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The effect of timing of the induction of ovulation on embryo production in superstimulated lactating Holstein cows undergoing fixed-time artificial insemination

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Abstract

Two experiments evaluated the effects of timing of the induction of ovulation in superstimulated lactating Holstein donor cows that were fixed-time artificially inseminated. Secondary objectives were to evaluate the effects of the timing of progesterone (P4) device removal (Experiment 1) or the addition of a second norgestomet implant (Experiment 2) during superstimulation. In Experiment 1, 12 cows were allocated to one of four treatment groups with the timing of P4 device removal (24 or 36 h) and pLH treatment (48 or 60 h), after the first PGF as main factors, in a *Latin Square* (cross-over) design. There was an interaction (P = 0.03) between time of P4 device removal and time of pLH treatment. Mean (± SEM) numbers of transferable embryos were higher when the P4 device was removed at 36 h and pLH was administered at 60 h after the first PGF (P36LH60 =6.3 ± 1.4) compared to other treatments (P24LH60 =3.7 ± 1.1; P24LH48 =2.4 ± 0.8; or P36LH48 =2.2 ± 0.7). In Experiment 2, 40 cows were randomly allocated into one of four treatments with the number of norgestomet implants (one or two) and the time of induction of ovulation with GnRH relative to the first PGF (48 vs. 60 h) as main effects. The mean number of transferable embryos was higher (P = 0.02) when GnRH was administered at 60 h (4.2 ± 1.3) compared to at 48 h (2.7 ± 0.8), and the number of freezable embryos was increased (P = 0.01) in cows receiving two (3.0 ± 1.0) rather than one norgestomet implant (1.5 ± 0.5). In summary, embryo production in lactating Holstein cows was increased when the ovulatory stimulus (pLH or GnRH) was given 60 h after the first PGF, particularly when the P4 device was removed 36 h after the first PGF and when two norgestomet ear implants were used during the superstimulation protocol.

Keywords: Cattle; Fixed-time artificial insemination (FTAI); Holstein; Induction of ovulation; Superovulation

1. Introduction

Although embryo transfer is used widely, variability in response to the superstimulatory treatments and the time and handling associated with estrus detection and AI remain important limitations [1]. Improved understanding of ovarian function has provided possibilities for a greater capability of controlling follicular development and ovulation. Current knowledge of ovarian function in different breeds and classes of cattle has allowed the development of superstimulatory treatments at a self-appointed time [2–5].

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Among the superstimulation protocols commonly used, estradiol (E2) and progestins/progesterone (P4) releasing devices are commonly used in South America to synchronize follicle wave emergence [4,5]. In these protocols, FSH treatment is initiated at expected time of follicular wave emergence, 4 days after the E2 plus P4 treatment. Ovulation time is controlled by delaying P4 device removal and administering GnRH or pLH at set times thereafter [4–10]. Fixed-time artificial insemination (FTAI) of superstimulated donors is normally done 12 and 24 h after the GnRH/pLH treatment.

The present studies were designed to test the hypothesis that delaying the time of induction of ovulation enhances ova/embryo production in superstimulated, lactating Holstein donor cows undergoing FTAI. The current studies also examined the effect of delaying the time of P4 device removal (Experiment 1) or adding a second norgestomet implant (Experiment 2) during the superstimulation protocol.

2. Materials and methods

2.1. Lactating cows

The two experiments were conducted on two farms located in the states of São Paulo and Minas Gerais, Brazil. In both experiments, cycling Holstein donors, with a body condition score between 2.5 and 3.5 (1–5 scale) were used [11].

Experiment 1 was conducted in an institutional herd (Unidade de Educacao e Producao- UEP, Zootecnia III da Escola Agrotecnica Federal de Muzambinho, Minas Gerais, Brazil). Twelve lactating Holstein donor cows housed in a free-stall facility, with milk production of 32.0 ± 1.3 kg/day (mean ± SEM) and >100 days in milk were used. Animals were fed according to the nutritional requirements of lactating dairy cows [12].

Experiment 2 was conducted in a commercial herd in southwest Brazil. Forty lactating Holstein cows housed in a free-stall facility were used. Donors were a mean (± SEM) 351.5 ± 39.5 days in milk and producing 30.9 ± 1.2 kg/day. Cows were milked three times daily at approximately 8 h intervals and fed silage and a corn and soybean meal-based concentrate diet, sufficient to exceed the nutritional requirements of lactating dairy cows [12].

2.2. Superstimulation treatment protocols

2.2.1. Experiment 1

Holstein donor cows (n = 12) were randomly allocated into one of four treatments groups with time of P4 device removal (24 or 36 h after the PGF administration) and the time of administration of 25 mg of pLH (48 or 60 h after the first PGF), in a *Latin Square* (cross-over) design. Cows were superstimulated four times at approximately 5-wk intervals. Cows received an intravaginal P4 device containing 1 g of P4 (DIB, Syntex SA, Argentina) 1 day before (Day −1) receiving and 2.0 mg estradiol benzoate im (estradiol benzoate (EB); RIC-BE, Tecnopec, Brazil; Day 0); superstimulation treatments were initiated on Day 4 with a total dose of 200 mg NIH-FSH-P1 (Folltropin-V, Bioniche Animal Health, Belleville, ON, Canada), divided into eight decreasing doses (40%, 30%, 20% and 10%) administered I.M. twice daily, over 4 days (i.e., Days 4–7). On Day 6, cows received two doses of PGF (150 μg D cloprostenol, Prolise, ARSA, Argentina) 12 h apart, and on Day 7, P4 devices were removed in the AM (P24) or in the PM (P36). Furthermore, cows were randomly reassigned to receive 25 mg of pLH (Lutropin-V, BionicheAnimal Health) at 48 h (LH48) or 60 h (LH60) after the first PGF. Cows were inseminated 12 and 24 h after pLH. On Day 15, nonsurgical (transcervical) ova/embryo collections and evaluations were consistently done by the same veterinarian. Treatment protocols are shown (Fig. 1).

2.2.2. Experiment 2

Holstein donor cows (n = 40) were randomly allocated into four treatments in a 2 × 2 factorial design with the number of norgestomet implants (one or two) utilized during the superstimulation protocol and the time of administration of GnRH (48 vs. 60 h after the first PGF) as main effects. On Day 0, cows received either one or two ear implants, each containing 3 mg of norgestomet (Crestar, Intervet, Boxmeer, Netherlands) and 3.0 mg of estradiol benzoate I.M. (EB; Index Farmaceutica, Brazil) and 100 mg of progesterone im (Afisterone, Hertape Calier, Saude Animal, Brazil) simultaneously. Superstimulation treatments were initiated on Day 4 with a total dose of 400 mg NIH-FSH-P1 (Folltropin-V, Bioniche Animal Health), divided into eight decreasing doses (40%, 30%, 20% and 10%) administered im twice daily im s over 4 days (Days 4–7). On Day 6, cows received two doses of PGF (Preloban, Intervet) 12 h apart. The norgestomet implants were removed in the PM of Day 7. Cows were randomly assigned to receive 200 μg of Gonadorelin (Fertagyl, Intervet) at 48 h (GnRH48) or 60 h (GnRH60) after the first PGF and were inseminated 12 and 24 h later. On Day 15, nonsurgical (transcervical) ova/embryo collections and evaluations were consistently done by the same veterinarian.

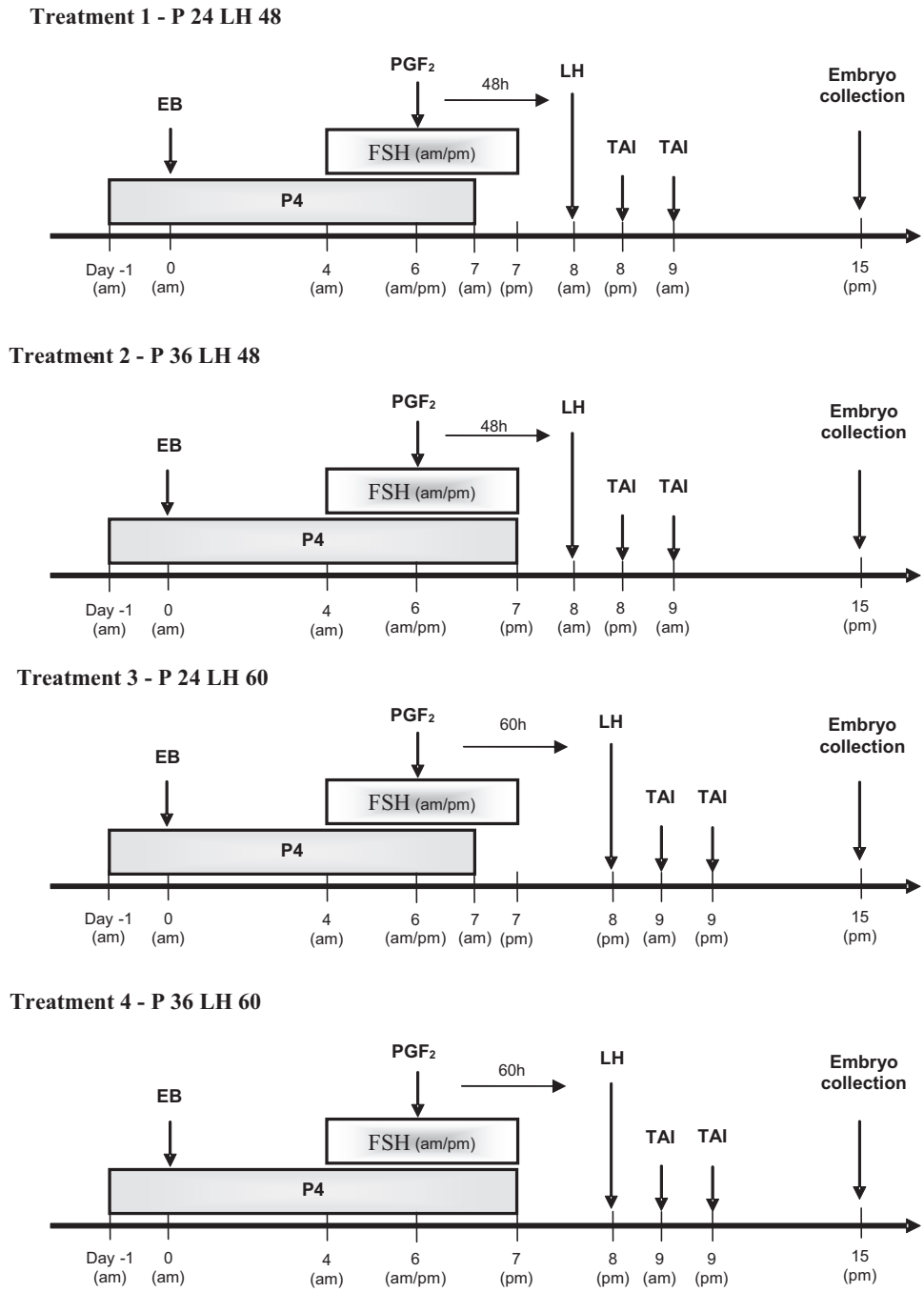

Fig. 1. Treatment protocols for Experiment 1. Cows received an intravaginal progesterone (P4) device on Day-1 and an injection of estradiol benzoate (EB) I.M. 24 h later (Day 0). All cows received twice daily injections of FSH I.M. over 4 d starting on Day 4. On Day 6, all cows received PGF concurrent with each FSH treatment. Cows in the P24 treatment had P4 devices removed 24 h after the first PGF, whereas cows in the P36 treatments had P4 devices removed 36 h after the first PGF. Cows in the LH48 treatment received 25 mg of pLH 48 h after the first PGF and were inseminated 12 and 24 h later. Cows in the LH60 treatment received pLH 60 h after the first PGF and were inseminated 12 and 24 h later. Ova/embryos were collected 7 d after the pLH treatment (Day 15) by a nonsurgical technique.

2.3. Ultrasound examinations

In both experiments, transrectal ultrasonography (Aloka SSD 500, 5 MHz linear transducer, Aloka, Inc., Tokyo, Japan) of both ovaries was done immediately before the ova/embryo collection on Day 15 in all cows) to determine the superovulatory response (number of CL). In Experiment 1, additional examinations were done in all cows on Day 4 (to quantify the number of follicles at the time of wave emergence) and on Day 8 (to quantify the number of follicles >10 mm of diameter; superstimulatory response). After pLH treatment, cows were ultrasonically examined twice a day until Day 11, to determine the occurrence of ovulations. Ovulations were considered to have occurred when large follicles (>10 mm), previously recorded were no longer present. The interval from the first to the last ovulation between Days 8 and 11 was also recorded.

2.4. Embryo collection and evaluation

In both experiments, ova/embryos were collected non-surgically on Day 15 with Dulbecco's phosphate-buffered saline (PBS, Nutricell Nutrientes Celulares, Campinas, SP, Brazil) supplemented with 1% fetal calf serum (Nutricell Nutrientes Celulares). Total ova/embryos, fertilized ova and Grades 1 (Excellent or Good), 2 (Fair) and 3 (Poor) embryos were classified according to the International Embryo Transfer Society (IETS) Manual [13]. Grades 1 and 2 embryos were considered suitable for freezing, whereas Grades 1, 2, and 3 embryos were considered transferable.

2.5. Statistical analyses

Data were analyzed by the SAS System for Windows, Version 9.2 (SAS Institute, Inc., Cary, NC). Data were tested for residual normality and homogeneity of variance by the Guided Data Analysis. Data transformation was employed whenever necessary.

A Poison distribution was assumed for the categorical response variables in both experiments. Procedure GLIMMIX was used, with the effect of donor as a random effect. In Experiment 1, the effects of donor, replicate, time of P4 device removal (24 or 36 h after the first PGF treatment), time of pLH treatment (48 or 60 h after the first PGF) and their interactions were included in the statistical model. In Experiment 2, dependent variables, including the effects of number of norgestomet implants (one or two), time of GnRH treatment (12 or 24 h after the first PGF) and their interactions were included in the statistical model.

Data are presented as means ± SEM. The level of significance to reject the null hypotheses (Ho) was 5%, and a variable was considered statistically different when $P \leq 0.05$, whereas $P < 0.10$ was considered to indicate a tendency for a difference.

3. Results

3.1. Experiment 1

Data for the end points examined in Experiment 1 are summarized in Table 1. The numbers of follicles on

Table 1
Superovulatory response (mean ± SEM) of lactating Holstein cows following fixed-time artificial insemination (FTAI) when P4 devices were removed 24 or 36 h after the first PGF and pLH was administered 48 or 60 h after the first PGF in the superstimulation protocol. Experiment 1.

	Treatments[a]				Effect (*P values*)[b]		
	P 24 h		P 36 h				
	LH 48 h	LH 60 h	LH 48 h	LH 60 h	P	LH	PxLH
No. cows	12	12	12	11	—	—	—
No. follicles at first FSH treatment	14.0 ± 1.8	13.5 ± 1.4	13.8 ± 0.8	12.4 ± 1.5	0.48	0.32	0.62
No. ≥ 10 mm follicles at pLH	10.2 ± 1.3	13.2 ± 2.9	8.8 ± 1.2	13.6 ± 3.3	0.39	0.005	0.27
Number CL at embryo collection	5.6 ± 1.4	7.2 ± 1.9	5.4 ± 1.1	8.8 ± 1.9	0.60	0.008	0.17
Ovulation rate (%)	49.7 ± 8.3[b]	50.0 ± 8.1[b]	56.5 ± 7.0[b]	65.7 ± 6.7[a]	<0.001	0.001	0.05
Total ova/embryos	4.0 ± 1.2[b]	4.8 ± 1.3[ab]	3.6 ± 0.9[ab]	6.5 ± 1.5[a]	0.87	0.01	0.07
No. transferable embryos	2.4 ± 0.8[b]	3.7 ± 1.1[b]	2.2 ± 0.7[b]	6.3 ± 1.4[a]	0.38	<0.001	0.03
Transferable embryos (%)	52.8 ± 10.1[c]	69.1 ± 8.9[b]	59.6 ± 12.6[c]	88.5 ± 8.9[a]	<0.001	<0.001	0.04
No. freezable embryos	2.3 ± 0.8[b]	3.5 ± 1.1[ab]	1.9 ± 0.7[b]	5.9 ± 1.3[a]	0.63	<0.001	0.02
Freezable embryos (%)	51.8 ± 10.1[c]	57.7 ± 10.2[b]	49.0 ± 12.6[c]	84.5 ± 8.8[a]	0.006	<0.001	<0.001
Interval from first to last ovulation (h)	17.5 ± 3.7[b]	15.6 ± 3.1[b]	17.5 ± 3.0[b]	6.5 ± 1.9[a]	<0.001	<0.001	<0.001

[a] Cows were superstimulated with 200 mg NIH-FSH-P1 (Folltropin-V) 4 days after the insertion of an intravaginal P4 device and injection of estradiol and progesterone; PGF was administered twice on Day 7.
[b] Effects of: P, Time of progesterone device removal (24 vs. 36 h); LH, Time of LH treatment (48 vs. 60 h); P xLH, P by LH interaction.

the day of first FSH treatment (Day 4) did not differ among treatments. However, the numbers of follicles >10-mm diameter at the time of pLH injection (13.4 ± 2.1 vs. 9.5 ± 0.9), CL at ova/embryo collection (8.0 ± 1.3 vs. 5.5 ± 0.9) were increased (P = 0.005) when pLH treatment was administered at 60 h rather than 24 h after the first PGF. The effects of time of P4 device removal on these response variables were not significant (P = 0.39). However, there were interactions (P < 0.04) between the time of P4 device removal and the time of pLH treatment with an increased ovulation rate, number and percentage of transferable and freezable embryos and a shorter interval from first to last ovulation after pLH treatment (most synchronous) in the P36LH24 treatment compared to other treatments (Table 1). In addition, the interaction between the time of P4 device removal and the time of pLH treatment on the total number of ova/embryos tended (P = 0.07) toward significance. Cows treated with P36LH24 treatment produced more ova/embryos than cows subjected to P24LH48 treatment.

3.2. Experiment 2

Data for end points examined in Experiment 2 are summarized (Table 2). Although the numbers of CL and ova/embryos did not differ between treatments, there was an interaction between the number of norgestomet implants and the time of GnRH administration on the number of follicles >10 mm of diameter on the day of ova/embryo collection (P < 0.01). The administration of GnRH 24 h after the first PGF resulted in fewer follicles >10 mm at the time of ova/embryo collection, mainly in those cows receiving two norgestomet implants during the superstimulation protocol (Table 2). The number of transferable embryos was increased (P = 0.02) GnRH was administered 60 h after the first PGF (4.2 ± 1.3) compared to 48 h (2.7 ± 0.8). In addition, the number of freezable embryos was increased (P = 0.02) in cows receiving two norgestomet implants (3.0 ± 1.0) compared to those receiving only one norgestomet implant (1.5 ± 0.5). There was also a tendency for a significant interaction (P = 0.07) between number of norgestomet ear implants and the time of GnRH treatment on the percentage of freezable embryos; cows treated with two norgestomet implants had an increased percentage of freezable embryos compared to cows treated with only one norgestomet implant. Among cows treated with one norgestomet implant, the administration of GnRH 60 h after the first PGF increased (P < 0.0001) the percentage of freezable embryos compared to GnRH at 48 h after the first PGF.

4. Discussion

In this study, superovulatory response and embryo production in lactating Holstein cows was increased when the ovulatory stimulus (GnRH or pLH) was given 60 vs. 48 h after the first PGF in the superstimulation protocol. In Experiment 1, embryo production was also increased in cows that had the P4 device removed at 36 h and received pLH 60 h after the first PGF. Simi-

Table 2
Superovulatory response (mean ± SEM) of lactating Holstein cows following FTAI when cow received one or two norgestomet implants during FSH treatment and 200 μg of gonadorelin (GnRH) 48 or 60 h after the first PGF in the superstimulation protocol. Experiment 2.

	Treatments[a]				Effect (*P values*)[b]		
	One implant		Two implants				
	GnRH 48 h	GnRH 60 h	GnRH 48 h	GnRH 60 h	Implant	GnRH	Implant x GnRH
No. cows	10	10	10	10	—	—	—
Number CL at embryo collection	11.3 ± 3.9	11.0 ± 2.5	9.9 ± 2.5	10.3 ± 3.2	0.31	0.95	0.73
No. ≥ 10 mm follicles at embryo collection	2.8 ± 0.6[ab]	2.3 ± 0.5[ab]	4.2 ± 0.8[a]	1.1 ± 0.3[b]	0.46	0.001	0.01
Total ova/embryos	8.0 ± 4.0	8.9 ± 2.5	8.6 ± 2.6	8.4 ± 3.2	0.95	0.71	0.55
No. transferable embryos	2.5 ± 1.1	3.6 ± 1.4	2.9 ± 1.1	4.7 ± 2.2	0.25	0.02	0.74
Transferable embryos (%)	14.7 ± 6.5[c]	28.2 ± 9.6[b]	40.4 ± 12.7[a]	41.2 ± 14.2[c]	<0.001	<0.001	<0.001
No. freezable embryos	1.1 ± 0.6	1.9 ± 0.9	2.1 ± 0.9	3.8 ± 1.9	0.01	0.02	0.92
Freezable embryos (%)	8.2 ± 5.1[c]	13.5 ± 5.5[b]	25.5 ± 7.6[a]	30.5 ± 11.6[a]	<0.001	<0.001	0.07

[a] Cows were assigned to receive one or two norgestomet implants and estradiol and progesterone on Day 0, FSH on Day 4, PGF twice on Day 6 and were reassigned to receive 200 μg of GnRH analog at 48 (GnRH 48 h) or 60 (GnRH 60 h) hafter the first PGF of the superstimulation treatment protocol.

[b] Effects of: Implant, one vs two implants; GnRH, Time of GnRH treatment (48 vs. 60 h); or Implant xGnRH, Implant by GnRH interaction.

larly, in Experiment 2, increased embryo production was achieved when the GnRH treatment was delayed from 48 to 60 h after the first PGF. There was also a significant effect of adding a second norgestomet implant on the number and percentage of freezable embryos in Experiment 2. These protocols apparently allow a larger number of follicles to acquire ovulatory capacity prior to the ovulatory stimulus, resulting in greater superovulatory responses and increased embryo production.

The occurrence of synchronous ovulations is a critical determinant for the efficacy of FTAI in superstimulated donors. For this, superstimulation protocols should control the timing of the LH peak in order to induce synchronous ovulations. During these protocols, the LH peak can be controlled by delaying P4 device removal and administering GnRH or pLH at set times thereafter [4–10]. In *Bos taurus* beef donors, the mean interval between the ovulation induction treatment (GnRH or pLH) and ovulations ranged from 28.2 to 41.4 h depending on the timing of treatments [5]. However, most ovulations occurred between 24 and 36 h after GnRH or pLH treatment. Therefore, FTAI at 12 and 24 h after the induction of ovulation was sufficiently close to ovulation to result in high fertilization rates in the *Bos taurus* beef donors.

In Red Angus (*Bos taurus* beef) donors, delaying the removal of the P4 device prevented the occurrence of early ovulations and the administration of GnRH 24 h after P4 device removal resulted in the most synchronous ovulations, and increased embryo production [10]. However, no differences were detected between the removal of P4 Devices 24 or 36 h after the first PGF [14]. It is noteworthy that in Experiment 1 ovulation time was most synchronous in Holstein cows when the P4 device was removed 36 h after the first injection of PGF and pLH was administered 24 h later. Conversely, in Nelore (*Bos indicus*) donors, delaying the time of pLH treatment to 24 h after P4 device removal adversely affected ova/embryo quality [4]. There are physiological differences between *Bos taurus* and *Bos indicus* breeds that should be considered in the development of superstimulation protocols for FTAI [15,16].

Therefore, differences in *Bos indicus* beef donors (previously reported) from *Bos taurus* dairy donors in the present study may be attributed to differences in the stage of follicle development at the time that ovulation was induced. The diameter at which the dominant follicle diverges from the subordinates (time of selection of the dominant follicle) has been shown to be earlier and at a smaller follicle diameter in *Bos indicus* cattle (6.0–6.3 mm) [16,17] than in *Bos taurus* cattle (8.5 mm) [18]. Furthermore, follicles acquired the capacity to ovulate at a smaller diameter (7.5–8 mm) in *Bos indicus* cattle than in *Bos taurus* dairy cattle (>10 mm) [16,17]. It is likely that delaying the pLH treatment was beneficial in *Bos taurus* cattle because it allowed more follicles to acquire the capacity to ovulate, whereas this was not necessary and may have been detrimental in *Bos indicus* cattle in which follicles acquire ovulatory capacity at a smaller diameter. Therefore, giving the pLH 60 h after the first PGF administration (24 h after P4 device removal) is necessary to allow more follicles in lactating Holstein cows reach an ovulatory-size and acquire the capacity to ovulate, which should in turn result in more synchronous ovulations and more transferable embryos.

In Experiment 2, utilization of two norgestomet implants resulted in more freezable embryos. It has been reported previously that superstimulation in an environment of low progesterone concentrations results in reduced ova/embryo quality [19,20]. In addition, progesterone concentrations are reported to be low in high producing dairy cattle, apparently due to high feed intake and increased steroid metabolism [21]. Inadequate regulation of LH pulsatility by low circulating concentrations of progesterone may interfere with oocyte maturation, ovulation, luteinization, and progesterone production by the ensuing CL [22]. In two recent studies in which cyclic Holstein cows [19] or cyclic Nelore beef cows [20] were superstimulated during the first follicular wave, embryo quality was compromised when exogenous progesterone was not provided during FSH treatments. In both experiments, embryo production and quality was improved when supplemental progesterone, through the insertion of two [19] or a single intravaginal P4 device [20] was added to the superstimulation treatment protocol. Therefore, use of two norgestomet implants would have a greater progestational effect, and may have regulated LH pulsatility, which would prevent the occurrence of premature nuclear maturation, accounting for the improved ova/embryo quality observed in the present study.

It is important to note that the positive effects of the addition of the second norgestomet implant during the superstimulation protocol and delaying the ovulatory stimulus observed in lactating Holstein cows in the current study should not be directly extrapolated for superstimulated Holstein heifers or beef (*Bos taurus*) breeds. There are several physiological differences of the estrous cycle between heifers and lactating dairy cows [23–25] that could alter the superovulatory re-

sponses and embryo production. Further studies are needed to confirm these observations in dairy heifers and *Bos taurus* beef cows.

In summary, results of this study confirmed that delaying the timing of an ovulatory stimulus (GnRH or pLH) resulted in an increased superstimulatory response and embryo production following FTAI of lactating Holstein cows. The highest embryo production occurred when the exogenous progestin/progesterone source was removed 36 h after the first PGF injection and the ovulatory stimulus was given 24 h later. Also, the addition of a second norgestomet implant during superstimulation of lactating Holstein cows increased numbers of freezable embryos following FTAI.

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