories in cows during a conventional 4-day *vs.* a 7-day superstimulatory protocol in cattle. From García Guerra *et al.* (2015).

Micro-array 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 (Dias *et al.*, 2013c, 2014). Expression of growth-related genes (even though LH had been administered), similar to the pre-LH stage of follicle growth, and those involved in oxidative stress response were up-regulated in granulosa cells of follicles

undergoing a 4-day FSH superstimulation protocol compared to a dominant follicle of an unstimulated follicular wave. Genes related to a disturbance in angiogenesis were also up-regulated in superstimulated follicles. We speculate that gene expression during a 7-day superstimulation protocol may be more similar to the naturally occurring dominant follicle.

The acquisition of LH receptors is commonly used as a marker for dominance and a prerequisite for the establishment of ovulatory capacity (Sartori *et al.*, 2001; Barros *et al.*, 2012). Recently, the evaluation of the expression of LHR mRNA in granulosa cells of superstimulated and unstimulated follicles 12 h after progesterone device removal revealed that expression of LHR was decreased following superstimulation (Lucacin *et al.*, 2013). As a result, following the conventional 4-day FSH superstimulation protocol, follicles lagged in maturational development and response to LH compared to a naturally occurring single dominant follicle (Dias *et al.*, 2013c, 2014). These findings further support the concept that following a 4-day superstimulation treatment some follicles do not have sufficient time to mature, up-regulate expression of appropriate genes, and acquire the capacity to ovulate.

The use of extended superstimulation protocols for OPU

Most protocols used to stimulate follicle growth prior to OPU are shortened superstimulation protocols with *in vitro* maturation following oocyte collection (Blondin *et al.*, 2002). Although we recognize that it is unlikely to be practical in a commercial setting, we have examined the use of an extended superstimulation treatment protocol on *in vitro* embryo production. *Bos taurus* beef heifers were superstimulated using a 4-day or a 7-day FSH protocol and cumulus oocyte complexes (COC) were collected 24 h after LH treatment (Dias *et al.*, 2013b). Compared to the traditional 4-day FSH protocol, the 7-day protocol resulted in a significantly greater number of follicles ≥9 mm at the time of COC collection (25.4 ± 5.3 *vs.* 10.6 ± 2.3), a tendency for a greater number of COC collected (17.8 ± 3.7 *vs.* 10.5 ± 2.4), and 2.5 times more transferable embryos at the end of *in vitro* embryo culture (9 days after IVF). Interestingly, >90% of the COC collected in the 7-day group were expanded, while in the 4-day group only 74% of the COC were expanded (ns). In another study, the 7-day protocol resulted in the collection of a greater proportion of mature oocytes (59 *vs.* 22%) compared to the 4-day protocol (Dadarwal *et al.*, 2014). Although the developmental potential of oocytes was similar between 4-day and 7-day protocols, embryo production was increased in the 7-day group because of a greater number of larger follicles from which COC could be aspirated.

Conclusions

An accumulating body of research suggests that the addition of 2 to 3 days to a traditional 4-day superstimulatory treatment protocol allows time for even the smallest follicles of a wave to be rescued from atresia and incorporated into the cohort that attains ovulatory capability. Conversely, the conventional 4-day superstimulatory treatment protocol results in an accelerated growth rate of follicles that may acquire the capacity to ovulate, but do so less synchronously and may produce oocytes of sub-optimal competence. The extended superstimulatory treatment protocol appears to result in a greater number of transferable embryos both *in vivo* and *in vitro*, because of a greater number of mature follicles. Based on these studies, we conclude that extending the period of follicular development during superstimulation (from 6 to 9 days between wave emergence and ovulation) increases follicular maturation, and the number and synchrony of ovulations without compromising ova/embryo competence, and in turn, the potential for more transferable embryos.

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