Synchronization of ovulation in crossbred dairy heifers using gonadotrophin-releasing hormone agonist, prostaglandin F2α and human chorionic gonadotrophin or estradiol benzoate

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Abstract

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Girolando (Gir x Holstein) is a very common dairy breed in Brazil because it combines the rusticity of Gir (*Bos indicus*) with the high milk yield of Holstein (*Bos taurus*). The ovarian follicular dynamics and hormonal treatments for synchronization of ovulation and timed artificial insemination were studied in Girolando heifers. The injection of a gonadotrophin-releasing hormone (GnRH) agonist was followed 6 or 7 days (d) later by prostaglandin F2α (PGF2α). Twenty-four hours after PGF2α injection either human chorionic gonadotropin (hCG, GPh-d6 and GPh-d7 groups) or estradiol benzoate (EB, GPE-d6 and GPE-d7 groups) was administered to synchronize ovulation and consequently allow timed artificial insemination (AI) 24 and 30 h after hCG and EB injection, respectively. Follicular dynamics in Girolando heifers was characterized by the predominance of three follicular waves (71.4%) with sizes of dominant follicles (10-13 mm) and corpus luteum (approximately 20 mm) similar to those for *Bos indicus* cattle. In the GnRH-PGF-hCG protocol, hCG administration induced earlier ovulation (67.4 h, $P<0.01$) compared to the control group (GnRH-PGF) and a better synchronization of ovulation, since most of it occurred within a period of 12 to 17 h. Pregnancy rate after timed AI was 42.8 (3/7, GPh-d6) to 50% (7/14, GPh-d7). In contrast, estradiol benzoate (GnRH-PGF-EB protocol) synchronized ovulation of only 5 of 11 heifers from the GPE-d7 group and of none (0/7) from the GPE-d6 group, which led to low pregnancy rates after timed AI (27.3 and 0%, respectively). However, since a small number of Girolando heifers was used to determine pregnancy rates in the present study, pregnancy rates should be confirmed with a larger number of animals.

Introduction

Zebu cattle (*Bos indicus*) is predominant in Brazil and other tropical and subtropical regions. Girolando (Gir x Holstein) is a very common dairy breed in Brazil because it combines the rusticity of Gir (*Bos indicus*) with the high milk yield of Holstein (*Bos taurus*). One of the most important factors for a successful artificial insemination (AI) program is the detection of estrus, which requires time and trained personnel. In the last decade the characterization of bovine follicular dynamics by ultrasonography has provided a rational basis for pharmacological manipulation of the estrous cycle in order to synchronize ovulation and allow AI at a predetermined time (timed AI) regardless of estrous behavior.

The gonadotrophin-releasing hormone (GnRH) analogs have been shown to induce follicle luteinization or ovulation, followed by the emergence of a new follicular wave (1). GnRH analog administration followed 7 days (d) later by prostaglandin F2α (PGF2α) is a synchronization system whereby the animals show a better homogeneity of follicular development at the time of induced luteolysis (2). If a second injection of GnRH agonist is administered 36 to 48 h following PGF2α administration the ovulation is synchronized (3-5) and timed AI 16 to 24 h after the second dose of GnRH results in pregnancy rates similar to those observed in cows bred during normal estrus (4-7). Pursley et al. (3) reported that pregnancy rates after timed AI were similar to those for cows bred 12 h after estrus detection (37.8 *vs* 38.9%). On the other hand, Roy and Twagiramungu (8), waiting only 6 days between the first GnRH injection and PGF2α administration, observed a high pregnancy rate (62.2%) in fixed-time AI beef heifers.

Therefore, the main objectives of the present study were to characterize follicular dynamics in Girolando heifers and to develop hormonal treatments for the efficient synchronization of ovulation to allow timed AI in heifers.

Material and Methods

Location and animals

The experiments were carried out on a private farm (Americana) located 50 km from Botucatu, São Paulo State, Brazil (latitude 22° 51' S, longitude 48° 26' W). During the last 20 years, the mean annual temperature in Botucatu was 20.6°C and the mean temperatures for the warmest and coldest months were 23.6° and 17.4°C, respectively.

Girolando heifers aged 20 to 30 months were used in four experiments (Exp.). Body weights were approximately 300 kg (Exp. I and II), 295 ± 6.6 kg (Exp. III) and 392 ± 9.4 kg (Exp. IV). The animals were maintained on pasture (*Brachiaria decumbens*) with *ad libitum* mineral salt supplementation. Heifers from Exp. I and II also had access to a ration of 30% napier grass, 50% corn and 20% soybean (4 kg heifer^{-1} day^{-1}) for the duration of the experiments.

Experiment I

Girolando heifers (N = 20) had their estrous cycle synchronized by two intramuscular (*im*) injections of PGF2α (dinoprost trometamine, 25 mg, Lutalyse®, Upjohn, São Paulo, SP, Brazil) administered 11 days apart. After estrus detection, follicle and corpus luteum (CL) development was monitored by daily ultrasonography (Aloka SSD-500, 7.5 MHz linear transducer) in 14 heifers during a complete estrous cycle. Ovarian maps were drawn to record the relative position of follicles (≥4 mm) and CL as described previously (9).

Experiment II

Approximately ten days after the end of Exp. I (i.e., 7 to 12 days after ovulation) the same heifers (N = 14) were treated intramuscularly with 8 μg of buserelin acetate, a GnRH agonist (Conceptal®, Hoechst Roussel

Veterinária, São Paulo, SP, Brazil, d 0), followed 7 days later by PGF2α (25 mg dinoprost trometamine, *im*, Lutalyse®, Upjohn, d 7), and 24 h after PGF2α administration they received human chorionic gonadotropin (hCG) (1,000 IU *iv*; 2000 IU *im*; Vetecor®, Serono Veterinária, São Paulo, SP, Brazil, d 8, GPh-d7 group). The hCG dose was split, and 1,000 IU was given *iv* to simulate the quick increase in luteinizing hormone (LH) that occurs at the time of the ovulatory surge, and 2,000 IU was given *im* to maintain LH-like activity for an extended period of time. All heifers were inseminated artificially 20 h after hCG injection without estrus detection. Pregnancy was diagnosed by ultrasonography at approximately 30 days postinsemination.

Follicular development was examined daily by ultrasonography until hCG injection and then every 6 h until ovulation during the following 6 days. The time of ovulation was considered as the average between the last time the ovulatory follicle was seen and the first time that it was not observed on the ultrasound screen.

Blood samples (10 ml) were collected into heparinized tubes just before each hormonal treatment and immediately placed on ice. Plasma was separated by centrifugation within 4 h and then stored at -20°C until the time for progesterone radioimmunoassay (RIA). The CL was considered to be undergoing regression when plasma progesterone concentration declined 50% or more from mid-luteal phase levels to concentrations ≤1.5 ng/ml (10).

Experiment III

Twenty-three cycling heifers (with a visible CL by ultrasonography) were allocated to two groups: GP-d7 (control, N = 12) and GPE-d7 (N = 11).

The animals were treated at random stages of the estrous cycle with a GnRH analog (8 μg Conceptal®, *im*, d 0) followed by PGF2α 7 days later (25 mg Lutalyse®, *im*, d 7). After

PGF2α injection heifers from the GP-d7 (control group) were observed three times a day to detect estrus and AI was performed 12 h later. Twenty-four hours after PGF2α injection heifers from the GPE-d7 group received estradiol benzoate (EB, 0.75 mg, *im*, Estrogin®, Farmavet, São Paulo, SP, Brazil, d 8) and were inseminated 30 h later without estrous detection. Blood samples and ultrasonography were performed as indicated in Exp. II.

Experiment IV

Twenty-one heifers at random stages of the estrous cycle were divided into three groups (GP-d6, GPE-d6 and GPh-d6) and received hormonal treatments similar to those described in Exp. II (group GPh-d7) and III (groups GP-d7 and GPE-d7), except that PGF2α injection occurred 6 days after GnRH administration instead of 7 days as in Exp. II and III. Heifers from groups GP-d6, GPE-d6 and GPh-d6 were inseminated approximately 12 h after heat detection, 24 h after EB injection and 30 h after hCG injection, respectively. Blood samples were obtained and ultrasonography was performed as indicated in Exp. II.

Progesterone radioimmunoassay (RIA)

Progesterone RIA was performed according to a method previously described (9). Antiserum to bovine progesterone was a gift from Dr. Magaly Manzo (Faculdade de Ciências Veterinárias Maracay, Venezuela) The intra- and the inter-assay coefficients of variation were 4.4 and 11.9%, respectively, and the sensitivity of the assay was 0.23 ± 0.02 ng/ml.

Statistical analysis

Data were analyzed using the Statistical Analysis System (11). ANOVA considering animal as a block and comparisons of aver-

ages (Tukey test) were used to analyze wave length, maximum diameter, growth and atresia rate of the dominant follicle in heifers with 3 follicular waves (Exp. I). The F-test was used to compare the synchronization of ovulation among groups in Exp. III and IV. The Wilcoxon-Mann-Whitney test (12) was used to compare the interval from PGF2α administration to ovulation.

Results

Experiment I

Follicular dynamics in Girolando heifers was characterized by three follicular waves (71.4%); two heifers exhibited 2 waves (14.3%), one showed 4 waves (7.1%) and another animal had a short estrous cycle. Consequently, statistical analysis was performed only in the animals with three follicular waves. The dominant follicle from the second wave was smaller when compared to dominant follicles from the first and third waves (P<0.01). The third wave was shorter (P<0.01) than the others and became the ovulatory follicle. Growth and atresia rates did not differ among dominant follicles. The maximum diameter of the CL was approximately 20 mm and the interovulatory interval was 20.6 ± 0.4 days (Table 1).

Experiment II

The injection of a GnRH agonist induced ovulation in 50% (7/14) of the animals and follicular atresia in the remaining ones. The emergence of a new follicular wave occurred 2.1 ± 0.1 days after GnRH injection in all heifers.

The sharp decline in progesterone concentrations (<1.5 ng/ml) indicates that PGF2α injection caused luteolysis in 11 of 14 heifers (Figure 1), i.e., 21% of the heifers did not respond to PGF2α administration.

PGF2α followed 24 h later by hCG administration induced ovulation in 12 of 14

(85.7%) heifers. The interval between hCG injection and ovulation was 31.6 ± 3.9 h and 10 of 12 ovulated within a period of 17 h. In two heifers the ovulation was not synchronized by the hormonal treatments and occurred 11.6 or 68.4 h after hCG injection. The pregnancy rate after timed AI was 50% (7 of 14).

Experiment III

The injection of GnRH induced ovulation in 43.5% (10/23) of the animals from the two groups, and emergence of a new follicular wave occurred 2.2 ± 0.2 days after GnRH administration in 74% of the heifers (17/23). In the heifers with no emergence of a new wave the follicles kept growing after GnRH injection.

The sharp decline in progesterone concentrations indicates that PGF2α injection caused luteolysis in all heifers from the GP-d7 and GPE-d7 groups (Figure 2).

In the control group (GP-d7) ovulation rate was 75% (9/12) while in the GPE-d7 group, PGF2α followed 24 h later by EB administration induced ovulation in 63.6% (7/11) of the heifers. Two heifers (one from GP-d7 and one from GPE-d7) ovulated 2 days before PGF2α injection and one ovulated on the day when PGF2α was administered (GPE-d7). Furthermore, three heifers from GP-d7 and four from GPE-d7 did not ovulate until 7 days after PGF2α administration. The injection of estradiol benzoate induced estrus behavior in all heifers from GPE-d7 in spite of the fact that 4 of 9 heifers did not ovulate after injection of EB. In the control group (GP-d7), only 58.3% of the heifers were observed in heat.

Excluding the animals that ovulated before (N = 2) or on the day (N = 1) of PGF2α administration, the intervals from PGF2α injection to ovulation were 103.7 ± 10.1 and 66.5 ± 3.5 h for GP-d7 and GPE-d7, respectively. Consequently, the administration of EB induced ovulation 37 h earlier when

compared to the control group (P<0.05) and caused a more precise synchrony of ovulation, which occurred within a period of 18 and 66 h, respectively, for GPE-d7 and GP-d7 (P<0.05). The pregnancy rate after timed AI was 27.3% (GPE-d7), as opposed to 41.6% in heifers inseminated after estrus detection (GP-d7).

Experiment IV

The injection of GnRH induced ovulation in 7 heifers (2 from GP-d6 and 5 from GPh-d6) and follicular atresia in 11 of 21 heifers (5, 2 and 4, respectively, from GP-d6, GPh-d6 and GPE-d6). The emergence of a new follicular wave occurred 2.2 ± 0.2 days after GnRH injection in 74% (17/23) of the heifers.

Progesterone concentrations indicate that 14, 71 and 100% of the heifers from GP-d6, GPh-d6 and GPE-d6 did not respond to PGF2α treatment, respectively (Figure 3). However, the percentage of animals detected in heat was 100, 0 and 85.7% for GP-d6, GPh-d6 and GPE-d6, respectively.

Table 1 - Comparison of follicular and luteal characteristics (mean ± SEM) between Girolando heifers with 2 or 3 follicular waves.

Values followed by different superscript letters within columns differed significantly (P<0.05* and P<0.01**) (Tukey test).

Characteristics	Follicular waves	
	2 waves (N = 2)	3 waves (N = 10)
Detection day		
First dominant follicle	–	0.2 ± 0.1
Second dominant follicle	10.5 ± 2.5	7.3 ± 0.4
Third dominant follicle	–	13.5 ± 0.5
Wave length (days)		
First dominant follicle	17.5 ± 0.5	14.9 ± 0.8[a]**
Second dominant follicle	8.5 ± 1.5	12.2 ± 0.6[a]
Third dominant follicle	–	6.9 ± 0.3[b]
Maximum diameter (mm)		
First dominant follicle	10.7 ± 1.3	10.6 ± 0.4[a]*
Second dominant follicle	11.8 ± 1.3	9.1 ± 0.3[b]
Third dominant follicle	–	11.3 ± 0.3[a]
Day of maximum diameter		
First dominant follicle	6.5 ± 0.5	6.3 ± 0.5
Second dominant follicle	15.5 ± 3.5	12.4 ± 0.7
Third dominant follicle	–	19.2 ± 0.4
Corpus luteum	19.2 ± 1.3	20.3 ± 1.0
Growth rate (mm/day)		
First dominant follicle	0.8 ± 0.3	0.9 ± 0.1[a]
Second dominant follicle	0.9 ± 0.2	0.8 ± 0.1[a]
Third dominant follicle	–	1.0 ± 0.5[a]
Onset of atresia		
First dominant follicle	12.5 ± 0.5	9.8 ± 0.3
Second dominant follicle	–	16.1 ± 0.8
Atresia rate (mm/day)		
First dominant follicle	1.0 ± 0.2	1.0 ± 0.1[a]
Second dominant follicle	–	1.0 ± 0.1[a]
Interovulatory interval (days)	19.0 ± 1.0	20.6 ± 0.4

After the hormonal treatment all heifers from the GP-d6 and GPh-d6 groups ovulated, as opposed to only one heifer from the GPE-d6 group (63.3 h after the EB injection).

The interval from PGF2α injection to ovulation in the GPh-d6 group (56.6 ± 1.9 h) was significantly shorter when compared to the GP-d6 group (101.9 ± 7.2 h, P<0.01). Therefore, injection of hCG induced ovulation 67.4 h earlier (P<0.01) when compared to the control group and induced a more precise synchrony of ovulation, which oc-

curred within a period of 12 and 52 h for GPh-d6 and GP-d6, respectively. The pregnancy rate was the same (42.8%) for heifers submitted to timed AI (GPh-d6) and for those that were bred after detection of estrus (GP-d6). None of the animals from GPE-d6 became pregnant.

Discussion

In the present study the follicular dynamics of Girolando heifers was characterized primarily by the presence of three follicular waves. These patterns of follicular growth are similar to those observed in *Bos taurus* (13,14) and *Bos indicus* (9,15) heifers. Other investigators have reported the rare occurrence of one or four follicular waves (9,13-16) and the predominance of two waves (17). The reasons for variations between two and three follicular waves are not clear. However, some factors such as pregnancy (16), puberty (18), diet (19), heat stress (20) and energy balance (21) may influence follicular dynamics.

The detection of the first, second and third follicular waves in 3-wave Girolando heifers (0.2 ± 0.1, 7.3 ± 0.4 and 13.5 ± 0.5 days, respectively) occurred a little earlier (especially the third wave) compared with those of Holstein heifers (1.9 ± 0.3, 9.4 ± 0.5 and 16.1 ± 0.7 days; -0.5 ± 0.3, 9.0 ± 0.0 and 16.0 ± 1.1 days, and approximately 4, 12 and 16 days; 13,14,17). The occurrence of follicular waves was very close to that observed in Nelore heifers (1.6 ± 0.2, 9.1 ± 0.5 and 15.1 ± 0.5 days; 9). As previously reported for European (6.1 days, 5.9 days, 6.8 days; 13,14,17) and Zebu breeds (6.9 days; 9), the length of the third follicular wave (6.9 days) was significantly (P<0.01) shorter than that of the other waves.

The maximum diameter of dominant follicles (10 to 13 mm) observed in Girolando heifers was smaller than that reported for *Bos taurus* heifers (14 to 20 mm, 13 to 18 mm, 14 to 16 mm; 13,17,22) and similar to

Figure 1 - Progesterone concentrations (ng/ml, mean ± SEM) in Girolando heifers during treatment with GnRH (8 µg, day 0), PGF2α (25 mg, day 7) and hCG (3000 IU, day 8, GPh-d7, N = 14).
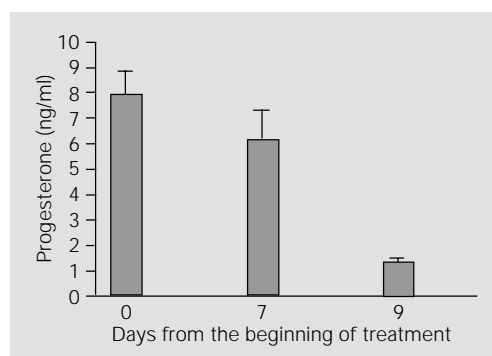

Figure 2 - Progesterone concentrations (ng/ml, mean ± SEM) in Girolando heifers during treatment with GnRH (8 µg, day 0), PGF2α (25 mg, day 7, GP-d7, N = 12) and estradiol benzoate (0.75 mg, day 8, GPE-d7, N = 11).
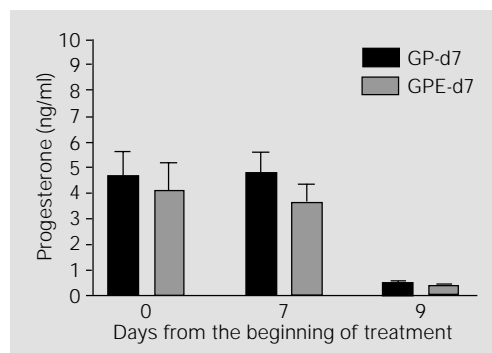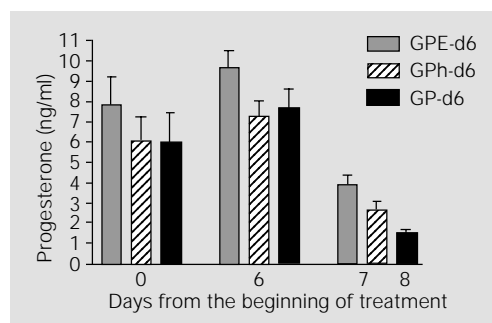

Figure 3 - Progesterone concentrations (ng/ml, mean ± SEM) in Girolando heifers during treatment with GnRH (8 µg, day 0), PGF2α (25 mg, day 6, GP-d6, N = 7) and hCG (3000 IU, day 7, GPh-d6, N = 7) or estradiol benzoate (0.75 mg, day 7, GPE-d6).

that reported for *Bos indicus* heifers (10 mm, 12 mm; 9,15). The second dominant follicle, developing during the luteal phase, was significantly smaller (P<0.01) than the others in 3-wave Girolando heifers. This result agrees with those obtained for European (14) and Zebu cattle (9).

The maximum CL diameter for 3-wave Girolando heifers (19 to 20 mm) was comparable to that obtained for Zebu heifers (17 to 18 mm; 9,15). However, the size tended to be smaller than that for *Bos taurus* (25 to 30 mm; 22). Thus, the size of dominant follicles and CL of Girolando heifers (Gir x Holstein) may be similar to those of Zebu cattle and smaller than those of *Bos taurus* breeds. However, the pattern of growth and turnover of dominant follicles is similar for Zebu and European breeds.

Injection of the GnRH agonist at random stages of the estrous cycle caused ovulation in 33 to 50% of Girolando heifers and induced a new follicular wave 2 to 3 days after its administration in 91% of the heifers (49/58). A similar ovulation rate has been reported for European heifers (54%; 6) and Zebu cows (33.3%; 23), while in European cows Pursley et al. (7) showed a higher percentage of ovulation (>85%) about 2 days after the first GnRH injection.

GnRH administration induces ovulation or atresia (24) depending on the stage of follicular development (4). Silcox et al. (25) reported that GnRH induced ovulation in 100% of growing follicles (>10 mm), 33% of follicles in the plateau phase and 0% (no ovulation) of atretic follicles. In addition, LH receptors decrease as the dominant follicle develops from growth to the plateau and regression phases (26) and as atresia is clearly being manifested (27).

In most Girolando heifers (100% Exp. II, 74% Exp. III and 86% Exp. IV) the GnRH agonist induced a new follicular wave at approximately 2 days after its administration, and as a consequence of this follicular synchronization, 86% of the heifers had a dominant follicle (>8.0 mm) at the time of PGF2α injection (day 6 or 7). The use of GnRH, in addition to inducing ovulation or atresia, stimulates recruitment of follicles directly by follicle-stimulating hormone (FSH) release within 2 to 4 h after its administration (28) and/or indirectly by an increase in FSH concentration occurring 1 to 2 days after removal of the dominant follicle (29) that contains FSH inhibitory factors such as inhibin (27).

Administration of PGF2α induced luteolysis in 85% (day 7, Exp. II), 100% (day 7, Exp. III) and 38% (day 6, Exp. IV) of the Girolando heifers. In Experiment IV, PGF2α injection did not cause luteolysis in 5 of 7 heifers from GPh-d6 or in the 7 heifers from GPE-d6, while most animals from GP-d6 presented luteolysis (6/7).

Injection of the primary GnRH agonist induced the formation of an accessory CL in 5, 0 and 2 heifers from GPh-d6, GPE-d6 and GP-d6, respectively. Considering that PGF2α is not efficient in causing luteolysis during the first 4 days after ovulation (30), a possible explanation for the reduced number of heifers that presented luteolysis in the GPh-d6 group is the presence of accessory CL (5/7) that are too young (4 days old) to respond to PGF2α administration. However, it is surprising that none of the animals from the GPE-d6 group presented luteolysis in spite of the absence of accessory CL and the presence of functional CL (progesterone >6.0 ng/ml). Pursley et al. (7) reported the absence of luteolysis in 6 of 24 Holstein heifers treated with PGF2α 7 days after GnRH agonist injection. In Nelore cows (31), PGF2α administration did not induce estrus in 54% of the animals even in the presence of a functional CL (progesterone >5.0 ng/ml) in 23 of 28 animals.

The administration of a second dose of GnRH 24 to 48 h after PGF2α causes a more precise synchronization of ovulation in both *Bos taurus* (7,32) and *Bos indicus* cows (33) and permits timed artificial insemination

(3,5,32,34,35). Pursley et al. (7) reported that all Holstein cows and 75% of the heifers ovulated within a period of 24 to 32 h after the second GnRH administration. In another study using an AI protocol of GnRH - 7 days - PGF2α - 30 to 36 h - GnRH - 16 to 24 h, the same authors (3) showed that the pregnancy rate after this treatment was similar to that for the control group (37.8 *vs* 38.9%). Additionally, Roy and Twagiramungu (8) observed a high pregnancy rate (62.2%) after timed AI in beef heifers treated with a similar protocol (GnRH-PGF-GnRH) except that GnRH was administered 6 days after PGF2α and the second dose of GnRH was injected 46 to 48 after PGF2α.

In the present study, the second dose of GnRH was replaced by estradiol benzoate or hCG. Estradiol benzoate in the absence of progesterone (<1.0 ng/ml) has been shown to induce an LH surge approximately 16 to 24 h after its administration (36). However, EB in the presence of a functional CL or when administered with exogenous progestagens decreases gonadotrophin secretion and induces atresia of the dominant follicle (37). In contrast, GnRH administration induces a short LH surge (approximately 5 h; 28), while plasma levels of hCG (LH-like activity) continue to be detectable up to 66 h after its *im* injection (32). In Girolando heifers treated with hCG (GPh-d6) ovulation occurred 67.4 h earlier (P<0.01) compared to control (GP-d6, Exp. IV) and was better synchronized since most of it occurred within a period of 12 (GPh-d6) or 17 h (GPh-d7). The pregnancy rate obtained after timed AI (50% in GPh-d7 and 42.8% in GPh-d6) was close to that obtained in Holstein heifers (56.1%) after the GnRH-PGF-hCG protocol (hCG was injected 48 h after PGF2α and all heifers were inseminated 16 h after hCG; 32). However, a small number of Girolando heifers was used. Consequently, these results must be confirmed with a larger number of animals.

The use of EB to synchronize ovulation in Girolando heifers was not as effective as hCG, because only one heifer ovulated in the GPE-d6 group and 2 of 11 heifers ovulated before the end of the hormonal treatments (GPE-d7). Excluding these 2 heifers, EB administration (GPE-d7) induced ovulation 37 h (P<0.05) earlier compared to control heifers (GP-d7) and induced a better synchrony of ovulation, which occurred within a period of 66 and 18 h for GP-d7 and GPE-d7, respectively. The low ovulation rate observed in GPE-d6 can be explained by the fact that none of the heifers presented luteolysis after PGF2α administration, showing that EB in the presence of elevated progesterone concentrations (>2.0 ng/ml) did not induce an LH surge and ovulation. On the other hand, although five of seven heifers from GPh-d6 did not undergo luteolysis, all of them ovulated after hCG injection. This may be explained by the fact that hCG, which has LH-like activity, induces ovulation acting directly on the follicles (32,38).

In the control groups the percentage of animals showing estrus was 58.3 and 100% for GP-d7 and GP-d6, respectively. These results agree with those obtained in other studies in which PGF2α administered 6 or 7 days after GnRH injection induced heat in 70 to 83% of the animals (4,5).

Estradiol benzoate injection induced estrus in most Girolando heifers (100% in GPE-d7 and 86% in GPE-d6) while none of them showed estrus after hCG injection (GPh-d6). It has been shown that administration of estrogens induces behavioral estrus in cattle (39), whereas GnRH agonist reduces the occurrence of spontaneous estrus due to functional alterations in the dominant follicles (1), which could lead to lower estradiol concentrations in the blood stream (4). Considering that both GnRH and hCG induce ovulation through LH-like activity, it is possible that hCG reduces spontaneous estrus in a similar manner as GnRH.

The pregnancy rates obtained after EB administration in groups GPE-d7 (27.3%)

and GPE-d6 (0%) were lower than those reported for Nelore cows treated with GPE-d7 protocol (45.5%; 35) and closer to those observed in Nelore heifers (N = 64) treated with the GPE-d6 protocol (33%; Barros CM, Figueiredo RA and Fernandes P, unpublished results). The high percentage of heifers showing estrus after EB administration (GPE-d7 and GPE-d6) followed by low pregnancy rates after timed AI indicates that the occurrence of heat in response to estradiol benzoate injection may not be linked to the actual time of LH surge or ovulation time. In spite of the small number of heifers used, the low pregnancy rates observed after GPE-d6 and GPE-d7 indicate that these treatments are not as promising for heifers as they are for adult cows (35).

In Experiment III, 14.3% of the animals (2 in GPE-d7 and 1 in GP-d7) ovulated before the injection of PGF2α, while no animal from GPE-d6 or GP-d6 exhibited estrus or ovulated before PGF2α administration. Results reported by Canadian researchers (8) indicate that a 6-day interval between GnRH and PGF2α injection improves estrus synchronization and reduces the occurrence of estrus before PGF2α administration.

Schmitt et al. (32) treated Holstein heifers with the GnRH-PGF-GnRH protocol for timed AI and reported a lower pregnancy rate in animals injected with GnRH 24 h after PGF2α (25.8%) when compared to those that received GnRH 48 h after prostaglandin (45.5%). Additionally, they observed that the low pregnancy rate was associated with a high incidence (34.8%) of heifers returning earlier to estrus (<16 days) after timed AI in the group of animals that received GnRH 24 h after PGF2α. In contrast, delaying the second GnRH injection (48 *vs* 24 h) allowed the ovulatory follicle to be exposed for a longer time to the increased basal pulsatile release of LH. This may have permitted the ovulatory follicle to differentiate into a normal CL with a higher steroidogenic capacity. This normal CL was capable of maintaining pregnancy and consequently the number of animals returning earlier to estrus decreased (32). Likewise, it may be possible to improve pregnancy rates in Girolando heifers treated with GnRH-PGF-EB by extending to 48 h the interval between PGF2α administration and EB injection.

Although hCG (GPh-d6 and GPh-d7 groups) and EB (GPE-d6 and GPE-d7 groups) were administered 24 h after PGF2α, the synchronization of ovulation and pregnancy rates after hCG treatment was much better than that observed after EB administration. At least two factors may have contributed to these results: hCG is capable of inducing ovulation even in the presence of a functional CL (32,40), and second the CL induced by hCG is larger and produces more progesterone than the GnRH-induced CL (40). Consequently, it may have a better capacity to maintain pregnancy.

In summary, hCG administration in both protocols, GPh-d6 and GPh-d7, was effective in synchronizing ovulation and allowed successful timed AI in crossbred heifers. On the other hand, estradiol benzoate synchronized ovulation in only 5 of 11 Girolando heifers from the GPE-d7 group and in none from the GPE-d6 group, which led to low pregnancy rates after timed AI. However, since a small number of Girolando heifers was used to determine pregnancy rates, pregnancy rates should be confirmed with a larger number of animals.

Acknowledgments

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