# Comparison of fertility following use of one versus two intravaginal progesterone inserts in dairy cows without a CL during a synchronization protocol before timed AI or timed embryo transfer

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## ARTICLE INFO

## ABSTRACT

The objective was to evaluate the effect of increased progesterone (P4) during pre-ovulatory follicle growth during timed AI (TAI) or timed embryo transfer (TET) protocols. Lactating dairy cows with no CL and low circulating P4 ($\leq$1.0 ng/mL) were submitted to a protocol using one or two intravaginal P4 implants (controlled intravaginal releasing device [CIDRs]), and were bred to TAI or TET. The low P4 cows for this experiment were identified on nine farms, four utilized TAI (n = 326 of 1160 cows examined), and five utilized TET (n = 445 of 1396). All cows were synchronized by insertion of P4 implant(s) (CIDR[s]) at start of protocol (Day $-11$) and simultaneous treatment with 2 mg of E2-benzoate. After 7 days, cows were treated with PGF (Day $-4$) and 2 days later treated with 1.0-mg E2-cypionate and CIDR(s) were removed (Day $-2$). Cows received TAI on Day 0 or TET on Day 7. Cows were randomly assigned to receive either one or two CIDRs on Day $-11$ until Day $-2$ (1CIDR vs. 2CIDR). Presence of CL was determined by ultrasound on Day $-11$ and Day 7 after protocol (to determine ovulation to protocol), P4 concentrations were determined on a subset of cows (Day $-11$, Day $-4$, Day 7), and ovulatory follicle diameter was evaluated on Day 0. Pregnancy success (P/AI or P/ET) was evaluated on Days 32 and 60. The 2CIDR treatment increased circulating P4 by Day $-4$ (1.77 $\pm$ 0.23 vs. 2.18 $\pm$ 0.24 ng/mL) but had no effect on ovulation at the end of protocol (83.6 vs. 82.6%) or ovulatory follicle diameter (15.6 $\pm$ 0.3 vs. 15.3 $\pm$ 0.3 mm). If only cows that ovulated to the protocol were included, 1CIDR tended to have lower P/AI than 2CIDR by Day 32 (42.8 vs. 52.6%; P = 0.10) and Day 60 (37.7 vs. 48.1%; P = 0.08) but there was no effect on pregnancy loss. There was an interaction (P = 0.05) between ovulatory follicle diameter and CIDR treatment on P/AI (Day 60). In cows ovulating larger follicles ($\geq$14 mm), 2CIDR treatment increased P/AI compared with 1CIDR (53.3 vs. 34.9%; P = 0.02) but not in cows ovulating small follicles (<14 mm). There was no effect of treatment on P/ET on Day 32 (30.0% vs. 32.0%) or Day 60 (24.7% vs. 25.6%). Thus, these results add evidence to the concept that increased circulating P4 during pre-ovulatory follicle development may improve P/AI, most likely due to improved oocyte

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quality in cows that ovulate larger follicles, since improvement was only in cows ovulating larger follicles and no effect of preovulatory P4 was observed in cows that received ET.

## 1. Introduction

Protocols that synchronize the time of ovulation can improve reproductive efficiency and profitability of dairy herds [1–3]. Along with precise synchronizing of the time of ovulation in cycling cows, independent of phase of the estrous cycle, these protocols can also induce cyclicity in anovular cows or cows that lack a CL at initiation of the protocol. At the end of the voluntary waiting period, about 24% of dairy cows do not have a CL or have low circulating progesterone (P4) concentrations [4–7]. In addition, at the time of pregnancy diagnosis, there are 15% to 46% of cows that do not have a CL [8–11] and the presence or absence of a CL may affect success in resynchronization strategies. Cows that do not have a CL at initiation of synchronization ovulation protocols, subsequently have reduced circulating P4 concentration during development of the preovulatory follicular wave [6,12–14]. Anovular cows or cows without a CL at protocol initiation typically have reduced pregnancies per AI (P/AI) following the timed AI (TAI) [13,15,16]. Likewise, greater circulating P4 concentrations during preovulatory follicle development is generally associated with improved embryo quality [17,18] and greater P/AI [12,13,15]. For example, Bisinotto et al. [6] evaluated circulating P4 7 days before and at the time of the first GnRH of the Ovsynch protocol. Cows were classified as anovular, cycling with high P4, or cycling with low P4 at the first GnRH of Ovsynch. Cycling cows with high P4 had greater P/AI (43.0%) than cycling cows with low P4 (31.3%) or cows that were anovular (29.7%). Thus, cows that are anovular or cycling cows that are at a stage of the cycle without a CL have similar low fertility, probably due to reduced circulating P4 during the synchronization protocol.

One method to increase circulating P4 during synchronization protocols is by using intravaginal P4 implants such as the controlled intravaginal releasing device (CIDR). During a 7-day period, the CIDR (1.9 g of P4) released 610 mg of P4 or, on average, 87 mg/day of P4 into the bloodstream of the treated cow [19]. This produced an average of 2 to 3 ng/mL in nonlactating, ovariectomized Holstein-Friesian heifers [19]. However, lactating cows metabolize P4 at extremely high rates that is directly proportional ($r^2 = 0.85$) to the rate of liver blood flow [20]. Liver blood flow and metabolic clearance rate of P4 are both dramatically elevated in high-producing lactating dairy cows. Thus, a single CIDR increased circulating P4 concentrations in lactating dairy cows by only 0.78 ng/mL after 7 days of treatment [21]. However, peak circulating P4 concentrations in lactating dairy cows are much greater at 4 to 5.8 ng/mL [21], highlighting the potential inadequacy of supplementing P4 in lactating dairy cows using only a single CIDR. Nevertheless, a recent meta-analysis [16] of 25 randomized controlled studies with a total of over 16,000 cows, found that P4 supplementation during the protocol prior to TAI increased risk of pregnancy (relative risk = 1.10

at 60 days after AI). The effect was mainly in cows lacking a CL at protocol initiation (1.18) and not in cows with a CL (1.06). Recent studies with lactating dairy cows lacking a CL at initiation of a GnRH-based synchronization protocol have reported that use of two CIDRs increased P/AI to similar values found in cows with a CL [13,16]. Thus, complete restoration of fertility in GnRH-based protocols may require supplementation of two rather than one CIDR during the protocol.

In many parts of the world, esters of estradiol (E2) are utilized at both the beginning and end of the synchronization protocol to synchronize follicular wave and ovulation [22–25]. These protocols use P4 implants, such as CIDR, to maintain P4 concentrations during the protocol and prevent premature ovulation. However, in contrast to GnRH-based protocols that synchronize follicular waves by inducing ovulation at the beginning of the protocol, E2/P4-based protocols are designed to induce regression of the dominant follicle and then initiate the new preovulatory follicular wave [26–28]. Therefore, the circulating P4 concentrations at PGF treatment are reduced in cows treated with E2/P4 protocols compared with GnRH protocols (E2: 2.29 ± 0.15 ng/mL; GnRH: 2.89 ± 0.15 ng/mL) [29] due to a reduced proportion of cows with a functional CL present at time of PGF (5dCOSynch = 73.6% [597] vs. E2/P4 = 44.3% [593]) [24]. Consequently, lactating cows that lack a CL at the start of E2/P4-based protocols are likely to also necessitate treatment with two rather than one CIDR to achieve optimal fertility, as is being done in GnRH-based protocols [13,16].

Thus, the objective of this study was to compare fertility in protocols with one or two intravaginal P4 implants (CIDR) in lactating dairy cows without a CL and with low circulating P4 concentrations (≤1.0 ng/mL) at the initiation of a protocol for TAI and timed embryo transfer (TET). The hypothesis was that the use of two rather than one CIDR would result in greater P/AI but would have no effect on P/ET.

## 2. Materials and methods

This experiment was conducted at nine commercial dairy farms in Minas Gerais and Paraná State, Brazil, from June 2010 to May 2012. All animal procedures followed the recommendations of the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* [30]. During the experimental period, cows were housed in free-stall barns, with access to an adjoining sod-based paddock. Throughout the experiment, cows were milked three times daily. All procedures, including injections, ovarian ultrasonography, pregnancy diagnosis, blood collection, TAI, and TET, were performed while cows were restrained in self-locking head gates at the feedline. Cows were fed *ad libitum* a total mixed ration (TMR) based on corn silage and Tifton hay as forages, with a corn and

soybean meal–based concentrate, and minerals and vitamins, balanced to meet or exceed the nutritional requirements of lactating dairy cows [31].

### 2.1. TAI study

#### 2.1.1. Animals and treatments

By evaluating a total of 1160 Holstein cows, there were 326 (28.1%) that had no CL and reduced circulating P4 concentrations ($\leq$1.0 ng/mL) at the beginning (Day $-11$) of the protocol. At the time of randomization, cows averaged ($\pm$SEM) 138.4 $\pm$ 6.34 days in milk (DIM), yielding 30.8 $\pm$ 0.40 kg of milk/day, with body condition score (BCS) of 2.71 $\pm$ 0.02 [in a 1 (emaciated) to 5 (obese) scale], lactation number of 1.9 $\pm$ 0.07 (primiparous [= 1] n = 200; multiparous [$\geq$2] n = 126), and had been bred 1.8 $\pm$ 0.13 times. Within each farm, cows were blocked by parity (primiparous and multiparous), all cows that completed the voluntary waiting period (60 days) and were not pregnant were utilized and randomized into the study, without regard to whether they had been previously utilized in the study. Within each block, cows were randomly assigned to receive the following protocol: I. 1CIDR treatment received an intravaginal P4 insert containing 1.9 g of P4 (CIDR, Zoetis, São Paulo, Brazil) and 2.0 mg (i.m.) estradiol benzoate (EB, 2.0 mL of Estrogin, Farmavet, São Paulo, SP, Brazil) on Day $-11$, 25 mg (i.m.) dinoprost tromethamine (PGF; 5.0 mL of Lutalyse, Zoetis, Brazil) on Day $-4$, CIDR withdrawal and 1.0 mg (i.m.) of estradiol cypionate (0.5 mL of E.C.P., Zoetis, Brazil) on Day $-2$, and TAI on Day 0; II. 2CIDR treatment received the same protocol used in the 1CIDR treatment, but instead had two CIDRs inserted on Day $-11$ and removed on Day $-2$. The TAI was performed (Day 0) by experienced technicians using commercial frozen-thawed semen from 32 different bulls.

Ovaries were evaluated by transrectal ultrasonography (US, Aloka SSD-500 with a 7.5-MHz linear-array transducer, Aloka, Tokyo, Japan) on Days $-11$, 0, and 7 to determine the presence of a CL, and at Day 0 to measure the diameter of the largest follicle present. The size of the ovulatory follicle was determined by the largest follicle present on the ovary on Day 0 that corresponded to an observed CL on Day 7. Cows with follicles less than 8 mm on Day 0 but with a CL on Day 7 were defined as "early ovulators" and were not used in analyses of ovulatory follicle diameters (Control 12.3% [17/138] vs. 2CIDR 6.0% [8/133]; P = 0.07).

#### 2.1.2. Sample collection

Milk production was measured daily between Days 0 and 7, and average daily production through this interval was used in the analysis. Blood samples were collected on Day $-11$, (n = 326), Day $-4$ (n = 198), and Day 7 (n = 321), by coccygeal venipuncture into commercial 10-mL blood collection tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). Tubes were immediately placed on ice, maintained at 4 °C for 12 hours, and then centrifuged at $\times$1500$g$ for 15 minutes at room temperature (20 °C–25 °C) for serum collection. Serum was stored at $-20$ °C for subsequent P4 analysis. Serum concentrations of P4 were analyzed using the Coat-A-Count solid phase $^{125}$I radioimmunoassay kit (Diagnostic Products Inc., Los Angeles, CA,

USA) that had been previously validated in our laboratory [32]. The intra-assay coefficient of variation was 5.9% and the interassay coefficient of variation was 12.7%, and the assay sensitivity was 0.01 ng/mL.

### 2.2. TET study

#### 2.2.1. Animals and treatments

After evaluation of 1396 Holstein or Gir-Holstein crossbred cows, a total of 445 had no CL (31.9%) and reduced circulating P4 concentration ($\leq$1.0 ng/mL) at the beginning (Day $-11$) of the protocol. At the time of randomization, cows averaged ($\pm$SEM) 169.5 $\pm$ 7.81 DIM, yielding 26.3 $\pm$ 0.56 kg of milk/day, with BCS of 2.7 $\pm$ 0.02 (in a 1 [emaciated] to 5 [obese] scale) lactation number of 2.2 $\pm$ 0.08 (primiparous [= 1] n = 202; multiparous [$\geq$2] n = 243) and had been bred 1.4 $\pm$ 0.08 times. Within each farm, cows were blocked by parity (primiparous and multiparous), all cows that were past the voluntary waiting period (60 days) and not pregnant were utilized and randomized into the study, without regard to whether they had been previously utilized in the study. Within each block, cows were randomly assigned to receive the same experimental design as performed with TAI. Cows (n = 323) received an embryo on Day 7 into the uterine horn ipsilateral to the CL, transferred by a veterinarian experienced in embryo transfer (ET). The embryos used in this study were fresh in vitro-produced embryos of excellent quality (G1), embryos consisted of morula (2% [n = 5]), early blastocyst (9% [n = 29]), blastocyst (31% [n = 101]), or expanded blastocyst (58% [n = 188]), according to the International Embryo Technology Society guidelines for grading embryos [33]. Milk production was measured daily from Day 0 to Day 7 and average daily production during this interval was used in the analysis.

### 2.3. Ovulation to the protocol, pregnancy success, and pregnancy loss

Cows with CL present on Day 7 were defined as having ovulated to the protocol. Pregnancy success (P/AI or P/ET) was calculated by dividing the number of pregnant cows at the pregnancy diagnosis at 32 days or 60 days of pregnancy by the total number of cows that received TAI or TET. Pregnancy loss was calculated by dividing the number of cows that lost their pregnancy by the number of pregnant cows at the 32 days pregnancy diagnosis.

### 2.4. Statistical analysis

The experiment was analyzed as a completely randomized design. The binomial variables (ovulation to the protocol, pregnancy on Days 32 and 60, and pregnancy losses from 32 to 60 days) were analyzed using the GLIMMIX procedures of SAS (SAS Institute Inc., Cary, NC, USA) with farm as a random effect and other variables were included in the models, if appropriate (P $\leq$ 0.20), including effects of treatment, parity and their interactions, as well as DIM, BCS, and milk yield as covariates. The continuous dependent variables (i.e., lactation number, DIM, BCS, milk yield, previous AI number, P4 concentration at Day 7, and

follicular diameter) were analyzed using the MIXED procedures of SAS, and other variables that were included in the models were the effects of treatment, parity and their interactions, as well as DIM, BCS, and milk yield as covariables. The GLM procedure of SAS was used to determine if each individual measurement influenced pregnancy values linearly, quadratically, or cubically. The LOGISTIC procedure was used to determine the intercept and slope(s) values according to maximum likelihood estimates from each significant continuous order effect and the probability of pregnancy was determined. Logistic curves were constructed using the minimum and maximum values detected for each individual measurement.

Satterthwaite approximation was used to determine the denominator degrees of freedom for tests of fixed effects with the random statement containing effect of group. Covariables that were found to be not significant ($P > 0.20$) were removed from the statistical model. The results are expressed as least square means $\pm$ SEM for continuous variables. In all analyses, differences were considered significant when $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered tendencies.

## 3. Results

### 3.1. TAI

There were no differences ($P = 0.59$) between treatment groups for lactation number (Primiparous: 1CIDR = 62% [105/165] vs. 2CIDR 57% [95/161] $P = 0.36$; Multiparous: 1CIDR = 38% [60/165] vs. 2CIDR 43% [66/161] $P = 0.30$), DIM (1CIDR = $164 \pm 6.4$ vs. 2CIDR $154 \pm 6.3$ days; $P = 0.24$), milk production (1CIDR = $31.0 \pm 0.59$ vs. 2CIDR = $30.6 \pm 0.56$ kg/day; $P = 0.59$), BCS (1CIDR = $2.72 \pm 0.03$ vs. 2CIDR = $2.69 \pm 0.03$; $P = 0.50$), and previous AI number (1CIDR = $1.97 \pm 0.18$ vs. 2CIDR = $1.57 \pm 0.16$; $P = 0.24$). Treatment increased ($P = 0.05$) P4 concentration on Day $-4$ (1CIDR = $1.77 \pm 0.23$ vs. 2CIDR = $2.18 \pm 0.24$ ng/mL).

There was no effect of treatment ($P = 0.81$) on ovulation to the protocol at the end of the protocol (Table 1). The 2CIDR treatment did not increase P/AI on Day 32 ($P = 0.18$) and Day 60 ($P = 0.14$) pregnancy diagnoses, when all cows that received AI were included in the analysis. In cows that ovulated to the protocol, the 2CIDR treatment tended to increase P/AI at Days 32 ($P = 0.10$) and 60 ($P = 0.08$) pregnancy diagnoses. There was no effect ($P = 0.54$) of treatment on pregnancy loss.

Treatment had no effect on follicle diameter on Day 0 ($P = 0.45$) in cows that ovulated to the protocol (1CIDR = $15.6 \pm 0.27$ vs. 2CIDR = $15.3$ mm $\pm 0.27$). There was an interaction between treatment and follicle diameter (Day 0) on P/AI on Day 60 ($P = 0.05$; Fig. 1). A comparison was made for treatment effects between cows with smaller (<14 mm) and larger ($\geq$14 mm) ovulatory follicles (Table 2). For cows ovulating smaller follicles, there was no effect of treatment ($P = 0.75$) on P/AI; however, in cows ovulating larger follicles, cows with 2CIDRs (53.3%) had greater ($P = 0.02$) P/AI compared with 1CIDR (34.9%). For 1CIDR treatment, the cows that ovulated larger follicles had increased ($P = 0.02$) circulating P4 concentration on Day 7

after TAI, whereas for 2CIDR, there was no difference ($P = 0.26$) in circulating P4 on Day 7 for cows ovulating larger or smaller follicles (Table 2). Thus analyzing only cows that ovulated at the end of protocol, 1CIDR cows had greater circulating P4 on Day 7 after AI ($2.93 \pm 0.10$ ng/mL) compared with 2CIDR cows ($2.5 \pm 0.10$ ng/mL). For cows ovulating a larger follicle, there was greater circulating P4 on Day 7 for 1CIDR than 2CIDR cows (3.04 vs. 2.56 ng/mL; $P < 0.01$), but there was no treatment difference in circulating P4 on Day 7 in cows ovulating smaller follicles ($P = 0.49$). There was no interaction between circulating P4 concentration on Day 7 and CIDR treatment on P/AI (Day 60; $P = 0.40$) in cows that ovulated to the protocol. There was no effect of circulating P4 concentration on Day 7 on P/AI in the 1CIDR ($P = 0.11$) and 2CIDR ($P = 0.83$) treatment (Fig. 2).

### 3.2. TET

There were no differences between treatment groups in lactation number (Primiparous: 1CIDR = 45.7% [106/232] vs.

**Table 1**
Effects of treatment with one or two CIDR on ovulation at the end of the protocol, P/AI, and pregnancy loss for all cows and for cows that ovulated after the protocol.

| Item[a] | Protocol | | P value |
|---|---|---|---|
| | 1CIDR | 2CIDR | |
| Ovulation to protocol | 83.6 (138/165) | 82.6 (133/161) | 0.81 |
| P/AI[b] | | | |
| Day 32, % (n/n) | 35.5 (59/165) | 42.6 (70/161) | 0.18 |
| Day 60, % (n/n) | 31.4 (52/165) | 39.1 (64/161) | 0.14 |
| Pregnancy loss, % (n/n) | 11.5 (7/59) | 8.3 (6/72) | 0.55 |
| P/AI[c] | | | |
| Day 32, % (n/n) | 42.8 (59/138) | 52.6 (70/133) | 0.10 |
| Day 60, % (n/n) | 37.7 (52/138) | 48.1 (64/133) | 0.08 |
| Pregnancy loss, % (n/n) | 11.9 (7/59) | 8.6 (6/70) | 0.54 |

Abbreviations: CIDR, controlled intravaginal releasing device; P/AI, pregnancies per AI.
[a] Least square means (n/n).
[b] All inseminated cows.
[c] Only cows with CL on Day 7 were used in the analysis. These cows were assumed to have ovulated to the protocol.
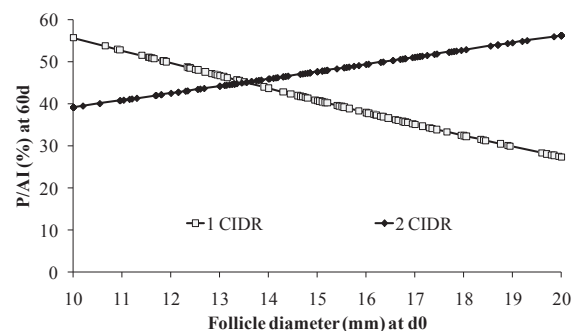


**Fig. 1.** Effect of follicle diameter at time of AI (Day 0) on P/AI at Day 60 pregnancy diagnosis in dairy cows that ovulated to the protocol (CL on Day 7). There was an interaction ($P = 0.05$) between treatment and follicle diameter on P/AI at Day 60 pregnancy diagnosis. CIDR, controlled intravaginal releasing device; P/AI, pregnancies per AI.

**Table 2**
Effects of treatment with one or two CIDR and follicle diameter at TAI on circulating P4 concentrations (ng/mL) on Day 7, and P/AI at Day 60 pregnancy diagnosis for cows that ovulated after the protocol.

| Item[a] | Follicle diameter on Day 0 | | P value |
|---|---|---|---|
| | <14 mm | ≥14 mm | |
| Distribution[b] | | | |
| 1CIDR | 31.0 (38/121) | 69.0 (83/121) | – |
| 2CIDR | 36.0 (45/125) | 64.0 (80/125) | – |
| P value | 0.24 | 0.24 | |
| P4 on Day 7 (ng/mL)[b] | | | |
| 1CIDR | $2.50 \pm 0.17$ | $3.04 \pm 0.13$ | 0.02 |
| 2CIDR | $2.34 \pm 0.15$ | $2.56 \pm 0.13$ | 0.26 |
| P value | 0.49 | <0.01 | |
| P/AI Day 60[b] | | | |
| 1CIDR, % (n/n) | 43.1 (19/38) | 34.9 (29/83) | 0.12 |
| 2CIDR, % (n/n) | 39.4 (20/45) | 53.3 (40/80) | 0.39 |
| P value | 0.75 | 0.02 | |

Abbreviations: CIDR, controlled intravaginal releasing device; P/AI, pregnancies per AI.
[a] Least square means (n/n) for P/AI at Day 60 pregnancy diagnosis.
[b] Cows with CL on Day 7 were designated as having ovulated to the protocol.

2CIDR 45.1% [96/213] P = 0.94; Multiparous: 1CIDR = 54.3% [126/232] vs. 2CIDR 54.9% [117/213] P = 0.94), DIM (1CIDR = 171 ± 9.6 vs. 2CIDR 168 ± 10 days; P = 0.85), previous AI number (1CIDR = 1.43 ± 0.17 vs. 2CIDR = 1.27 ± 0.16; P = 0.34), and BCS (1CIDR = 2.76 ± 0.10 vs. 2CIDR = 2.71 ± 0.03; P = 0.15). There was a difference between treatment groups in milk production (1CIDR = 25.6 ± 0.73 vs. 2CIDR = 27.9 ± 4.8 kg/day; P = 0.03).

There was no effect of treatment (Table 3) on ovulation to the protocol (P = 0.73), P/ET at Day 32 (P = 0.70), or P/ET at Day 60 (P = 0.87) pregnancy diagnoses, and no effect of treatment on pregnancy loss (P = 0.73).

## 4. Discussion

Absence of CL and low P4 at initiation of protocol represents a substantial problem for dairy herds (∼24%) [4–7]. Similarly in our study, the percentage of cows without a CL at protocol initiation was 30.2% (771/2556) of all lactating dairy cows, with 28.1% of cows without a CL at protocol initiation in the TAI herds (326/1160) and 31.9% in the TET herds (445/1396).
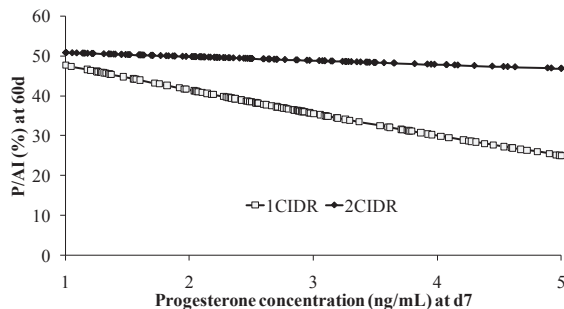


**Fig. 2.** Effect of progesterone concentration at Day 7 on P/AI at Day 60 in dairy cows that ovulated to the protocol (CL on Day 7). 1CIDR P = 0.11; 2CIDR P = 0.83. CIDR, controlled intravaginal releasing device; P/AI, pregnancies per AI.

**Table 3**
Effects of treatment with one or two CIDR on P/ET and pregnancy loss for all cows and for cows that ovulated at the end of the protocol.

| Item[a] | Protocol | | P value |
|---|---|---|---|
| | 1CIDR | 2CIDR | |
| Ovulation to protocol P/ET[b] | 73.3 (170/232) | 71.8 (153/213) | 0.73 |
| Day 32, % (n/n) | 30.0 (51/170) | 32.0 (49/153) | 0.70 |
| Day 60, % (n/n) | 24.7 (42/170) | 25.6 (39/153) | 0.87 |
| Pregnancy loss, % (n/n) | 17.7 (9/51) | 20.4 (10/49) | 0.73 |

Abbreviation: CIDR, controlled intravaginal releasing device.
[a] Least square means (n/n) at Days 32 or 60 pregnancy diagnosis or pregnancy loss from 32 to 60 days.
[b] Cows with CL on Day 7 were designated as having ovulated at the end of the protocol.

Although over 2500 cows were evaluated in this study, the use of only cows without a CL at the start of the protocol (∼30% of enrolled cows) reduced our statistical power. Therefore, the results should be viewed as preliminary as only tendencies were detected even with the ∼10% improvement in P/AI at both Days 32 and 60 pregnancy diagnoses. The relative increase in P/AI was 22.9% on Day 32 (9.8 difference/42.8) and 27.6% on Day 60 (10.4/37.7) indicating about a 25% improvement in number of cows pregnant to the protocol when two CIDRs were utilized rather than one CIDR. Although studies have not been reported that directly compared two CIDR versus 1CIDR in E2/P4-based protocols, a recent study evaluated the use of two CIDRs [13] in a 5-day CO-Synch protocol (Day −8 GnRH, Day −3 and Day −2 PGF, Day 0 GnRH + TAI). If no CL was present, the cows either received two CIDR or remained untreated (CON), and the cows with a CL were designated as diestrus. Supplementation of P4 with two CIDR implants increased plasma P4 from 0.51 ng/mL (CON) to 2.65 ng/mL (2CIDR) or over 2 ng/mL and restored fertility similar to that of cows in diestrus (CON 28.6%, 2CIDR 43.7%, Diestrus 47.3%). Similarly, a recent study [34] utilizing a 7 days Ovsynch protocol, found an increase from 0.92 ng/mL (CON) to 2.77 (2CIDR) and restoration of normal fertility in cows that initiated the protocol without a CL (CON 31.3%, 2CIDR 42.2%, Diestrus 38.4%). Thus, the results of our study are consistent with the improvements that have been reported in previous studies that utilized GnRH-based protocols but still need to be repeated with greater number of cows to assure that greater P4 supplementation results in the observed improvements in P/AI in cows with no CL at protocol initiation.

One unique aspect of our study was that the effect of increased P4 supplementation was evaluated in cows that received both TAI and TET in the same treatment protocols. This allowed us to provide some insight into the mechanisms that underlie the fertility enhancement due to increased P4 supplementation. Previous studies have demonstrated that increased P4 concentration during follicular development can alter subsequent early embryo development and embryo quality. For example, Cerri et al. [35] collected embryos 6 days after AI in cows submitted to protocols with low versus high P4. There was no effect of increased P4 on fertilization rate, but there was a tendency to have greater embryo quality in cows submitted to

protocols with increased P4 during the protocol. Rivera et al. [17] reported a greater percentage of transferrable embryos (78.6% vs. 55.9%) when comparing superovulated donors in the first follicular wave (low P4) that received or did not receive two CIDRs during the protocol. These results are in agreement with our study suggesting that the major effect of increasing P4 during the protocol is likely to be associated with improved oocyte quality or early embryo development (prior to Day 7), since no effect on fertility was observed in TET protocols.

In addition, previous studies have indicated that cows with low circulating P4 concentrations during development of the ovulatory follicle are more likely to have premature secretion of PGF by the endometrium causing short luteal phases [13,36]. Sá Filho et al. [37] detected that treatments with P4 (3 or 6 days) prior to induction of ovulation in anestrous Nelore, B. indicus, cows increased the percentage of animals with a normal luteal lifespan. Nevertheless, we did not observe an increase in short luteal phases and there was no effect of 2CIDR treatment on P/ET, suggesting that short luteal phases are unlikely to be an explanation for the greater P/AI with increased P4 supplementation.

There was an interaction between treatment and follicle diameter on P/AI on Day 60 (Fig. 1). In small follicles (<14 mm), there was no effect of treatment on P/AI; however, in cows with larger follicles (≥14 mm), the 2CIDR treatment increased P/AI. In a similar fashion, Pereira et al. [23] observed that cows, synchronized with an E2/P4 protocol, that had a CL at the time of PGF had greater P/AI as the ovulatory follicle diameter increased (linear effect; $P < 0.01$), but no effect on follicle diameter was observed in cows without a CL at PGF. They speculated that development of larger follicles is positive for fertility if these follicles are growing in the presence of high circulating P4. The results of our study are somewhat consistent with this idea and the corresponding hypothesis that in the presence of low circulating P4, there is decreased P/AI as the follicle size increases. Cows with low circulating P4 concentration during follicle development have greater number of LH pulses [38,39], and this could be more detrimental in cows with larger or older follicles that have greater LH receptors in granulosa cells [40,41]. In addition, a recent study [42] observed that reduced circulating P4 concentration can increase LH receptor mRNA expression in granulosa cells of dominant follicles from Nelore heifers. Thus, increased numbers of LH pulses and increased follicular responsiveness to LH could underlie an effect of reduced P4 concentrations on oocyte and embryo quality [17,35,36]. In some previous studies using E2/P4 protocols [24,43], there was a bimodal effect of follicle size on P/AI with cows ovulating smaller or larger follicles having a reduction in P/AI; however, P4 concentrations at PGF were not evaluated in these studies.

The 2CIDR treatment resulted in lower circulating P4 concentration during the subsequent luteal phase, specifically on Day 7. In general, greater P4 concentrations after AI have been associated with greater fertility in TAI [44,45], but not in TET [45] programs. In contrast, Vasconcelos et al. [44] reported that cows ovulating smaller follicles had reduced circulating P4 concentrations on Day 7 after Ovsynch protocols and had reduced P/AI compared with cows ovulating larger follicles that had greater circulating P4 on Day 7. In our study, the effects of P4 supplementation were only observed in cows ovulating larger follicles (>14 mm) and not in cows ovulating smaller follicles. It seems possible that the key factors limiting fertility are different in cows ovulating smaller compared with larger follicles. For example, cows with low circulating P4 (1CIDR) and ovulating larger follicles may have problems with overstimulation of the preovulatory follicle due to excessive LH pulses, whereas cows ovulating smaller follicles may have more of a problem with insufficient or nonoptimal circulating P4 concentrations after AI due to a smaller CL and this may limit embryo development or pregnancy maintenance in these cows. Thus, this study supports the idea that there are interactions among the dynamics of follicle growth and circulating hormone concentrations prior to AI that have effects on fertility to the AI.

### 4.1. Conclusion

The use of two intravaginal P4 implants rather than one P4 implant in cows without CL and low P4 concentration at the beginning of the protocol tended to improve fertility in lactating dairy cows that were submitted to TAI, but not in cows receiving TET. The results of this study are consistent with the idea that the fertility effects of greater P4 supplementation are primarily on either oocyte quality or early embryo development since improvement was only in cows ovulating larger follicles and no effect of preovulatory P4 was observed in cows that received ET.

### Acknowledgments

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