

# Follicular characteristics and luteal development after follicle-stimulating hormone induced multiple ovulations in heifers<sup>1</sup>

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**ABSTRACT:** A protocol based on small doses of FSH was examined for the induction of double or triple (multiple) ovulations in cattle. Ovulation rate, follicular characteristics, and luteal responses were determined. In Exp. 1, three groups of estrous-synchronized, cyclic Holstein heifers were treated once daily, on d 3 to 6 of the cycle, with a FSH product (Folltropin-V): large FSH dose (total of 150 mg;  $n = 18$ ), medium FSH dose (total of 130 mg,  $n = 12$ ), and small FSH dose (total of 80 mg;  $n = 7$ ). Controls received saline ( $n = 6$ ). Prostaglandin  $F_{2\alpha}$  was injected on d 6, ultrasound-guided aspiration of surplus follicles (if needed) was performed on d 7, and GnRH was injected on d 8 to induce ovulation. The large FSH dose induced growth of more ( $2.6 \pm 0.3$ ,  $P < 0.05$ ) large follicles than controls on d 8; medium and small FSH doses insufficiently stimulated growth of  $<2$  large follicles. Ovulation rates were determined in subgroups of heifers ( $n = 10, 13, 4$ , and  $6$ , respectively). The large FSH dose induced greater rates ( $P < 0.01$ ) of mostly double and triple ovulations (90% multiple ovulations, 70% double ovulations), most of which (89%) were bilateral, with only 2 out of 10 heifers requiring aspiration of surplus follicles. Medium

and small FSH doses induced fewer multiple ovulations (38% and 25%, respectively). Estradiol concentrations on d 8 did not differ among treatments, but the concentration per large follicle in controls was greater ( $P < 0.05$ ) than in FSH treatments. Mean corpus luteum (CL) volume in single-ovulation controls was greater ( $P < 0.05$ ) than that of multiple ovulations in the large FSH group and total CL volume and progesterone concentrations were numerically greater in multiple ovulations. In Exp. 2, the characteristics of follicles aspirated on d 7 from large FSH ( $n = 11$ ) and control heifers ( $n = 10$ ) were compared. Based on estradiol-to-progesterone ratio, 57% of the large FSH-treated follicles were classified as codominant/healthy follicles and 43% as subordinate/early atretic. Although concentrations of estradiol and androstenedione in FSH-treated codominant follicles were less ( $P < 0.05$ ) than in controls, estradiol-to-progesterone ratio indicated that those follicles were steroidogenically active. Finely tuned small doses of FSH administered during the first follicular wave can induce a large incidence of double/triple, mainly bilateral, ovulations in cattle, which may serve as a basis for treatment aimed at promoting twinning in beef cattle.

**Key words:** corpus luteum, double ovulation, follicle, follicle-stimulating hormone, heifer

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## INTRODUCTION

Twinning rates in dairy cows can reach 10% (Kinsel et al., 1998; Lopez-Gatius et al., 2005), whereas in beef cattle, similar to dairy heifers, they range from 0.4 to 1.1% (Rutledge, 1975). Most cattle twins are dizygotic, resulting from multiple ovulations (Silva Del Rio

et al., 2006). Twinning in dairy cows is considered undesirable (Nielen et al., 1989), whereas in beef cattle it increases herd profitability (de Rose and Wilton, 1991; Echternkamp et al., 2002), despite the greater associated costs (Guerra-Martinez et al., 1990; Echternkamp et al., 2007). Assistance during calving and nutritional support can decrease the deleterious effects of twinning and increase calf survival and birth weight (Knight et al., 2001; Kirkpatrick, 2002). Importantly, increased calving performance of twins has been recorded for bilateral vs. unilateral pregnancy (Penny et al., 1995; Echternkamp et al., 2007).

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Twins have been induced in beef cattle by genetic selection (Echternkamp et al., 2007), embryo transfer (Guerra-Martinez et al., 1990), and immunization against inhibin (Takedomi et al., 1997). Ovarian manipulation, consisting of follicular ablation, luteal regression, and resulting induction of premature LH surge, increases double ovulations in beef cows and dairy heifers (Musard et al., 2006; Palhao et al., 2009). Follicle-stimulating hormone treatment is also used to induce double ovulation. An extended, endogenous rise in FSH has been associated with follicular wave emergence, resulting in the growth of 2 or 3 large follicles and establishment of follicular codominance, leading to double ovulation (Wiltbank et al., 2000; Kulick et al., 2001; Hunter et al., 2004; Lopez et al., 2004). Unlike superovulation treatments, in which large doses of FSH are used to induce growth of several large follicles, a few studies have used small FSH doses to induce double ovulation (Davis and Bishop, 1992; Murphy et al., 1998). Common to those studies is the large variation in individual response of cows in terms of induced ovulation numbers. We present a procedure for the induction of double or triple, preferably bilateral, ovulations by small-dose FSH treatment, which may serve as a basis to promote twin production in beef cattle. The quality of the FSH treatment-induced codominant preovulatory follicles was examined.

## MATERIALS AND METHODS

The study was conducted in accordance with the guidelines of the local ethics committee of Hebrew University.

### Animals

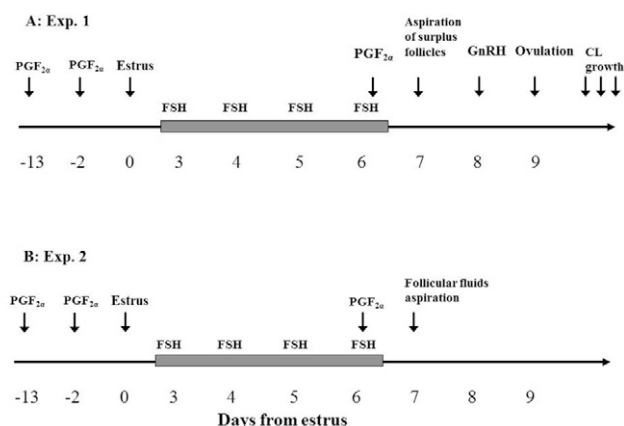
The experiment was conducted using 12- to 15-month-old nulliparous, cyclic Holstein heifers. The heifers were kept in an open shed with access to an adjacent yard, and had ad libitum access to a total mixed diet containing 1.37 Mcal/kg DM and 13.2% protein. The experiments were conducted at the experimental dairy farm of the Agricultural Research Organization in Bet-Dagan, Israel, from November to June, when mean maximal and minimal air temperature and relative humidity were 22.8 and 12.6°C, and 89% and 46%, respectively.

### Experimental Protocol

Estrous cycles of the heifers were synchronized with 2 doses of PGF<sub>2α</sub> analog (2 mL Cloprostenol, Estropan, Parnell Labs, Alexandria, Australia), administered intramuscularly (i.m.) 11 d apart. Estrous behavior was monitored visually 4 times daily for 2 d, starting 24 h after the second PGF<sub>2α</sub> injection. Only heifers that exhibited standing estrus (day of estrus = d 0) were included in

the experiment. A schematic illustration of the protocol is presented in Fig. 1. Heifers were assigned randomly to receive 3 different regimens of FSH doses or saline (control). All FSH-treated heifers were given daily i.m. doses on d 3 to 6 of the cycle. On d 6, all heifers received PGF<sub>2α</sub> to induce luteolysis and start a follicular phase (Fig. 1A). On d 7 of the cycle, heifers were assigned to either the ovulation (Fig. 1A; Exp. 1) or follicular characterization experiment (Fig. 1B; Exp. 2).

**Ovulation Experiment (Exp. 1).** Heifers received 3 different regimens of FSH doses. The treatments were large FSH (150 mg; 100, 20, 20, 10 mg/d; *n* = 18), medium FSH (130 mg; 100, 10, 10, 10 mg/d; *n* = 12), or small FSH (80 mg; 20, 20, 20, 20 mg/d *n* = 7; Follitropin-V, Bioniche, Ontario, Canada), given on d 3 to 6, respectively. Control heifers received saline (*n* = 6; Fig. 1A). On d 7 of the cycle, the FSH-treated heifers underwent selective aspiration (described later on) of surplus follicles (if needed) to ensure ovulation of the remaining 2 to maximum 3 large follicles. On d 8, the heifers received an i.m. dose of 200 µg GnRH analog (Gonadorelin-acetate, Gonabreed; Parnell Laboratories) to induce ovulation of the large follicles. Heifers were monitored daily, starting on d 3 of the cycle, by ultrasound (Aloka, SSD-900; Tokyo, Japan), equipped with a 7.5-MHz transrectal linear transducer, to determine the effect of FSH on follicular growth. Sizes and numbers of medium (6 to 9 mm) and large (≥10 mm) follicles were recorded. After GnRH administration, ovulation was determined in subgroups of heifers (*n* = 10, 13, 4, and 6 heifers, respectively) by transrectal ultrasound and later it was confirmed by monitoring the number of corpora lutea (CL) developed (Fig. 1A). Size of CL was moni-



**Figure 1.** Experimental protocol. Estrous cycles were synchronized in heifers with 2 doses of PGF<sub>2α</sub>. After estrus, FSH doses were given on d 3 to 6, and PGF<sub>2α</sub> was given on d 6. (A) In Exp. 1, three FSH treatment doses (small, medium, and large) and a saline control were tested. Surplus follicles were aspirated (if needed) on d 7 and GnRH was given on d 8 to induce ovulations; later, corpus luteum growth rates were determined. (B) In Exp. 2, on d 7, follicular fluids were aspirated from large FSH and saline control.

tored and 8-mL blood samples were collected into heparinized tubes from subgroups of heifers before and after ovulation. Samples were stored immediately on ice until centrifuged at  $1,200 \times g$  for 20 min at  $20^{\circ}\text{C}$ , and plasma stored at  $-20^{\circ}\text{C}$  for analysis of hormone concentrations.

**Follicular and Luteal Characterization (Exp. 2).** A schematic illustration of this experiment is presented in Fig. 1B. The large FSH treatment from Exp. 1 was selected as the optimal treatment in the ovulation experiment. Therefore, in this part of the study, we compared follicles that were obtained from the large FSH-treated heifers ( $n = 11$ ) with those from control heifers ( $n = 10$ ). On d 7, 24 h after  $\text{PGF}_{2\alpha}$  administration, follicular fluids were aspirated *in vivo*. To obtain maximum information, 2 to 3 large follicles were selected for follicular aspiration from each FSH-treated heifer (usually from a pool of 2 to 4 large follicles). A single dominant follicle was aspirated from control heifers. Collection of follicular fluids was performed as previously described (Roth et al., 2001). Briefly, heifers were sedated (14 mg 2% xylazine *i.m.*, Sedaxylan; Eurovet Animal Health, Bladel, The Netherlands) and caudal epidural anesthesia was induced with 5 mL of 2% lidocaine. Follicles were aspirated using an ultrasonic scanner (Pie Medical, Maastricht, The Netherlands) connected to a 7.5-MHz vaginal sector transducer equipped with a needle guide attached to a 21-gauge aspiration needle that was connected by a silicon tube to a 5-mL syringe. Follicular fluid was aspirated and stored at  $-20^{\circ}\text{C}$  for hormone analyses.

### Analysis of Hormones

Plasma and follicular fluid progesterone concentrations were analyzed with a solid-phase RIA kit (Diagnostic Product Corp., Los Angeles, CA) against a standard curve prepared with ovariectomized cow plasma (Shaham-Albalancy et al., 2000). The minimal detectable amount was 0.2 ng/mL and the intra-assay and interassay CV were 8.6% and 9.9%, respectively. Estradiol was measured in extracted plasma samples using an ultra-sensitive RIA kit (DSL-4800, Diagnostic System Laboratory, Webster, TX). The standard curve was prepared with charcoal-stripped bovine plasma (Turzillo and Fortune, 1990). Cross reactivity of the assay was 2.4% for estrone and 0.4% for estriol. The minimal detectable amount was 0.5 pg/mL and the intra-assay and interassay CV were 3% and 5%, respectively. Follicular fluid estradiol concentrations were analyzed by means of a solid-phase RIA kit (Diagnostic Products Corp.). The minimal detectable amount was 8 pg/mL and the intra-assay CV was 3%. The follicular fluid androstenedione concentration was analyzed by means of a kit (Androstenedione RIA DSL-4200, Diagnostic System Laboratory), following manufacturer instructions. The minimal

detectable amount was 0.02 ng/mL and intra-assay CV was 4.3%.

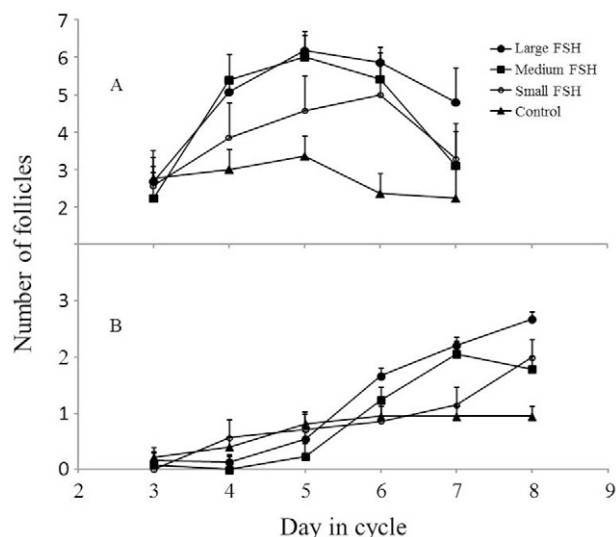
### Statistical Analyses

For plasma progesterone concentrations and follicular dynamics, heifers served as experimental units with repeated measurements within heifers and data were analyzed using the PROC MIXED analyses (SAS Inst. Inc., Cary, NC). The experimental model included experimental treatments, heifers within treatments, days, and day-by-treatment interaction. Other data were tested by 1-way ANOVA. This included the number of large follicles and plasma estradiol concentrations on d 8 (pg/mL and  $\text{pg follicle}^{-1}\cdot\text{mL}^{-1}$ ), day in the cycle when the number of medium-sized follicles declined, CL volume at the midluteal phase in the cycle after ovulation, and steroids in the follicular fluids aspirated on d 7. Aspirated follicles from FSH-treated heifers were sorted into healthy or subordinate, according to estradiol-to-progesterone ratio:  $>1$  (healthy) or  $<1$  (atretic, subordinate). For 2 or 3 large follicles aspirated from a single FSH-treated heifer, which were sorted similarly into the healthy or subordinate subgroup, the mean value of follicular steroid concentration was taken. All single control follicles were sorted as healthy. The significance between groups was tested by Tukey-Kramer test. The distribution of heifers between those that exhibited multiple ovulations and a single ovulation was tested by  $\chi^2$  test. The volume of CL was calculated from ultrasound measurements, according to the formula:  $\text{volume} = 4/3\pi R^3$ , using a radius calculated by the formula  $\text{radius} = (L/2 + W/2)/2$  where  $L$  = length and  $W$  = width. For a CL with a fluid-filled cavity, the volume of the cavity was calculated and subtracted from the total volume of CL. Data are presented as least square means and SEM.

## RESULTS

### Exp. 1: Follicular Dynamics

The effects of large, medium, or small FSH treatments, given on d 3 to 6 of the cycle, on growth dynamics of follicles are presented in Fig. 2. The overall mean number of medium (Fig. 2A) and large (Fig. 2B) follicles was greater ( $P < 0.05$ ) in the large FSH treatment compared with the control treatment; medium and small FSH treatments did not differ from controls. The number of large follicles present on the day of GnRH injection (d 8) indicates the ovulatory potential of the specific hormonal treatment. Obviously, the minimum requirement for double ovulation is the presence of at least 2 large follicles. As shown in Fig. 2 and Table 1, the large FSH treatment was the only one that exceeded the minimum



**Figure 2.** Effects of different FSH treatments on the number of (A) medium-sized follicles (6 to 9 mm) and (B) large follicles (>10 mm). Daily doses of FSH (Folltropin-V, Bioniche, Ontario, Canada) given on d 3 to 6 of the cycle were: large ( $n = 18$ ; 100, 20, 20, 10 mg/d), medium ( $n = 12$ , 100, 10, 10, 10 mg/d), and small ( $n = 7$ , 20, 20, 20, 20 mg/d). Control heifers received saline ( $n = 6$ ). Pooled SEM values for medium and large follicles were 0.53, 0.36, 0.71, 0.44, and 0.15, 0.11, 0.20, 0.13 follicles, respectively.

threshold of 2 large follicles on d 8 ( $2.6 \pm 0.3$  follicles;  $P < 0.05$ ). Notably, the number of medium-sized follicles declined 1 d later in the large FSH treatment than in the control (d  $5.6 \pm 0.2$  vs.  $4.5 \pm 0.2$  of the cycle;  $P < 0.05$ ), possibly indicating a delayed gain of dominance in the former group. Plasma estradiol concentrations on d 8 did not differ among treatments (Table 1). In contrast, the concentration per large follicle presented on d 8 was less ( $P < 0.05$ ) in all heifers treated with FSH than in the control.

### Ovulation Rates

In 2 of 10 (20%) heifers receiving the large FSH dose, surplus follicles were aspirated on d 7. In 1 heifer, 1 follicle was aspirated; and in the other heifer, 5 were aspirated. Surplus follicles ( $n = 3$ ) were aspirated in

**Table 1.** Effects of different FSH treatments on the number of large follicles developed and plasma estradiol concentrations determined on the day of GnRH injection (d 8 of cycle)

Item	Control	Small FSH	Medium FSH	Large FSH
$n$	6	7	12	18
Large follicles, $n$	$1 \pm 0^b$	$2.0 \pm 0.3^{ab}$	$1.75 \pm 0.2^{ab}$	$2.61 \pm 0.3^a$
Estradiol, pg/mL	$11.7 \pm 2.0^a$	$5.6 \pm 3.6^a$	$8.3 \pm 1.6^a$	$9.0 \pm 2.0^a$
Estradiol per large <sup>1</sup> follicle, pg/mL	$11.7 \pm 2.0^a$	$4.3 \pm 3.9^b$	$4.2 \pm 0.7^b$	$3.7 \pm 0.8^b$

<sup>a,b</sup>Within a row, means without a common superscript differ ( $P < 0.05$ ).

<sup>1</sup>Based on 6, 4, 10, and 14 subsamples, respectively.

**Table 2.** Effect of different FSH treatments on ovulation rates and bilateral distribution

Item	Control	Small FSH	Medium FSH	Large FSH
$n$	6	4	13	10
No ovulation, $n$	0	1	1	0
Single ovulation	6/6 (100%)	2/4 (50%)	7/13 (54%)	1/10 (10%)
Double ovulation	0	1/4 (25%)	5/13 (38%)	7/10 (70%)
Triple ovulation	0	0	0	2/10 (20%)
Double/triple ovulations <sup>1</sup>	0/6 (0%)	1/4 (25%)	5/13 (38%)	9/10 (90%)
Bilateral ovulations	0	1/1	3/5 (60%)	8/9 (89%)

<sup>1</sup>Large FSH treatment differs from other treatments ( $P < 0.01$ ).

only 1 out of 13 heifers (8%) from the medium FSH dose treatment and none were aspirated from heifers in the small FSH dose. The large FSH treatment induced the greatest rate (90%,  $P < 0.01$ ) of multiple ovulations, most of which were bilateral (Table 2, 89%). Most multiple ovulations (70%) were double ovulations and none of the heifers exhibited more than 3 ovulations. Nevertheless, in the large FSH treatment, 14% of the large follicles present in the ovaries on GnRH injection (d 8) did not ovulate. The other FSH doses induced lesser rates of multiple ovulations that did not differ from the single-follicle-ovulating control heifers. All control heifers ovulated a single follicle.

### Corpus Luteum Growth

The total volume of luteal tissue in the midluteal phase of large FSH heifers that ovulated double or triple follicles was numerically greater but did not differ significantly from that of controls (Table 3). Similarly, plasma progesterone concentrations in multiple-ovulating large FSH heifers were  $\sim 2$  ng/mL greater numerically but were not significantly different from those of single-ovulating control heifers (Fig. 3). The average size of a CL in the multiple-ovulating, large FSH-treated heifers was smaller ( $P < 0.05$ ) than that of a single CL in controls. Note that the diameter of ovulating follicles on the day of GnRH injection (d 8; data not shown) in the large FSH treatment did not differ from that of its control counterparts ( $12.8 \pm 0.4$  vs.  $13.6 \pm 0.8$  mm, respectively).

### Exp. 2: Follicular Steroids

Follicular fluids were aspirated from  $2.15 \pm 0.15$  large follicles in the high FSH-treated heifers and from a single follicle in controls. Follicles were assigned to the healthy or subordinate subgroup (Table 4). Twelve out of 28 follicles aspirated from the large FSH treatment were categorized as subordinate, based on an estradiol-to-progesterone ratio of  $< 1$ . Estradiol and androstenedione concentrations in the follicular fluid of the con-



**Table 3.** Total luteal tissue volume and mean corpus luteum (CL) volume at midluteal phase (d 10 to 16 of the cycle) in large FSH-treated double/triple-ovulating heifers and single-ovulating controls

Item	Control Single ovulation	Large FSH Double/triple ovulation
<i>n</i>	5	5
Total luteal volume, cm <sup>3</sup>	5.45 ± 0.79	7.37 ± 1.27
Mean CL volume, cm <sup>3</sup>	5.45 ± 0.79 <sup>a</sup>	3.22 ± 0.56 <sup>b</sup>

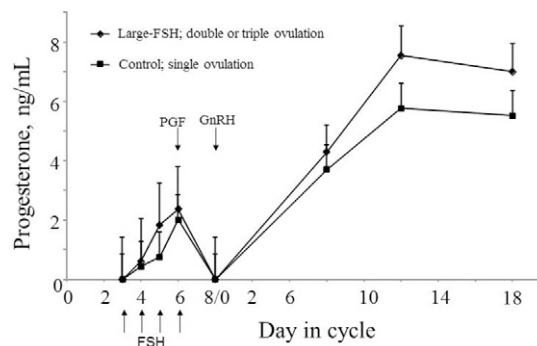
<sup>a,b</sup>Within a row, means without a common superscript differ ( $P < 0.05$ ).

control follicles were greater ( $P < 0.05$ ) than those in both healthy and subordinate follicles from the large FSH-treated heifers (Table 4). Progesterone concentrations did differ among groups (Table 4).

## DISCUSSION

We examined an FSH-based protocol that induces a high rate of double or triple ovulation in heifers, most of which are bilateral. The FSH treatment induced the growth of large follicles that varied markedly in their quality; 60% were healthy, steroidogenic follicles and 40% were subordinate, early atretic follicles. The protocol was based on the assumption that extension of the FSH surge, associated with follicular wave emergence, may establish codominance that will eventually lead to multiple ovulations. Accordingly, large FSH concentrations are found around the time of expected follicular deviation in the circulation of multiple-ovulating, compared with single-ovulating cows (Wiltbank et al., 2000; Lopez et al., 2004). In the current study, we pooled double and triple ovulations into 1 group. This is because currently we do not have any information on the success rate of embryo production in double- vs. triple-ovulating heifers, which would obviously be less than the number of ovulating follicles.

In the current study, the 3 FSH doses examined were much less than the commercial dose used routinely in ovarian superstimulation protocols. We chose to treat the heifers during the first follicular wave because it is more predictable than the second follicular wave. The basic strategy for induction of double or triple ovulations was based on 4 steps. First, the emergence of a larger than normal pool of medium-sized follicles was stimulated, similar to that recorded for cows selected for twin births (Echternkamp et al., 2004). This was achieved by a single (100 mg) dose of FSH given on d 3 of the cycle. A smaller (20 mg) dose in the small FSH treatment on d 3 was insufficient to induce emergence of a large enough number of follicles to yield more than 2 codominant ovulatory follicles later, on d 8. Second, increased concentrations of FSH were maintained on d 4, 5, and 6 with smaller doses (10 to 20 mg), aimed to establish codominance. Third, surplus follicles were aspirated from the ovaries to



**Figure 3.** Plasma progesterone concentration of single-ovulating control and double/triple-ovulating large FSH-treated heifers during the treatment cycle and after ovulation in the subsequent cycle. Pooled SEM values of the large FSH and control groups were 0.60 and 0.73 ng/mL, respectively.

avoid multiple ovulations of more than 3 follicles. This approach (although not feasible for use under commercial farm conditions) limits multiple ovulations to 2 or 3 follicles. The aspiration of surplus follicles in only 20% (2/10) and 8% (1/12) of the heifers in the large and medium FSH treatments, respectively, is encouraging. The fourth step was induction of ovulation with GnRH. Collectively, our data showed high sensitivity of the ovarian pool of follicles to the FSH doses used. Note that a slight difference (13%) in FSH dose (150 vs. 130 mg) was enough to obtain sufficient vs. insufficient follicular growth. More specifically, the small difference in doses between the large and medium groups occurred on d 4 and 5 of the cycle, corresponding to about d 3 to 4 of the first follicular wave (assuming emergence on d 1 to 1.5 after estrus and a deviation ~2.5 d later; Lopez et al., 2004).

About 40% of the aspirated follicles were categorized as subordinate. The progesterone concentration in the follicular fluid of the subordinate follicles remained low, similar to control or healthy FSH-treated follicles, and did not accumulate to the very high concentrations noted in large estrogen-inactive follicles (Echternkamp

**Table 4.** Concentration of steroid hormones (ng/mL) in the follicular fluid of follicles aspirated from large FSH and control heifers on d 7 of the cycle, 24 h after PGF<sub>2α</sub>. Individual follicles were sorted to healthy or subordinate subgroups, according to estradiol-to-progesterone ratio

Item	Control	Large FSH healthy	Large FSH subor- dinate
<i>n</i>	10	6	5
Estradiol, ng/mL	668.8 ± 69.1 <sup>a</sup>	246.4 ± 148.7 <sup>b</sup>	13.4 ± 3.3 <sup>b</sup>
Progesterone, ng/mL	42.9 ± 10.0	29.1 ± 7.1	19.6 ± 3.6
Androstenedione, ng/mL	40.5 ± 11.2 <sup>a</sup>	9.6 ± 2.3 <sup>b</sup>	1.8 ± 0.4 <sup>b</sup>
Estradiol: progesterone	24.4 ± 8.0 <sup>a</sup>	7.1 ± 2.0 <sup>ab</sup>	0.8 ± 0.3 <sup>b</sup>

<sup>a,b</sup>Within a row, means without a common superscript differ ( $P < 0.05$ ).

et al., 2004). Nevertheless, low follicular estradiol was associated with an estradiol-to-progesterone ratio of <1. The large subpopulation of subordinate follicles recorded on d 8 could be associated with the absence of FSH treatment support on d 7 and 8 of the cycle. Presence of a large subordinate follicle population might explain the fact that a small subpopulation of the large follicles that developed in the large FSH treatment did not respond to GnRH and did not ovulate. It also explains why most of the multiple ovulations in this study were actually double ovulations (70%), even though the number of large follicles present on the day of GnRH injection was greater than 2 (2.6 follicles). Because functional atresia precedes morphological atresia (Bao et al., 1997), it is hard to predict, before the aspiration of surplus follicles, which of the large follicles will be healthy or will have already begun an apoptotic degradation process.

The decreased calculated plasma estradiol concentration per large follicle in FSH-treated heifers vs. controls suggests low production of the steroid in the FSH-treated follicles. This is strengthened by the decreased estradiol concentrations in the follicular fluid in the large FSH treatment, assuming that follicular fluid concentrations of steroids correlate with their production rates by the theca or granulosa cells. This situation resembles that reported by Mihm et al. (1997) in which exogenous FSH given to heifers on d 2 to 3 of the cycle delayed the process of follicular selection and later the FSH-treated follicles exhibited low follicular estradiol. It is possible that the reduction in follicular estradiol concentrations observed here is related to decreased LH secretion. Supporting this is the reduction in pulsatile LH found in several studies that used FSH in superovulation treatments (Ben Jebara et al., 1994; Roberge et al., 1995; Gosselin et al., 2000). Normal LH secretion has been shown to be crucial for estradiol production (Crowe et al., 2001). Moreover, LH stimulates both androgen production in thecal cells and the production of follicular IGF-I, which has numerous stimulating effects on steroidogenesis, including proliferation of granulosa and theca cells (Spicer, 2004; Lopez et al., 2004). In our study, the reduced concentration of follicular androstenedione in the FSH-treated follicles suggests an association between decreased estradiol and decreased androgen production, which serves as a substrate for aromatization to estradiol in the granulosa cells.

The greater proportion of bilateral double ovulation is an important finding because it has been shown that the success rate of bilateral twin pregnancies is greater than that of unilateral ones (Hanrahan, 1983; Gordon, 1994). A rate of bilateral pregnancy that surpasses the predicted 50% (Cushman et al., 2005) is beneficial (compared with unilateral twin pregnancy) because of the marked rise in survival rate of the embryo at calving,

greater weight of calves, embryonic weight gain, and ease of calving (Echternkamp et al., 2007, 2009).

The average volume of a CL in the multiple-ovulation, large FSH treatment was smaller than that of a single CL in controls. Reduced CL size has been reported in double-ovulating cows (Lopez et al., 2004; Mann et al., 2007; Echternkamp et al., 2009). This finding is in agreement with the previously discussed low steroidogenic capacity of the FSH-treated follicles because a small number of proliferating follicular-wall cells will likely subsequently form suboptimal CL. However, the total luteal tissue volume in the FSH-treated group was similar and even tended to be greater than that in controls. Similarly, plasma progesterone concentrations at midluteal phase were similar and even tended to be slightly greater in the double/triple-ovulating large FSH heifers than in controls. Sufficient progesterone secretion from the CL is essential for maintaining normal embryo development and a high rate of embryo survival. Our findings indicate that the total progesterone output, as reflected by its circulating concentration, is sufficient to support twin pregnancies.

### Conclusions

This study describes an efficient protocol in which high rates of double/triple ovulations (90%) were recorded, with a minimal need for aspiration of surplus follicles to avoid ovulation of more than 3 follicles. Most of the double/triple ovulations were bilateral. Most of the follicles exhibited characteristics of viable and active follicles, though their steroidogenic capacity was lower than that of controls. A relatively large proportion (40%) of follicles obtained from FSH-treated heifers was subordinate in the early stages of atresia. Nevertheless, results of this study may serve as a basis for treatment aimed to promote twinning in beef cattle.

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