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Effect of induction of ovulation with estradiol benzoate at P4 device removal on ovulation rate and fertility in *Bos indicus* cows submitted to a TAI protocol

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ABSTRACT

This study aimed to minimize the number of times cattle need to be confined during protocols for TAI in beef cows treated for induction of ovulation with EB at the time of P4 device removal (P4r). In Experiment 1, cows were treated with P4 plus EB (Day 0; AM) and were allocated to one of three groups at P4r: EB8.5, EB at P4r on Day 8.5 (PM; three confinements); EB9, EB 24 h after P4r on Day 8 (AM; four confinements) and EC8, EC at P4r on Day 8 (AM; positive control; three confinements). At P4r, cows were treated with PGF_{2α} plus eCG. Ultrasonography was performed from D8 to D12. The interval from P4r to ovulation was less in the EB8.5 compared to EB9 and EC8 group. There was no difference in the ovulation rate between groups. The variability of ovulation was greater in the EB8.5 and EC8 compared to EB9 group. In Experiment 2, cows of EC8 and EB9 groups were submitted to TAI 48 to 52 h (AM) or 54 to 58 h (PM) after P4r (D10). Cows of the EB8.5 group were submitted to TAI 38 to 42 h (AM) or 44 to 48 h (PM) after P4r (D10). There was no interaction between treatments and timing of AI and no treatment effect and timing of AI on P/AI. In conclusion, the delay compared to what typically occurs by 10 h of P4r concomitant with EB administration (Day 8.5) reduced the frequency of animal confinement for the TAI protocol without affecting the reproductive efficiency and the flexibility to perform the TAI in suckled beef cows.

1. Introduction

Estradiol plus progesterone-based estrous synchronization protocols are well established for use in *Bos indicus* females (Bó et al., 2003; Sá Filho et al., 2009a; Baruselli et al., 2012). With these protocols, the administration of a progesterone (P4) device and estradiol has been used to synchronize time of emergence of a new wave of ovarian follicular development in cows (Baruselli et al., 2012) and heifers (Carvalho et al., 2008; Sá Filho et al., 2011a). The subsequent removal of the P4 device (exogenous source) and administration of prostaglandin to promote corpus luteum (CL) regression (endogenous source) leads to a decrease in circulating P4 concentrations allowing ovulation to occur. Growth of the ovulatory follicle is stimulated by using eCG (Sá Filho et al., 2010b; Sales et al., 2011) and a synchronized time of ovulation among animals is induced with a second injection of estradiol (Wiltbank and

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Pursley, 2014), which allows for insemination at a pre-determined time (Baruselli et al., 2012).

Estradiol esters, such as benzoate (Baruselli et al., 2004) and cypionate (Meneghetti et al., 2009), have been used for inducing synchronized times of ovulation in beef cows. The administration of EC at the time of P4 device removal on Day 8 or EB 9 resulted in ovulation 68.5 and 70.2 h after of P4 device removal, respectively (Sales et al., 2012). In addition, the P/AI percentage was similar between the cows treated with EB on Day 9 (57.5%; 277/482) and EC on Day 8 (61.8%; 291/471; (Sales et al., 2012). The traditional protocol in which EB is administered on Day 9, however, requires confinement of cows in facilities for treatment administrations at least four times to perform TAI (Day 0, EB + P4 device insertion; Day 8, PGF + P4 device removal; Day 9, EB; and Day 10, TAI; (Macmillan and Burke, 1996), implying a disadvantage for reproductive management. To decrease the number of times cows have to be managed in confinement facilities, EB was administered at the time of P4 device removal. The administration of EB at the time of P4 device removal on Day 8 resulted in anticipated ovulation (59.4 h after P4 device removal) and the resulting pregnancy rates were satisfactory when TAI was performed 48 h after P4 device removal (Ayres et al., 2008). The TAI 54 h after P4 device removal resulted in a decreased conception rate, probably because TAI was performed near the time of the synchronized ovulation period. Another report (Cavalieri et al., 2002) also verified that pregnancy rates to AI are less following administration of EB at P4 device removal (Day 8) compared to EB administrated 24 after device removal (Day 9).

Based on the interval from P4 device removal to ovulation and with the aim of reducing the number of times there is TAI-related management of females without decreasing reproductive efficiency and flexibility to perform the TAI (AM or PM on Day 10), two experiments were conducted. These studies were conducted to evaluate the effect of delaying, as compared to the typical timing, by 10 h P4 device removal and administration of EB (Day 8.5) for the induction of ovulation using a TAI protocol for suckled beef cows. The hypothesis was that the later than typical time of EB administration at the time of P4 device removal (on Day 8.5) would decrease the number of times cows had to be confined for treatment administrations (three times) without affecting fertility and flexibility to perform the TAI of beef cows submitted to TAI programs, compared to when EB is administered 24 h after P4 device removal (P4 device removal on Day 8 and EB treatment on Day 9; 4 managements) and EC administration at the time of P4 device removal on Day 8 of the treatment period.

2. Materials and methods

2.1. Experiment 1 – ovarian follicular dynamics in cows treated with EB or EC at different times

2.1.1. Animals and handling procedures

The first experiment was conducted at a commercial farm located in northwestern Brazil, during the spring season. Suckled multiparous Nelore (*Bos indicus*) cows ($n = 60$) with body condition score (BCS) 2.6 ± 0.1 [scale 1–5, where 1 = very thin and 5 = very fat; (Ayres et al., 2009)] were used. All cows were maintained on *Brachiaria brizantha* pastures and provided mineralized-salt and free access to water.

2.1.2. Experimental design

Hormonal treatments started between 30 and 45 days post-partum. All cows received an intravaginal progesterone device (P4; 1.0 g; DIB®, MSD Animal Health, Brazil) plus 2.0 mg im of estradiol benzoate (EB; Gonadiol®, MSD Animal Health) at random stages of the estrous cycle (designated Day 0; AM). Cows were randomly allocated to one of three groups at the time of P4 device removal and treated as follows: EC8 ($n = 14$), 1.0 mg im of estradiol cypionate (EC) at device removal on Day 8 (AM); EB9 ($n = 17$), 1.0 mg im of EB 24 h after P4 device removal on Day 8 (AM); EB8.5 ($n = 19$), 1.0 mg im of EB at the time of P4 device removal on Day 8.5 (PM). At the time of P4 device removal, cows were treated with PGF_{2a} (sodium cloprostenol; 500 µg im) plus eCG (equine chorionic gonadotropin; 300 IU im).

2.1.3. Ultrasonic examinations

Ovaries were scanned using ultrasonography with a 5.0-MHz linear-array transrectal transducer (Chison 600 VET, China). Ovarian ultrasonic examinations were performed daily from Days 8 to 10, and then twice daily from Day 11 until 12 to evaluate ovarian follicular dynamics and the interval from P4 device removal to ovulation. The largest follicles from each ovary were identified, recorded and measured (largest diameter). The time of ovulation was defined as the time of disappearance of a previously identified largest follicle (LF) from one ultrasonic examination to the next. The maximum diameter of the LF was defined as the greater diameter of the largest follicle recorded within 96 h of removal of P4 devices.

2.2. Experiment 2 – pregnancy per AI in cows treated with EB or EC at different times

2.2.1. Animals and management

The experiments were conducted on two commercial farms located in northwestern Brazil, during the spring season. A total of 1034 suckled Nelore (*Bos indicus*) cows 30–60 d postpartum and with a body condition score (BCS) of 2.9 ± 0.1 (scale 1–5, where 1 = very thin and 5 = very fat) were used. Cows were maintained on *Brachiaria brizantha* pastures and provided mineralized-salt and free access to water.

2.2.2. Experimental design

Cows were treated with estradiol plus there was administration of a P4-based TAI estrous synchronization protocol as described

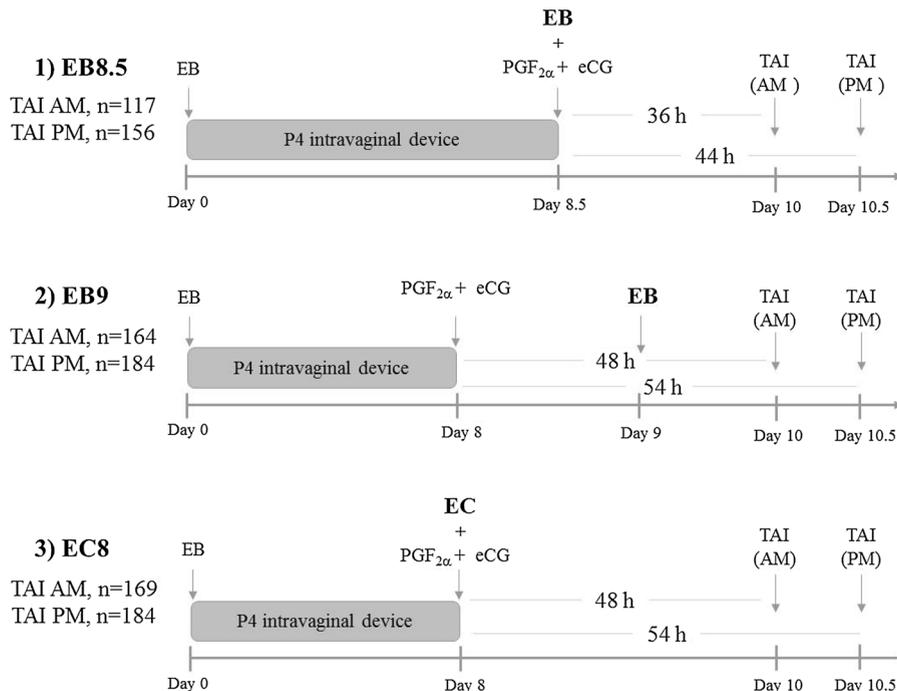


Fig. 1. Experimental design for the treatments to induce ovulation in Nelore (*Bos indicus*) cows; EB = 2.0 mg estradiol benzoate, P4 = 1.0 g progesterone, PGF_{2α} = 500 µg sodium cloprostenol, eCG = 300 IU equine chorionic gonadotropin, EC = 1.0 mg estradiol cypionate, EB = 1.0 mg estradiol benzoate, TAI = Timed artificial insemination.

for Experiment 1 and were divided using the same criteria in one of three groups: EC8 ($n = 353$), EB9 ($n = 348$) and EB8.5 ($n = 333$; Fig. 1). There was TAI 48 to 52 h (AM) or 54 to 58 h (PM) after P4 device removal (Day 10) in cows of the EC8 and EB9 groups. There was TAI 38 to 42 h (AM) or 44 to 48 h (PM) after P4 device removal (Day 10) in cows of the EB8.5 treatment group. The TAI from EC8 and EB9 groups, therefore, were performed 24 to 20 h (AM) and 18 to 14 h (PM) before the predicted time for the synchrony of ovulations and for the EB8.5 group TAI were performed 23 to 19 h (AM) and 17 to 13 h (PM) before the expected time for synchrony of ovulations. Inseminations were performed by the same technician (two replicates) who had no knowledge of treatment group. Frozen-thawed semen from three bulls that were previously proven fertile was equally distributed among the treatment groups.

2.2.3. Pregnancy diagnosis

Pregnancy diagnosis was performed by ultrasonography (Chison D600VET, Chison Medical Imaging Co., China) 30 days after TAI. The pregnancy per TAI protocol (P/AI percentage) was defined as the number of pregnant cows divided by the total number of cows mated using TAI. The detection of an embryonic vesicle with a viable embryo (presence of a heartbeat) was used as an indicator of pregnancy.

2.3. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System for Windows-SAS. In Experiment 1, the variables evaluated were diameter of LF on Day 8 (mm), maximum diameter of the LF (mm), maximum diameter of the ovulatory follicle (OF; mm), interval from P4 device removal to ovulation (h) and ovulation rate (%). Continuous data were tested for normality of the residuals and analyzed using the UNIVARIATE procedure (transformed when necessary) and were subjected to the Bartlett's test to assess homogeneity of variances. All values were expressed as mean \pm SEM. The GLIMMIX procedure with Tukey adjustment was used to determine significant differences among groups. In Experiment 2, values for the binomial variable, P/AI, were analyzed using the PROC GLIMMIX procedure of SAS. Explanatory variables such as treatment, farm, timing of AI, BCS at Day 0 and sire were included in the model as classes. All two-way interactions were tested in logistic regression models. Data were analyzed by a multivariate logistic regression using the LOGISTIC procedure of SAS. Variables were removed by backward elimination, based on the Wald statistics criterion when $P > 0.20$ for the final model. In Experiment 2, variables included in the final model for analysis of P/AI percentages were treatment (EB8.5, EB9 and EC8), timing of AI (AM and PM) and BCS at Day 0 (categorized as ≤ 2.5 or > 2.5). An adjusted odds ratio (AOR) and a 95% confidence interval (CI) were generated when conducting the logistic regression procedures. Results are presented as proportions and AOR. The P/AI percentage was analyzed using the GLIMMIX procedure of SAS.

Table 1

Ovarian follicular responses (mean \pm SEM) of suckled Nelore (*Bos indicus*) cows submitted to an estradiol plus P4-based TAI protocol with differing times and types of ovulation induction (EB8.5, EB9 and EC8 groups).

Item or Variable	EB8.5	EB9	EC8	P value
No. animals	19	17	14	
Diameter of LF on Day 8 (mm)	10.2 \pm 0.6	9.8 \pm 0.4	10.7 \pm 0.5	0.72
Maximum diameter of the LF (mm)	12.6 \pm 0.4 ^b	13.3 \pm 0.5 ^{ab}	14.9 \pm 0.6 ^a	0.01
Maximum diameter of the OF (mm)	12.5 \pm 0.4 ^b	12.9 \pm 0.5 ^{ab}	14.5 \pm 0.5 ^a	0.01
Interval from P4 device removal to ovulation (h)	60.6 \pm 1.7 ^b	72.8 \pm 0.8 ^a	72.9 \pm 1.6 ^a	0.01
Ovulation rate (%)	100.0 (19/19)	94.1 (16/17)	92.9 (13/14)	0.98

LF = largest follicle; OF = ovulatory follicle.

3. Results

3.1. Experiment 1 – ovarian follicular dynamics in cows treated with EB and EC at different times

Of the 60 cows initially used in these experiments, 10 (16.7%) did not respond (absence of a LF \geq 6 mm at P4 device removal) to the TAI protocol and were, therefore, removed from the study. The data for effects of treatments are summarized in Table 1. Interval from P4 device removal to ovulation ($P = 0.001$), maximum diameters of the LF ($P = 0.01$) and of the ovulatory follicle ($P = 0.01$) were different between the EC8 and EB8.5 groups. Ovulation rate after the TAI protocol was similar between the EB8.5 (100.0%; 19/19), EB9 (94.1%; 16/17) and EC8 (92.9%, 13/14) groups. The variability of ovulation, however, was greater ($P = 0.001$) in the EB8.5 and EC8 compared to the EB9 group (Fig. 2).

3.2. Experiment 2 – pregnancy per AI in cows treated with EC and EB at different times

There was no interaction between treatment groups and timing of AI ($P = 0.40$) and between treatments and BCS ($P = 0.20$) on P/AI percentages (EB8.5 AM = 64.8 and EB8.5 PM = 55.1%; EB9 AM = 66.5 and EB9 PM = 66.9%; and EC8 AM = 68.4 and EC8 PM = 63.6%; Table 3). There was no effect of treatment for induction of ovulation (EB8.5, EB9 and EC8; $P = 0.15$) and timing of AI ($P = 0.11$) on P/AI percentages (Table 2). A greater BCS on the first day of administration of the estrous synchronization protocol was associated with increases in P/AI percentages ($P = 0.05$; Table 2).

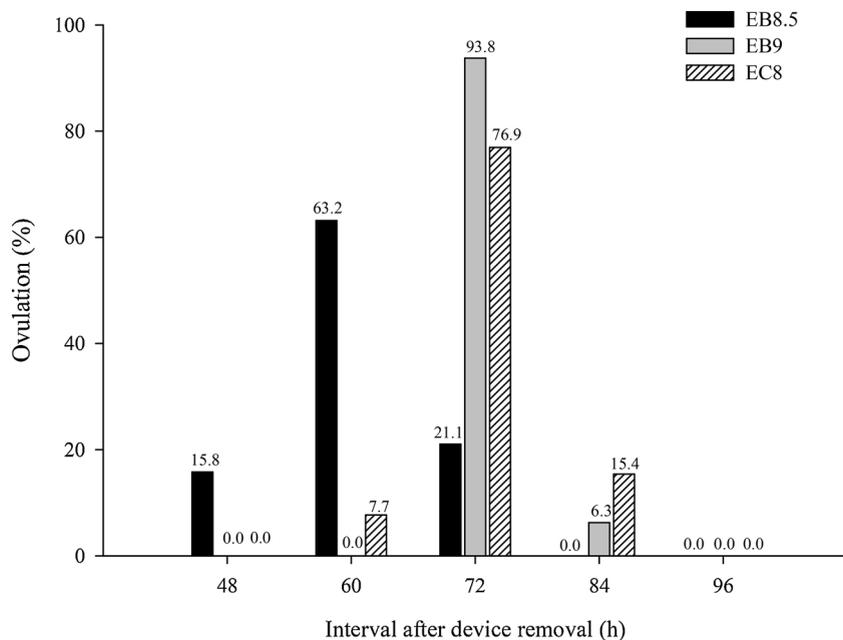


Fig. 2. Distribution of the time of ovulation (hours) after P4 device removal in Nelore cows (*Bos indicus*) submitted to TAI in the EC8 ($n = 14$) and EB8.5 ($n = 19$) treatment groups at the time of P4 device removal or in the EB9 group 24 h after device removal ($n = 17$); Homogeneity of variance in the interval to ovulation differed between treatments, being greater in the EB8.5 and EC8 compared to the EB9 group ($P = 0.001$).

Table 2

Risk factors for P/AI percentage with the use of three estradiol/progesterone-based estrous synchronization protocols in suckled Nelore (*Bos indicus*) cows.

Variable	P/AI, % (n/n)	Adjusted OR (95% CI) [*]	P
Treatment			
EB8.5	60.1 (200/333)	–	0.15
EB9	66.7 (232/348)	–	
EC8	66.0 (233/353)	–	
Timing AI			
AM	66.5 (339/510)	–	0.11
PM	62.2 (326/524)	–	
BCS category			
Low (2.0 to 2.5)	59.6 (127/213)	Referent ^{**}	0.05
Moderate to High (> 2.5)	65.5 (538/821)	1.32 (0.97-1.80)	

* OR = odds ratio; CI = confidence interval.

** Referent = reference group for adjusted risk ratio.

Table 3

P/AI percentage in Nelore cows after imposing different treatments for induction of ovulation with estradiol benzoate (EB) at time of P4 device removal (Day 8.5, three confinements, EB8.5) or 24 h after device removal (Day 9, four confinements, EB9) and with estradiol cypionate (EC) at P4 device removal (Day 8, three confinements, EC8); TAI was performed on Day 10 AM or PM.

	EB8.5		EB9		EC8		Treat	TAI	Treat* TAI
	AM	PM	AM	PM	AM	PM			
P/AI, % (n/n)	64.4% (114/177)	55.1% (86/156)	66.5% (109/164)	66.9% (123/184)	68.4 (116/169)	63.6% (117/184)	0.15	0.11	0.40

4. Discussion

This study, to the best of our knowledge, is the first where there was assessment of a program to minimize need for animal confinement for TAI treatment administrations without compromising fertility in beef cows using EB at a later time of P4 device removal for TAI at either AM or PM than that typically imposed. There was comparison of the effects of three protocols for synchronizing time of ovulation among animals using treatments based on the use of estradiol, intravaginal release of P4 and TAI and comparison of follicular responses and fertility in Nelore (*Bos indicus*) cows. Even though there were differences in time of EB administration, the ovarian response and P/AI percentages of *Bos indicus* cows were similar among treatment groups in the present study. Furthermore, there were no differences in the P/AI percentage when AI was performed on Day 10 AM or PM. The experimental hypothesis was confirmed because the fertility in cows treated with EB at a delayed time of P4 device removal, as compared that which typically occurs, was similar to when EB was administered 24 h after P4 device removal and EC was administered at the time of P4 device removal when there was use of a P4-based TAI protocol in *Bos indicus* beef cows.

The main contribution of the present study was the reduction in the need for animal confinement for imposing the TAI protocol without decreasing the flexibility of timing of AI and P/AI percentage with imposing of an estradiol and P4-based TAI protocol in *Bos indicus* cows using EB for induction of ovulation at the time of P4 device removal. In a previous study in which there was EB administration at the time of P4 device removal (Day 8, AM) and consequently a reduction in need for animal confinement for imposing the TAI protocol resulted in a lesser P/AI percentage as a result of TAI performed 54 h after P4 device removal. There was the assumption that in this previous study there was an associated reduction in pregnancy rates when there was a relatively short interval from AI to ovulation, with AI being performed 2 to 6 h before ovulation (Ayres et al., 2008). In the present study, the delay by 10 h, as compared to that typically imposed, for EB administration and P4 device removal allowed more flexibility in timing of AI on D10 of the protocol, which could be performed between 38 (D10 AM; around 22 h before the predicted ovulation) and 48 h (D10 PM; around 12 h before ovulation) after P4 device removal without compromising fertility.

There were the outcomes in the present study due to the distinct response for induction of a preovulatory LH surge release when estradiol esters are used in the treatment protocol. In a previous study, cows submitted to TAI that were treated with EC for induction of ovulation had a preovulatory surge release of LH 31 h later than cows treated with EB (Sales et al., 2012). This difference in timing of the preovulatory LH surge release resulted in an expected delay of 10 h in the time of ovulation. The delay by 10 h in EB administration and P4 device removal, as compared with the timing when there is typically administrations of EB in the EB8.5 group, resulted in a day and timing of ovulation (Day 11 AM) similar to that for the EC8 (administered on Day 8 AM) and EB9 (administered on Day 9 AM) groups. This adjustment in time of EB administration and P4 device removal allowed the TAI to occur on the morning and afternoon of day 10 of the treatment protocol without there being a reduction in the reproductive efficiency with use of the TAI protocol.

In the present study, even though there was no difference in ovulation rate, cows in the EB8.5 group had a greater variability in

time of ovulation compared to EB9 group and a lesser diameter of the ovulatory follicle (OF) compared to cows in the EC8 group. Although these characteristics of follicular dynamics could be associated with a lesser fertility in cows (Thatcher et al., 2001; Vasconcelos et al., 2001; Sá Filho et al., 2010a), in the present study the P/AI percentage was similar among the treatment groups, even when AI was performed AM or PM. Macfarlane (2003) reported that when using highly fertile bulls, the pregnancy rate was similar when TAI was performed 12 or 36 h before ovulation (Macfarlane, 2003). Semen of high fertility bulls contain sperm with relatively greater motility, viability and resulting fertility rates with use of this semen is greater (Alves et al., 2016). The semen used in the present study was determined, previous to the time the present study was conducted, to result in highly acceptable fertility rates when used for AI and this may explain the absence of differences in P/AI percentages among groups in the present study.

An estrous synchronization protocol (5-day Co-Synch) was developed with the aim being reduction in the period the P4 device was in place in animals and to prolong the proestrous period [reviewed in (Day, 2015)]. The prolonged proestrus has been associated with greater circulating estradiol concentrations prior to ovulation and greater P4 concentrations in the ensuing luteal phase (Bridges et al., 2014). Furthermore, greater estradiol concentrations during the preovulatory period are important for modulation of the uterus for being regulated by the hormonal milieu during the subsequent luteal phase and were also associated with lesser embryonic losses in the time period between maternal recognition of pregnancy and placental attachment (Madsen et al., 2015). In the present study, the treatments imposed on the EB8.5 group resulted in a relatively shorter interval from P4 device removal to ovulation. Even though there was a reduction in the duration of proestrus, there was no difference in P/AI percentage. Cows treated with estradiol have greater concentrations of estradiol in plasma during proestrus and estrus than non-treated cows (Perry and Perry, 2008), regardless the estradiol production from the dominant follicle. The supplementation with estradiol at the onset of proestrus in anestrus beef cows positively affected ovarian responses, global and specific endometrial gene expression (Sa Filho et al., 2017). The negative effects of lesser concentrations of estradiol during proestrus, therefore, may have been minimized with the treatment with EB at the P4 device removal. More studies, however, need to be conducted to assess the effect of a reduction in the duration of the period of proestrus when using P4/E2 protocols.

Other factors may affect the outcomes when there is TAI synchronization protocols imposed in *Bos indicus* cows, such as body condition score (BCS) at the beginning of TAI protocol, parity (primiparous and multiparous), breed and bull used for AI (Bó et al., 2003; Meneghetti et al., 2009; Sá Filho et al., 2011b; Sales et al., 2012). In the current study, there were no interactions between treatment with estradiol and BCS. The risk for a lesser P/AI percentage, however, was greater in cows with a moderate to high BCS. Similarly, results of some studies with beef cows indicate that cows with a relatively lesser BCS (less than 2.5 on a 1–5 scale) may have a lesser fertility when submitted to TAI (Sá Filho et al., 2009b, Sá Filho et al., 2011b; Sales et al., 2012). These findings reinforce the need for nutritional supplementation during late gestation and the early postpartum period, when the quantity and quality of pasture in tropical regions does not meet nutritional needs for maintenance or improvement of BCS before there is imposing of a TAI program.

In conclusion, the delay in 10 h in P4 device removal and administration of EB, as compared with what typically is used in TAI programs, for the induction of ovulation (Day 8.5) reduced the frequency of need for animal confinement for imposing the TAI protocol (four to three confinements) without affecting the reproductive efficiency and the flexibility to perform the TAI (AM or PM on Day 10) in suckled *Bos indicus* beef cows.

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