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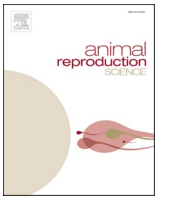

Postponing TAI in beef cows with small preovulatory follicles

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ABSTRACT

The aims of this study were (1) to establish an optimal cut-off point for evaluating the effect of the preovulatory follicle (POF) diameter in timed AI (TAI) programs, and (2) to evaluate the effect of postponing TAI in cows with smaller follicles on pregnancy per AI (P/AI). In Study 1, Nelore cows ($n = 426$) were subjected to an estradiol-progesterone (P4) based TAI protocol. The diameter of POF was measured at TAI, 48 h after P4 insert removal. From the ROC curves for determining the relationship between the POF diameter and pregnancy, the cut-off was 11 mm ($P < 0.001$). Cows with larger POF had greater ($P < 0.05$) P/AI (62.5%) than cows with smaller POF (34.8%). In Study 2, Nelore cows ($n = 1257$) were subjected to the same TAI protocol as in Study 1. Before TAI, cows were separated according to POF diameter in three groups: (1) Larger POF (LP, $n = 873$; POF ≥11 mm), (2) Smaller POF 0 h (SP0, n = 195; POF <11 mm), and (3) Smaller POF 24 h (SP24, n = 189; POF <11 mm). Cows in the LP and SP0 were TAI at 48 h and cows in the SP24 group were TAI at 72 h after P4 insert removal. Cows in the LP group had the greatest ($P < 0.05$) P/AI (60.25%), followed by cows in the SP24 group (41.8%), while cows in the SP0 group had the lowest P/AI (31.8%). In conclusion, cows with smaller follicles have lower P/AI, but postponing the TAI by 24 h in these cows increases P/AI.

1. Introduction

There is an optimal size of the ovulatory follicle at the time of insemination that significantly influence pregnancy rates in beef heifers (Perry et al., 2007) and cows (Perry et al., 2014; Sa Filho et al., 2010) and also affects embryonic/fetal mortality after timed artificial insemination (TAI) in dairy cows (Santos et al., 2004). In *B. indicus* cattle, the ovulatory response increases linearly with follicle size (Gimenes et al., 2008). *B. indicus* heifers ovulated 90% of the preovulatory follicle (POF) > 10 mm. In contrast, when the largest follicle was of 7–8.4 mm, only 33% of the heifers ovulated. Moreover, there is a strong correlation between the POF diameter and the time to ovulation. *B. indicus* cows included in an estradiol-progesterone based protocol with POF >15 mm ovulated ~48 h earlier than cows with POF <10 mm (Pfeifer et al., 2015). Fertilization rates are increased when cows are inseminated closer to the moment of ovulation, but embryo quality was decreased when compared with insemination at the onset of estrus (Saacke, 2008).

Smaller POF develops into smaller corpus luteum after ovulation, which secretes less progesterone in dairy (Vasconcelos et al., 2001) and beef cows (Pfeifer et al., 2009). Cows with lower serum progesterone post-ovulation have lower pregnancy rate (Lamb et al., 2001) and increased embryo loss (Perry et al., 2005). Similarly, in *B. indicus* beef cows, the presence of larger follicles on the day of TAI improved ovulation rate and P/AI (Sa Filho et al., 2010). Oocyte developmental competence continues to increase with increased

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follicular diameter in cattle (Arlotto et al., 1996), due to the continuous production and accumulation of mRNA and proteins that are produced and stored in the oocyte (Brevini Gandolfi and Gandolfi, 2001).

The effect of POF diameter on the fertility of cows inseminated after estrous detection (Perry et al., 2007) or TAI (Meneghetti et al., 2009; Pfeifer et al., 2020; Sa Filho et al., 2010) has been clearly described. However, one question remains to be answered: how to increase the fertility of cows with small follicles at the moment of TAI? In an attempt to increase P/AI in cows with different follicles sizes at TAI, our group developed a protocol based on POF diameter as the criteria to decide the best moment of TAI in *B. indicus* cows (Pfeifer et al., 2015). Termed as artificial insemination in blocks (AIB), this technique has provided greater P/AI than cows TAI conventionally (63% x 49%). However, AIB demands many cattle handlings which may limit the adoption by farmers and technicians. Therefore, a more suitable alternative to increase P/AI in cows with smaller POF at TAI needs to be established.

Based on these considerations, the aims of this study were (1) to establish an optimal cut-off point for evaluating the effect of POF diameter in P/AI, and (2) to evaluate the effect of postponing TAI in cows with smaller POF in the P/AI. The hypothesis of this study is that postponing TAI by 24 h increases P/AI in cows with smaller POF (<11 mm).

2. Materials and methods

The Committee for Ethics in Animal Experimentation from Embrapa approved all the procedures described in this manuscript (Protocols F02.2014 / F04.2017).

2.1. Study 1

This retrospective observational study was performed to characterize the optimal cut-off point for evaluating the effect of POF diameter in P/AI of cows subjected to estradiol-progesterone (E2-P4) based protocols. For this purpose, data from diameter of POF measured 48 h after P4 insert removal from a previous study was used (Pfeifer et al., 2015). The studies were performed in two farms located in the Rondonia State, Brazil. Suckled (30–80 days postpartum) Nelore cows (*B. indicus*; n = 426), 3–10 years old, 450–600 kg of body weight, and with 2.75–3.5 of body condition score (BCS; range 1–5, where 1 = emaciated and 5 = obese) were used (Table 1). Cows were kept in an outdoor grazing system (*Brachiaria brizantha* pasture) with ad libitum access to trace mineral salt and water.

Cows with unknown cyclicity status and stage of the estrous cycle received an intravaginal P4-releasing insert (1.9 g P4, CIDR®, Zoetis Animal Health, São Paulo, Brazil) plus 2 mg i.m. of estradiol benzoate (EB; Bioestrogen®, Biogénesis-Bagó, Curitiba, Brazil) at the beginning of the protocol (Day 0). The P4 inserts were removed on Day 8, and all cows received 150 μg i.m. of D-cloprostenol (PGF-analogue; Croniben®, Biogénesis-Bagó, Curitiba, Brazil), 300 IU i.m. of eCG (Novormon®, Zoetis, São Paulo, Brasil), and 1 mg i.m. of estradiol cypionate (ECP®, Zoetis, São Paulo, SP). All cows were inseminated 48 h after P4 insert removal. Semen from 2 bulls, one per farm, was used for TAI.

The POF diameter was assessed by ultrasonography (SIUI CTS-900, equipped with a 5 MHz linear-array transducer, Guangdong, China) before TAI. The ovaries of each cow were examined until detect the images of the largest follicle, focusing on its widest diameter. Thus, the diameter of the follicle was measured using the internal calipers of the ultrasound machine. All cows were examined by a single operator (LFMP). Ultrasound examinations were also performed 30 d post-TAI to assess pregnancy status. Visualization of the embryonic vesicle and detection of the embryo were the positive criteria for determining pregnancy.

2.2. Study 2

Suckled (30 – 100 days postpartum) Nelore cows (*B. indicus*; 347 primiparous and 910 multiparous) 3–14 years old, 450–650 kg of body weight, and with 2.75–3.75 of BCS included in TAI programs from 2018 to 2020 breeding seasons, from 3 commercial beef farms in Rondônia - Brazil, were used in this study (Table 1). All cows were maintained on *Brachiaria brizantha* pasture and given mineralized-salt and free access to water. All cows in all farms were treated with the same TAI protocol as in Study 1. On the morning of Day 10 (07:00 am), the POF diameter was assessed by ultrasonography, as in Study 1. Cows with larger POF (≥11 mm) were assigned to Larger POF group (LP, $n = 873$); and cows with smaller POF (<11 mm) were randomly assigned to one of the two groups: Smaller

Table 1
Descriptive statistics in the Study 1 and 2.

Item	Farms		
Study 1	1	2	
Number of cows	98	426	
Number of cohort of cows enrolled in the TAI programs	1	4	
Breeding season (Nov – Jan)	2014	2014	
Number of bulls (semen used for TAI)	1	1	
Study 2	1	2	3
Number of cows	784	192	281
Number of cohort of cows enrolled in the TAI programs	7	2	3
Breeding season (Nov – Jan)	2018–2019	2019	2019–2020
Number of bulls (semen used for TAI)	3	1	2

POF 0 h (SP0, n = 195), and Smaller POF 24 h (SP24, *n* = 189). Cows in the LP and SP0 groups were TAI at 48 h after P4 insert removal, while cows in the SP24 were TAI 24 h later (at 72 h after P4 insert removal). Semen from six bulls was used for TAI and were equally distributed among treatments (Table 1). As in Study 1, all cows were examined by a single operator (LFMP). Pregnancy status was assessed by ultrasound examination 30 d post-TAI, as in Study 1.

2.3. Definitions

In Study 1, the optimal cut-off point for POF diameter effects in pregnancy was calculated using receiver operating characteristic (ROC) curve and was determined based on the highest sensitivity (Se), and specificity (Sp). Area under the curve (AUC), Se, Sp, and P-value of the ROC test are reported.

The cut-off value for the diameter of the preovulatory follicle was 11.0 mm. Therefore, we separated the cows in Study 2 according to POF diameter in two groups: cows with smaller follicles (<11 mm), and cows with larger follicles (≥11 mm). As we previously observed (Pfeifer et al., 2015), cows with a larger POF ovulate earlier than cows with a smaller POF. Therefore, in Study 2, we used the cut-off of 11 mm for POF to further divide cows in the SP0 and SP24 groups, based on the moment of TAI.

2.4. Statistical analysis

All statistical analyses were performed using the SAS 9.0 software (SAS Institute Inc., Cary, NC, USA), except for ROC calculations, which was performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). In the Study 1, the initial analysis included the effect of farm, age, BCS, and days postpartum in the statistical model. The variables age, days postpartum, and farm had no significant effect and were, therefore, excluded from the final statistical model. Optimal cut-off points for determining the relationship between the diameter of the POF and pregnancy status was calculated using receiver operating characteristic (ROC) curve. Therefore, cows were separated according the diameter of POF in smaller and larger POF and the pregnancy per AI (P/AI) was compared between these groups by Chi-square test. To evaluate the effect of the diameter of the POF on P/AI and the number of cows according to the diameter of POF, polynomial regression analysis was used, in which the statistical models were selected according to the significance of the coefficient of determination.

In Study 2, the initial analysis included the effect of sire, age, parity, BCS, farm, and days postpartum in the statistical model. The variables sire, age, days postpartum, and farm had no significant effect and were, therefore, excluded from the final statistical model. Thus, the maximum diameter of the ovulatory follicle at TAI was analyzed using two-way ANOVA to test the effects of group (LP, SP0, and SP24) and parity (primiparous and multiparous) and their interaction. Means were compared among groups using the Tukey's post hoc test. Logistic regression analysis was used to examine the effects of group and parity on P/AI. The proportion of cows with smaller follicles were compared among parity by chi-square test. Differences among groups were considered significant based on a *P*-value lower than 0.05. *P*-values between 0.05 and 0.10 were considered trends.

3. Results

3.1. Study 1

From the ROC analysis for determining the relationship between the POF diameter and pregnancy, the cut-off was 11 mm (Sp, 86.1%; Se, 40.1%; AUC, 0.62; $P < 0.001$; Fig. 1). Quadratic associations were observed between diameter of the POF at TAI and the P/AI (Fig. 2; $R^2 = 0.96$; $P < 0.001$) and between the number of cows according to the diameter of the POF at TAI (Fig. 2; $R^2 = 0.87$; $P < 0.001$).

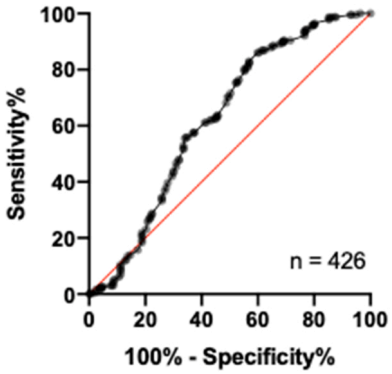

Fig. 1. Receiver Operating Characteristic (ROC) curve for determining the relationship between the diameter of preovulatory follicle and pregnancy in postpartum beef cows (n = 426) subjected to an estradiol-progesterone based TAI protocol in Study 1. The preovulatory follicle was measured 48 h after P4 insert removal. The cut-off for POF diameter was 11 mm (40.1% sensitivity and 86.1% specificity; AUC 0.62; $P < 0.001$).

Cows with larger POF (≥11 mm) had greater ($P < 0.05$) P/AI (62.5%) than cows with smaller POF (34.8%; Fig. 3).

3.2. Study 2

Ovarian response and P/AI data are summarized in Table 2. Cows in the LP group had the greatest ($P < 0.05$) and cows in the SP0 group had the lowest ($P < 0.05$) P/AI. Cows from SP24 group had greater ($P < 0.05$) P/AI than cows from SP0 group but lower compared to cows from LP group. Multiparous had larger ($P = 0.02$) POF and greater ($P < 0.001$) P/AI than primiparous cows (Table 2). However, an interaction group*parity ($P < 0.01$) was detected in the diameter of the POF. In that regard, the difference was observed in the SP0 group, in which primiparous cows had POF follicles of 9.5 ± 0.23 mm, and multiparous cows had POF follicles of 9.1 ± 0.22 mm. A greater ($P < 0.05$) proportion of primiparous cows had smaller follicles in comparison to multiparous cows (47% and 24.2%, respectively). The P/AI of cows from different groups according to parity is shown in Fig. 4.

4. Discussion

The present study examined the relationship between POF diameter and fertility in suckled Nelore beef cows in an E2-P4 based TAI protocol. Study 1 was performed to determine the optimal POF diameter in which cows are more likely to become pregnant. Therefore, the POF diameter detected in the ROC analyses was used to define a group of poor responders, with smaller follicles at TAI, in Study 2. Our hypothesis was confirmed, as postponing TAI in cows with smaller (<11 mm) POF increased P/AI in suckled beef cows, especially for multiparous cows. However, the P/AI of cows with smaller POF in which TAI was postponed by 24 h, was still lower than that of cows with larger POF, indicating further optimization of the protocol is needed in future studies.

Positive effects of POF diameter at TAI on the percentage of cows ovulating and getting pregnant were reported in *B. taurus* (Vasconcelos et al., 1999) and *B. indicus* cows (Meneghetti et al., 2009; Sa Filho et al., 2009). However, to the best of our knowledge there is only one previous study that presented an alternative protocol to increase P/AI in cows with smaller POF at TAI (Pfeifer et al., 2015). In this previous study our group suggested that the time of insemination can be set according to the diameter of the POF (Pfeifer et al., 2015). Termed as AIB, this technique provided greater P/AI than conventional TAI, because cows with smaller follicles were TAI later. In the present study, cows with smaller follicles were inseminated 24 h later and had increased P/AI, which was proven effective and reduced handling in comparison with our previous AIB protocol.

Although all cows enrolled in this study were treated with the same TAI protocol, a high amplitude in the size of the largest follicle detected at 48 h after P4 insert removal was observed (ranging from 6 to 20 mm). There is no certainty about the nature of this high variation among individuals; however, some studies observed that the interval between follicular emergency and the progestin removal (Utt et al., 2003), the proestrus length (Dadarwal et al., 2013), the occurrence of ovulation during progesterone treatment (Kastelic et al., 2004; Pfeifer et al., 2019), might affect the diameter of the follicle in TAI programs. Therefore, the success of programs for synchronization of the estrous cycle and ovulation depends on an orchestrated manipulation of the physiological process such as recruitment of a new wave of follicles for development, control of follicular dominance, and the interval between induction of

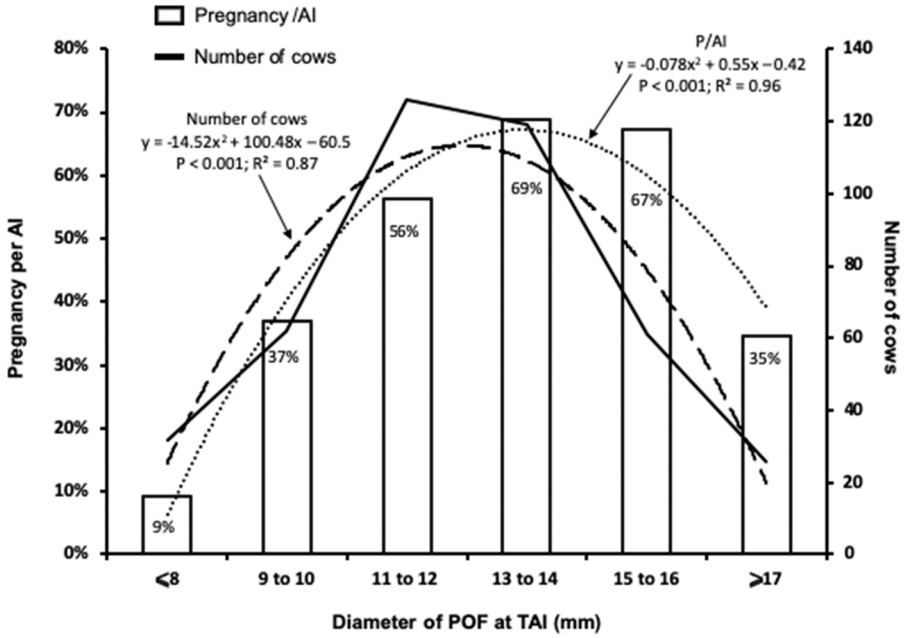

Fig. 2. Pregnancy per AI, and number of cows according to the diameter of the POF, measured 48 h after P4 insert removal, in postpartum beef cows (n = 426) subjected to an estradiol-progesterone based TAI protocol in Study 1.

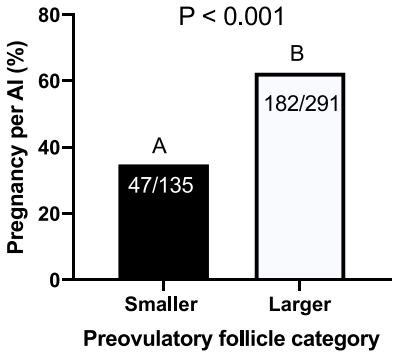

Fig. 3. Pregnancy per AI in postpartum beef cows (n = 426) subjected to an estradiol progesterone-based protocol with smaller (< 11 mm) and larger POF (≥ 11 mm). The preovulatory follicle was measured 48 h after P4 insert removal in Study 1.

Table 2
Ovarian response and pregnancy per AI in primiparous and multiparous postpartum beef cows with larger POF (≥11 mm) inseminated 48 h after P4 insert removal (LP), and cows with smaller POF (<11 mm) inseminated 48 h (SP0) or 72 h (SP24) after P4 insert removal in Study 2.

	Groups			Parity		P-Value		
	LP	SP0	SP24	Primiparous	Multiparous	Group	Parity	Group*Parity
Preovulatory follicle diameter, mm	14.2 ± 0.08^{A}	9.3 ± 0.15^{B}	9.7 ± 0.16^{B}	11.6 ± 0.1	13.6 ± 0.1	<0.001	0.02	<0.01
Pregnancy per AI, %	60.25^{A} (526/873)	31.8^{B} (62/195)	41.8^{C} (79/189)	44.1 (153/347)	56.5 (514/910)	<0.01	<0.001	0.7

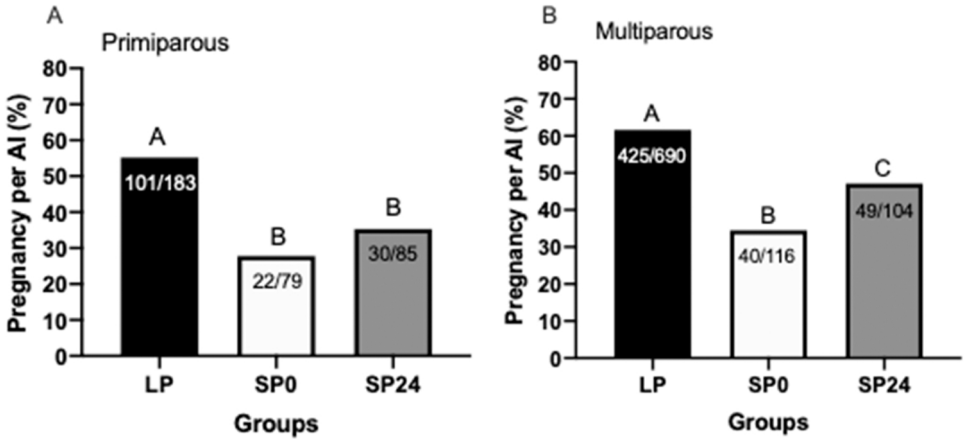

Fig. 4. Pregnancy per AI in primiparous (A; n = 347) and multiparous (B; n = 910) postpartum beef cows subjected to an estradiol-progesterone based TAI protocol with larger POF (≥11 mm) inseminated 48 h after P4 insert removal (LP), and cows with smaller POF (<11 mm) inseminated 48 h (SP0) or 72 h (SP24) after P4 insert removal in Study 2.

ovulation and AI (Bisinotto et al., 2010).

Similar to our findings, earlier studies observed that a critical follicle diameter threshold for ovulation and pregnancy in *B. indicus* (Sa Filho et al., 2010) and *B. taurus* (Perry et al., 2005) cows subjected to TAI programs was at least 11 mm. Gimenes et al. (2008) reported that in *B. indicus* cattle, the ovulatory response was 33%, 80%, and 90% in heifers with POF of 7–8.4 mm, 8.5–10 mm, and >10 mm, respectively. Additionally, cows with a larger POF can ovulate 24–30 h earlier than cows with smaller POF, since dominant follicles increase in diameter as the time of ovulation approaches (Pfeifer et al., 2015). Moreover, the ovulation rate positively influences the P/AI in cows subjected to TAI programs. These evidences led us to hypothesize that the synchrony between ovulation and AI may be optimized to improve the establishment of pregnancy in cows subject to TAI. Insemination of cows close to the moment of ovulation resulted in increased fertilization rate, but decreased embryo quality compared with insemination at the onset of estrus (Dalton et al., 2001; Saacke, 2008). Therefore, in the present study, cows with smaller POF, in which AI was postponed by 24 h had greater P/AI than cows with smaller follicles inseminated conventionally, at 48 h after P4 insert removal. These results suggest that smaller follicles detected at TAI need a greater interval to mature and reach ovulatory size before AI; thus, when performing TAI in

conventional protocols (at 48 h after ECP), ovulation of smaller follicles can occur when the fertilization potential of the semen in the female tract is no longer optimal.

The success of gestation establishment is directly associated to the competence and diameter of the ovulatory follicle. Moreover, the relationship between diameter of POF and diameter of CL and P4 concentrations post ovulation have been described (Dadarwal et al., 2013; Mann and Lamming, 1999; Pfeifer et al., 2009). Cows induced to ovulate small follicles (<11.5 mm) developed small corpus luteum, which secreted less progesterone (Vasconcelos et al., 2001), and consequently, had lower pregnancy rate (Lamb et al., 2001) and an increase in embryonic death (Perry et al., 2005). Similarly, as observed in the present study, the cows with smaller POF at TAI had lower P/AI than cows with larger POF. Although the delay in the time of AI increase P/AI in cows with smaller follicles, this procedure did not provide adequate P/AI as we observed in cows with larger POF.

Postponing the time of AI according to the POF diameter may be an alternative to increase the P/AI in cows with smaller follicles at TAI. Overall, the P/AI of the SP24 group was 10% greater than cows from the SP0 group. Considering all cows from Study 2, only 30.54% had smaller follicles. However, technicians may evaluate the feasibility of the ultrasonographic evaluation to detect the POF diameter of every single cow in the TAI protocol and the extra animal handling necessary to perform the inseminations 24 h later than the conventional TAI program. A similar approach was tested in buffaloes during the breeding season treated with two doses of PGF2alpha separated by an interval of 13 days. Animals with a POF measuring at least 10 mm were inseminated 16–22 h later, while animals without a POF at the time of the ultrasound exam were inseminated two days later. To ensure ovulation, GnRH was given at the time of insemination, which resulted in similar pregnancy rates between groups (De Rensis and López-Gatius, 2007).

Although in some studies there were no differences in fertility between multiparous and primiparous *B. indicus* postpartum cows (Marques et al., 2015; Meneghetti et al., 2009; Sa Filho et al., 2010), others have found that multiparous cows are more likely to become pregnant in TAI programs (Ferraz Junior et al., 2016; Sa Filho et al., 2013). Similarly, in the present study, multiparous cows had greater P/AI than primiparous. Moreover, the difference on the P/AI between SP24 and SP0 was detected only in multiparous cows. A greater proportion of primiparous cows had smaller follicles in comparison to multiparous cows. The lack of statistical difference observed for primiparous cows could be an effect of the low number of cows of this category included in the experiment. However, more studies are necessary before concluding that the effect of postponing AI in primiparous cows with small POF is marginal.

In conclusion, the results of the present study demonstrate that POF diameter determines P/AI in suckled *B. indicus* cows. In addition, postponing TAI in cows with smaller POF (<11 mm) increase P/AI, but still lower than for cows with larger POF. More studies must be performed to verify if postponing AI in cows with smaller POF is a feasible and economical alternative to increase fertility of suckled beef cows.

Declaration of Competing Interest

On behalf of all co-authors, I declare that this manuscript has no conflict of interest.

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