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Use of injectable progesterone at the beginning of the TAI protocol is not necessary in super-early resynchronization started 14 days after artificial insemination in *Bos indicus* beef heifers

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ABSTRACT

In a protocol for the resynchronization of ovulation starting 14 days (D14) after timed artificial insemination, the effects of short-action injectable progesterone (P4i) administration and the length of treatment with an intravaginal progesterone (P4) device on follicular dynamics, the conception rate (P/AI) and the percentage of false positives were evaluated in 1065 Nelore heifers previously timed-inseminated. On D14, P4 devices were inserted, and heifers allocated, based on a 2 × 2 factorial design, to receive (P4i) or not 50 mg of P4i (NoP4i), and to remove the P4 device after 6 (6Day) or 8 days (8Day). At the time of P4 device removal (D20 and D22), non-pregnancy diagnosis was performed using vascularization of the corpus luteum (VCL) evaluated. At this time, non-pregnant heifers received 150 μg of D-cloprostenol, 0.6 mg of estradiol cypionate and 300IU of eCG. TAI was performed 48 h after P4 device removal. For these heifers, ultrasound examinations were performed at P4 device removal and at TAI to evaluate follicular dynamics and at 30 days after TAI to evaluate the P/AI. Pregnant heifers based on VCL were evaluated using B-mode ultrasonography 10 days after Doppler ultrasound to evaluate the percentage of false positives. Statistical analyses were performed using the GLIMMIX procedure of SAS. There were no interaction effects between P4i and duration of treatment with a P4 device. The P/AI was diagnosed by Doppler, 1st TAI, 2nd TAI, total and percentage of false positives did not differ between heifers receiving or not P4i. Similarly, duration of treatment with a P4 device did not influence the P/AI by Doppler, 1st TAI, 2nd TAI and total. However, the percentage of false positives diagnoses was higher in 6Day heifers ($P = 0.01$). The diameter of largest follicle at P4 device removal was greater in the 8Day heifers ($P = 0.001$), and at TAI was higher in the P4i-treated heifers ($P = 0.03$). Additionally, the percentage of false positives diagnoses was lower in heifers that ovulated to the 1st TAI protocol ($P = 0.001$). In conclusion, for resynchronization 14 days after TAI, it is not necessary to inject P4i at the beginning of the protocol. In addition, P4 device removal after 6 instead of 8 days increases the percentage of false positives because of the earlier diagnosis (20 days after TAI), but does not interfere in P/AI of resynchronization protocol. Furthermore, the percentage of false positives is higher in heifers that did not ovulate to 1st TAI protocol.

1. Introduction

Timed artificial insemination (TAI) using estradiol and progesterone is well established in suckled *Bos indicus* cows and substantially increases the reproductive efficiency of beef herds,

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leading to numerous economic benefits related to beef production [1]. TAI programs reduce the calving interval, increase conception at the beginning of the reproductive period and increase the number of pregnant cows during the breeding season and the number of calves with greater genetic merit from artificial insemination [2]. Due to these characteristics, interest has increased in the use of TAI in beef cows using resynchronization.

In beef cows, resynchronization consists of performing a new TAI protocol in cows in a herd within a short time interval before or after pregnancy diagnosis [3]. For resynchronization after a pregnancy diagnosis (RESYNC30), cows diagnosed as not being pregnant undergo an ovulation resynchronization protocol, usually 30 days after insemination, with an interval of 40 days between two inseminations. For resynchronization before a pregnancy diagnosis (RESYNC22), the ovulation resynchronization protocol begins 8 days (22 days after insemination - D22) before a pregnancy diagnosis in all females inseminated in the previous TAI without prior knowledge of the pregnancy status. On the day of progesterone device removal for the protocol started on D22, the pregnancy status is determined. Cows that are not pregnant on that day are inseminated again 2 days later, with an interval of 32 days between two inseminations. Thus, in cows under RESYNC22, the interval between inseminations is reduced from 40 to 32 days without compromising embryonic survival [4]. However, resynchronization in *Bos indicus* beef cows is still not widely used, with most breeders preferring the use of a single TAI, followed by natural mating until the end of the breeding season [5].

Recently, a new resynchronization method using Doppler ultrasound has been developed for *Bos indicus* cows [6]. This new method consists of performing an early diagnosis of non-pregnancy by evaluating luteal vascularization (VCL) from the 20th day after TAI [7,8]. With this early diagnosis using Doppler ultrasound, it is possible to start the resynchronization protocol 14 days (D14) after previous TAI, without knowing the pregnancy status of the animals (RESYNC14) [5]. However, at 13 days after TAI, the use of 1.5 mg of estradiol benzoate compromises the pregnancy rate in dairy cows [9], due to early luteolysis estrogen-induced [10,11]. In contrast, in beef heifers the administration of 1 mg of estradiol benzoate at 14 days after TAI did not compromise the conception rate [18]. Thus, due to inconsistent results of estradiol ester on RESSINC14 and their ability to induce early luteolysis [9–11], the synchronization of the emergence of the follicular wave in RESYNC14 is synchronized due to the high progesterone concentration on the first day of the TAI protocol [12] that is induced by the intramuscular administration of injectable progesterone combined with the insertion of an intravaginal P4 [5]. After 8 days (D22), the P4 device is removed, and non-pregnancy is diagnosed by evaluating luteal vascularization using Doppler ultrasound [13]. Diagnostically, females with low or no luteal vascularization are considered non-pregnant [8], and the protocol continues with the administration of equine chorionic gonadotropin (eCG), estradiol cypionate and PGF2α, with TAI being performed 48 h later (D24) [5]. Thus, RESYNC14 reduces the interval between inseminations and increases the reproductive efficiency of the herd [5,7,13]. However, using injectable progesterone to synchronize the emergence of a follicular wave may not be necessary because females may be near the beginning of the second or third follicular wave of the estrous cycle [14]. Thus, the hypothesis of this study is that in RESYNC14, it is not necessary to use injectable progesterone at the start of the super-early resynchronization protocol when a P4 device remains inserted for fewer days (6 days; TAI protocol lasting 8 days). The objective of this study was to evaluate the effect of the duration of treatment with a progesterone device and the administration of injectable progesterone at the start of the RESYNC14 protocol on follicular dynamics, the conception rate and the percentage of false positives diagnoses in Nelore heifers (*Bos indicus*) undergoing TAI.

2. Materials and methods

2.1. Animals and management

This study was conducted in a commercial farm located in northwestern Brazil. A total of 1065 Nelore heifers (24.5 ± 0.1 months old) with a body condition score (BCS) of 3.5 ± 0.1 (scale of 1–5 [15]) were used in the study. The heifers were kept on *Brachiaria brizantha* pastures and had free access to water and mineral salt.

2.2. Experimental design

All heifers were previously synchronized using a progesterone/estradiol-based protocol [16]. Thus, the heifers received an intravaginal P4 device containing 1 g of progesterone (Primer, Agener, SP, Brazil) previously used for 16 days and 2 mg of estradiol benzoate (RIC-BE, Agener, SP, Brazil) and 500 μg of cloprostenol (Estron, Agener, SP, Brazil) were administered 10 days before TAI (D-10). Eight days later (D-2), the progesterone device was removed, and 300IU of eCG (Folligon, MSD, SP, Brazil), 0.6 mg of estradiol cypionate (ECP, Zoetis, SP, Brazil) and 500 μg of cloprostenol (Estron, Agener, SP, Brazil) were administered. TAI was performed 48 h after removal of the progesterone device (D0). Fourteen days after TAI (D14), heifers again received an intravaginal device containing 0.75 g of progesterone (Prociclar, CEVA, SP, Brazil) and were distributed based on a 2 × 2 factorial design for the administration (P4i; n = 520) or not (NoP4i; n = 545) of 50 mg of short-action injectable progesterone (Afisterone, CEVA, SP, Brazil) on D14 and removal of the intravaginal P4 device after 6 (6Day; n = 543) or 8 days (8Day; n = 522). Thus, removal of the P4 device was performed at D20 for the heifers in the 6Day group and at D22 for the heifers in the 8Day group. For both groups, at the time of P4 device removal, ultrasound examinations were performed with color Doppler ultrasound (Mindray M5, China) to assess luteal vascularization and identify non-pregnant heifers based on the criteria published by Pugliesi [8]. At this time, non-pregnant heifers received 300IU of eCG (Folligon, MSD, SP, Brazil), 0.6 mg of estradiol cypionate (Cipionato HC, CEVA, SP, Brazil) and 500 μg of cloprostenol (Estron, Agener, SP, Brazil) and underwent TAI 48 h later (Fig. 1).

2.3. Ultrasound exams

B-mode ultrasound examinations (Mindray M5, 5-MHz linear

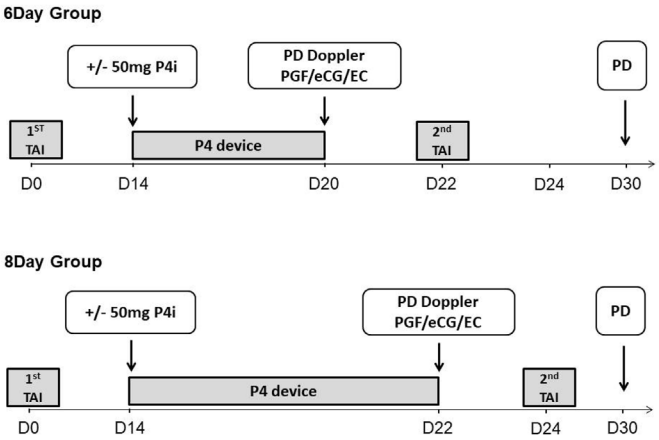

Fig. 1. Experimental design. TAI - Timed artificial insemination; P4i - short-action injectable progesterone; PGF - 500 μg of cloprostenol sodium; eCG - 300IU of equine chorionic gonadotropin; EC – 0.6 mg of estradiol cypionate; P4 device - progesterone device; PD Doppler - luteal vascularization evaluation; PD - pregnancy diagnosis.

transducer, China) were performed to evaluate the ovulation rate in the first TAI protocol at D4 (presence of CL at D4), to measure the diameter of the largest follicle at the time of progesterone device removal (D20 and D22) and the time of the second TAI (D22 and D24) and to evaluate the early ovulation rate in the second TAI (presence of the largest follicle at the time of progesterone device removal and absence of this follicle at the time of the 2nd TAI). The follicular growth rate was calculated based on the difference in the diameter of the largest follicle between day of progesterone device removal (D20/22) and day of the second TAI (D22/24) divided by 2.

The diagnosis of non-pregnancy 20–22 days after first artificial insemination was based on luteal vascularization. For this purpose, color Doppler ultrasound examinations were performed to evaluate the blood perfusion of the CL at the time of progesterone device removal (D20 and D22). In this evaluation, heifers were considered non-pregnant when they did not have a CL or when less than 25% of the CL area was vascularized [8]. In the heifers in the 8Day group with functional CL, the late luteolysis rate was calculated based on the difference in the number of heifers with functional CL between D20 and D22 divided by the number of heifers in this group with functional CL at D20.

Diagnosis of pregnancy with embryo visualization was performed by B-mode transrectal ultrasound 30 days after the first (D30) and second (D54) TAIs. The false positive diagnosis rate was calculated as the difference between heifers with functional CL at D20/22 and pregnant heifers at D30 divided by the total number of heifers with functional CL at D20/22.

2.4. Statistical analyses

Statistical analyses were performed using the Statistical Analysis System for Windows. The variables evaluated were diameter of the largest follicle at progesterone device removal, at second TAI and final follicular growth and model including treatments [Duration of progesterone device (6Day and 8Day groups) and Injectable progesterone on D0 (P4i and NoP4i groups)], BCS at the first day of the synchronization protocol and interactions. Continuous data were tested for normality of the residues and analyzed by the UNIVARIATE procedure (transformed when necessary) and subject to the Bartlett test to assess the homogeneity of variances. The GLIMMIX procedure was used to determine significant differences between groups. All values are expressed as mean ± SEM. For binomial datas (P/AI Doppler, P/AI at 1st TAI, False positives, P/AI at 2nd TAI and P/AI 1st + 2nd TAI), the variables initially included in the models were treatment, 6 or 8-day duration, and whether progesterone was injected (P4i) or not (NoP4i) at the time of insertion P4 device, bull, synchronization at 1st TAI and BCS on the first day of the synchronization protocol, and their interactions. Data were analyzed by multivariate logistic regression using the LOGISTIC procedure of SAS. Variables were removed by backward elimination based on the Wald statistic criterion when $P > 0.20$. Variables included in the final model for the analysis of P/AI were treatment and BCS. The P/AI was analyzed using the GLIMMIX procedure of SAS. Significant differences were indicated by the probability of $P \leq 0.05$.

3. Results

There was no interaction between the duration of treatment with an intravaginal P4 device and the administration of short-acting progesterone in the studied variables ($P > 0.05$). The diameter of the largest follicle at the time of progesterone device removal was similar between the heifers that received and those that did not receive injectable progesterone ($P = 0.79$) and greater in the heifers whose intravaginal P4 devices were left in place for 8 days ($P = 0.001$; Table 1). In addition, the diameter of the largest follicle at the time of the 2nd TAI was larger ($P = 0.03$) in the heifers that received short-acting injectable progesterone at the start of the resynchronization protocol (P4i group). However, the diameter of the largest follicle at the time of the 2nd TAI was similar between heifers whose intravaginal P4 devices were left in place for 6 or 8 days ($P = 0.24$; Table 1). The rate of follicular growth between the removal of the intravaginal P4 device and the 2nd TAI was similar between heifers that received and those that were not administered the P4i ($P = 0.16$). However, it was higher in heifers in the 6Day group ($P = 0.01$). The rate of early ovulation was similar between the P4i and NoP4i ($P = 0.13$) groups and between the 6Day and 8Day groups ($P = 0.14$; Table 1).

The rate of heifers with a functional CL at intravaginal P4 device removal ($P = 0.88$), the conception rate at the 1st TAI ($P = 0.37$) and at the 2nd TAI ($P = 0.47$), cumulative conception ($P = 0.65$) and percentage of false positives diagnoses [P4i - 25.9% (76/293) vs NoP4i - 31.3 (97/310); $P = 0.27$] were similar between heifers that received and those that did not receive injectable progesterone at the start of the resynchronization protocol (Fig. 2). Likewise, there was no difference in the rate of heifers with a functional CL ($P = 0.36$), the conception rate at the 1st TAI ($P = 0.20$) and at the 2nd TAI ($P = 0.84$) or cumulative conception ($P = 0.43$; Fig. 3) between the heifers whose intravaginal P4 device was left in place for 6 or 8 days. However, early Doppler ultrasound evaluation of VCL (20 days after TAI) increased the percentage of false positives diagnoses [6Day group - 33.6% (106/315) vs 8Day group - 23.3% (67/288); $P = 0.01$; Fig. 3]. In addition, the false positive diagnosis rate was lower in heifers with CL on D4 ($P = 0.001$; Table 2). Finally, the late luteolysis rate was 5.8% in the 8Day group.

4. Discussion

To our knowledge, this is the first study evaluating the need of administering short-acting injectable progesterone at the start of the super-early resynchronization protocol in *Bos indicus* beef heifers. The administration of short-action injectable progesterone at the beginning of the TAI protocol did not affect the pregnancy rate of *Bos indicus* heifers undergoing the super-early resynchronization protocol. In addition, the reduction in the time the P4 device remained in place, from 8 to 6 days, in super-early resynchronization protocols had no effect on the fertility of *Bos indicus* heifers. However, this reduction in the duration of intravaginal P4 treatment increased the rate of false positive diagnoses because the evaluation of luteal blood perfusion occurred two days earlier. Furthermore, when the P4 device was left in place for 6 days, there was a reduction in follicular diameter at the time of P4 device removal due to a decreased development time for the dominant follicle. However, the P4 device treatment duration did not affect the follicular diameter at the time of the second TAI. Thus, the hypothesis of this study was partially supported.

In the present study, follicular wave emergence and the development of persistent follicles were not evaluated. However, in super-early resynchronization protocols starting 14 days after artificial insemination, the emergence of a new follicular wave is synchronized using high progesterone concentrations [13,17], as there are controversies regarding the possible negative effects of the use of estradiol esters on the maintenance of pregnancy close to maternal recognition of pregnancy [18]. The synchronization of follicular wave emergence can be promoted by the administration of injectable progesterone [12], insertion of a progesterone device [12,19] or by the use of a combination of intravaginal P4 device with injectable progesterone [5]. The synchronization of follicular emergence with high concentrations of progesterone occurs by a direct action on the hypothalamus, reducing the production and release of GnRH and, consequently, a dose-dependent tonic release

Table 1
Effect of progesterone device treatment duration and injectable progesterone administration on follicular dynamics and early ovulation (before TAI) in Nellore heifers undergoing super-early resynchronization.

	Duration of progesterone treatment		*P*	Injectable progesterone D0		*P*
	6Day	8Day		P4i	NoP4i	
Ø LF at P4 device removal (mm)	10.3 ± 0.2	11.2 ± 0.2	0.01	10.8 ± 0.2	10.7 ± 0.2	0.79
Ø LF - 2nd TAI (mm)	12.5 ± 0.2	12.8 ± 0.2	0.24	12.9 ± 0.2	12.3 ± 0.2	0.03
Follicular growth rate (mm/day)	1.28 ± 0.1	0.98 ± 0.1	0.01	1.18 ± 0.1	1.07 ± 0.1	0.16
Early ovulation% (n/n)	3.57 (8/224)	7.23 (17/235)	0.14	3.24 (8/226)	7.3 (17/233)	0.13

Ø LF - diameter of the largest follicle; TAI - Timed artificial insemination.

of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary [20].

The conception rate at resynchronization was similar between heifers that received and those that did not receive 50 mg of short-acting injectable progesterone at the beginning of the resynchronization protocol. Thus, it is possible that only the progesterone released by the intravaginal P4 device is able to block the pulsatile release of LH and consequently promote the atresia of LH-dependent follicles [19,21]. Another possibility that justifies the similar conception rates between heifers that received and those that did not receive 50 mg of short-action P4i, although in the study we did not evaluate the synchronization of follicular wave emerge, could be that the beginning of the super-early resynchronization protocol (12–14 days after ovulation in the first TAI) may have coincided with the beginning of a new physiological follicular wave in heifers that normally exhibit a 3-follicular wave pattern [22]. In *Bos indicus* (Nelore) heifers, follicular growth typically occurs in 3 waves during the estrous cycle [14], increasing the likelihood that this may have in fact occurred, given that heifers not receiving short-action P4i at the beginning of the ovulation synchronization protocol had conception rates similar to those of heifers in the short-action P4i-treated. However, higher conception rates were expected in heifers on the shorter TAI protocol (6Day) that did not receive injectable progesterone at the beginning of the resynchronization protocol than in heifers on the 10-day TAI protocol (8Day), given that the possible negative effects on fertility promoted by the prolongation of follicular development [23]. In cows with prolonged follicular development, the premature activation of oocyte maturation (activation of meiosis with development until metaphase II, before the LH peak [24]) is observed due to increased exposure to estradiol [25] and a higher frequency of LH pulsatility [26]. This condition has been observed in cows with high milk production in which there is a delay in the time of ovulation after luteolysis, lengthening the proestrus phase [27]. Thus, similar conception rate in the 6Day and 8Day groups may be due fact that both groups had and did not have cows that had turnover after D14. However, in the present study was not possible verify this, because the emergence of follicular wave was not evaluated. Thus, in super-early resynchronization protocols, it is possible that the emergence of the follicular wave is not synchronized with the administration of drugs because the beginning of the resynchronization protocol coincides with the physiological period of follicular wave emergence [14]. As a result, there is no need to pharmacologically promote the synchronization of the follicular wave emergence.

The diagnosis of non-pregnancy using Doppler ultrasound is based on the evaluation of the vascularization of the CL and not the visualization of the embryo presents in the uterus. Thus, this Doppler ultrasound evaluation can lead to a false positive diagnosis due to delayed luteolysis, potential embryo losses [6,8,13,17] or misdiagnosis of pregnancy. The false positive rate was higher in heifers with the shortest progesterone device usage (6day group), and was not affected by the administration of injectable progesterone at the beginning of the resynchronization protocol. Independently of the treatment, the false positive diagnosis rates were similar to those observed for *Bos indicus* heifers [17] and were higher than those observed for *Bos indicus* cows [13]. Studies have shown that in *Bos indicus* heifers, there is a predominance of 3 follicular waves [14] and up to 4 follicular waves can occur [28]; this results in a longer diestrus. As a result, in heifers with longer estrous cycles, there is an increase in the time that the active CL is retained and a delay in the luteolysis mechanism, increasing the rate of false positives [17]. Thus, determining a pregnancy diagnosis

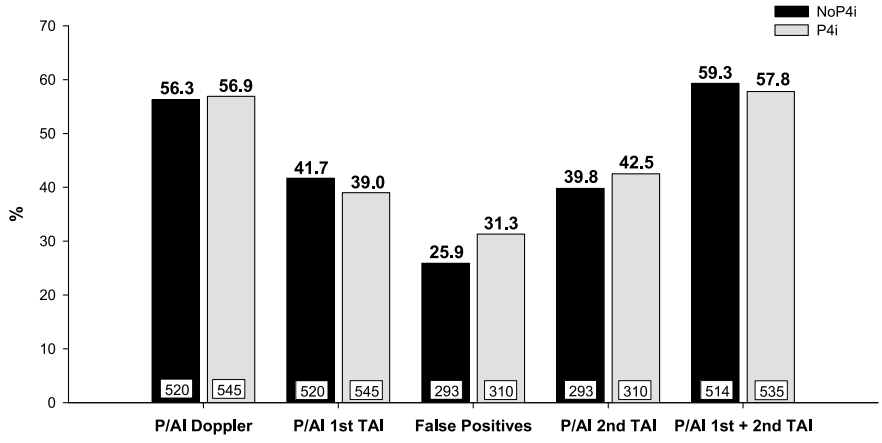

Fig. 2. Effect of injectable progesterone administration at the beginning of the super-early resynchronization protocol on the conception rate based on luteal vascularization (P/AI Doppler), at the 1st TAI, 2nd TAI and cumulative (P/AI 1st TAI + 2nd TAI) and on the false positive diagnosis rate.
Abbreviations: P4i - group of heifers that received 50 mg of injectable progesterone at the beginning of the super-early resynchronization protocol. NoP4i - group of heifers that did not receive 50 mg of injectable progesterone at the beginning of the super-early resynchronization protocol. P/AI- conception rate. TAI - Timed artificial insemination.

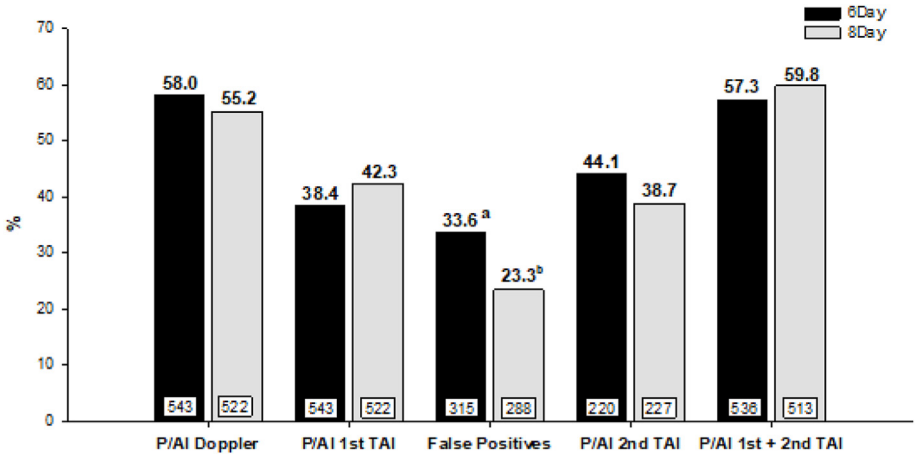

Fig. 3. Effect of duration of treatment with a P4 device on the conception rate based on luteal vascularization (P/AI Doppler), at the 1st TAI, 2nd TAI and cumulative (P/AI 1st TAI + 2nd TAI) and on the false positive diagnosis rate. [a-b] Different superscripts are different ($P \leq 0.05$).
Abbreviations: 6Day - group of heifers whose progesterone device was left in place for 6 days. 8Day - group of heifers whose progesterone device was left in place for 8 day. P/AI - conception rate; TAI - Timed artificial insemination.

Table 2
Presence of corpus luteum 96 h after 1st TAI (synchronization at 1st TAI) and conception rate at 22 Days (P/AI Doppler) and at 30 days (P/AI TAI) after 1st TAI and false positive diagnosis rate (False positive) in Nelore heifers.

	Synchronization at 1st TAI		*P*
	Yes	No	
P/AI Doppler% (n/n)	60.8 (549/903)	33.5 (51/152)	0.001
P/AI TAI% (n/n)	44.9 (406/904)	15.1 (23/152)	0.001
False Positive% (n/n)	26.0 (143/549)	54.9 (28/51)	0.001

Abbreviations: TAI - Timed artificial insemination; P/AI - conception rate.

using Doppler ultrasound two days earlier for the 6Day group may have increased the number of non-pregnant heifers that had not yet started the process of luteolysis and had functional CLs at the time of diagnosis. Luteolysis was found in approximately 6% of the animals between D20 and D22, as determined by evaluating CL vascularization, thus reinforcing the thesis that there is a delay in luteolysis in heifers. Another possible explanation for the high rates of false positives in heifers is the greater pregnancy loss observed between 22 and 30 days of gestation, when pregnancy is detected by observing the embryo in the uterus [29]. In addition, the false positive diagnoses may have been influenced by the response to the first TAI protocol because, as demonstrated in this study, the percentage of false positives diagnoses was higher in heifers that did not respond to the first TAI protocol (absence of the CL 4 days after 1st TAI). Some of these heifers probably ovulated after this evaluation, resulting in a still-developing CL at the time of luteal vascularization evaluation by Doppler ultrasound, thus increasing the number of false positive diagnoses.

It is concluded that in super-early resynchronization (14 days after TAI), it is not necessary to administered short-action injectable progesterone at the beginning of the ovulation synchronization protocol. In addition, the reduction from 8 to 6 days in the use of the progesterone device does not affect the conception rate; however, it increases the percentage of false positives pregnancy diagnosis based on luteal vascularization evaluation by Doppler ultrasonography.

CRediT authorship contribution statement

L.M. S. Simões: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, and, Writing – review & editing. **E.A. Lima:** Investigation. **A.P.C. Santos:** Investigation. **R.E. Orlandi:** Investigation. **M.P. Bottino:** Investigation. **L.A. Scandiuzzi:** Investigation, and, Resources. **J.P.M. Massoneto:** Investigation, and, Resources. **L. Inague:** Investigation. **J.C. Souza:** Methodology, and, Writing – original draft. **P.S. Baruselli:** Methodology. **J.N.S. Sales:** Methodology, Supervision, Writing – original draft, and, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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