Archives of Veterinary Science **ISSN 1517-784X**
v.26, n.3, p. 47-58, 2021 ww.ser.ufpr.br/veterinary

COMPARISON OF PROTOCOLS FOR TIMED ARTIFICIAL INSEMINATION BY ONE OR TWO ARTIFICIAL INSEMINATIONS AND BY RESYNCHRONIZATION OF OVULATION IN BOS TAURUS INDICUS COWS

(Comparação de protocolos para inseminação artificial em tempo fixo com uma ou duas inseminações artificiais e por ressincronização da ovulação em vacas Bos taurus indicus)

Francisco Romano Gaievski[1], Luiz Ernandes Kozicki[1], Rafaela Talini[1], Ana Paula Kaminski[1], Tácia Gomes Bergstein-Galan[2], Romildo Romualdo Weiss[3]

[1]Programa de Pós-Graduação em Ciência Animal, Pontifícia Universidade Católica do Paraná, Curitiba, Brasil; [2]Universidade Positivo, Curitiba, Paraná, Brasil; [3]Universidade Federal do Paraná, Curitiba, Paraná, Brasil.

*Corresponding author: rafatalini@gmail.com

Editor: Gilson Pessoa

ABSTRACT - The goals were to compare the conception rate in cows subjected to timed artificial insemination (TAI) with one AI, two AIs, or resynchronization (RES), and verify the effects of POF (preovulatory follicle) diameter on the CR. Two hundred and seventy-four cows (*Bos taurus indicus*) were assigned. In the group with one AI (1AIG; *n*=88), cows were subjected to intravaginal P4 and EB (2mg, IM) on day zero (D0); $PGF_{2\alpha}$ (500µg, IM) on D7; P4 removal on D8; EB on D9; TAI and ultrasonography for POF diameter measurement on D10; PD (pregnancy diagnosis) and natural service on D41; bull removal on D90; and final PD on D110. The same protocol was used in the two AI (2AIG; *n*=88), except a second AI 20h after the first AI. The resynchronization group (RESG; n=98) was subjected to the same protocol as 1AIG, except a PD was performed on D40, and the non-pregnant cows received the P4 device and EB; $PGF_{2\alpha}$ on D47; P4 removal on D48; EB was administered on D49; TAI was performed on D50; PD was performed, and bulls were introduced on D80; bulls' removal on D110; final PD on D160. CR of 50, 58, and 73.4% were observed in 1AIG, 2AIG, and RESG, respectively, increasing to 85.2, 88.6, and 87.7% after natural service. The POF diameter at the first TAI was 12.8, 12.1, and 12.7mm in 1AIG, 2AIG, and RESG, respectively, and 12.6mm in RESG and 13.6mm in 2AIG (*P*=0.031) at the second AI. Groups 1 and 02 inseminations remained with bulls for 49 days and resynchronization for 30 days. In conclusion, RESG yielded a higher CR than TAI with one or two AIs. AI with follicles larger than 12.1mm resulted in a higher CR.

Key words: timed artificial insemination. cattle. progesterone. two artificial inseminations. resynchronization. preovulatory follicle.

RESUMO - Os objetivos foram comparar a taxa de prenhez (TP) em vacas *Bos taurus indicus* submetidas à IATF com uma IA, duas IA ou ressincronização e verificar os efeitos do diâmetro do folículo pré-ovulatório sobre a taxa de prenhez. Foram utilizadas 274 vacas (Bos taurus indicus). No grupo uma inseminação (1AIG; n=88), as vacas foram submetidas a inserção de progesterona intravaginal e benzoato de estradiol (im) no dia zero (D0); prostaglandina $F_{2\alpha}$ no dia 7 (D7); remoção da progesterona em D8; administração de benzoato em D9. A IATF e a ultrassonografia ovariana foram feitas no dia 10 (D10). O diagnóstico de prenhez e a colocação das vacas com os touros ocorreu no D41; a remoção dos touros no D90; e o diagnostico final no D110. Idêntico protocolo foi usado no grupo 2 inseminações (2AIG; n=88), exceto uma segunda inseminação 20 horas após a primeira. O grupo ressincronização (RESG; n=98) foi submetido ao mesmo protocolo que 1AIG, exceto que o diagnóstico de prenhez foi feito em D40, e as vacas não-prenhes receberam o dispositivo com P4 e benzoato neste dia, $PGF_{2\alpha}$ no D47;

Received in 12/08/2020
Approved in 05/24/2021

remoção da P4 no D48 e benzoato no D49. A IA foi feita no D50; o diagnóstico de gestação e os touros foram colocados no D80; remoção dos touros no D110; diagnostico final no D160. A taxa de prenhez observada foi de 50, 58, e 73.4% no 1AIG, 2AIG, e RESG, respectivamente, atingindo 85.2, 88.6, e 87.7% após a monta natural. O diâmetro do folículo pré-ovulatório após a primeira IA foi de 12.8, 12.1, e 12.7mm no 1AIG, 2AIG, e RESG, respectivamente, e de 12.6mm no RESG e 13.6mm no 2AIG (P=0.031) após a 2ª. IA. Os grupos 1 e 02 inseminações permaneceram com touros por 49 dias e o ressincronização 30 dias. Em conclusão, a ressincronização resultou em maior taxa de prenhez que os protocolos de IATF com uma ou com duas IAs; a inseminação artificial executada perante folículos maiores que 12,1mm resultou em maiores taxas de prenhez.
Palavras-chave - inseminação artificial em tempo fixo. bovino de corte. progesterona. dupla inseminação artificial. ressincronização. folículo pré-ovulatório.

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INTRODUCTION

Artificial insemination in fixed time (TAI) is presently employed in nearly 15.9% of the Brazilian cattle herd (Boletim Eletrônico FMVZ/USP, 2020). TAI allows hundreds of animals to be inseminated at a predetermined time, without the need for estrus observation or compromising reproductive efficiency, further allowing parturition to occur at a desired period (Baruselli et al., 2004; Ayres et al., 2006; Torres et al., 2009).

Hormonal protocols for TAI became prominent after the development of Ovsynch (Pursley et al., 1995). This protocol underwent several modifications, such as inclusion of intravaginal devices with progesterone in association with esters, luteolytic agents, and ovulation inducers, for greater control over the estrous cycle and synchronizing ovulation in the herd (Bó et al., 2002; Souza, 2013).

Of the TAI protocol improvements (such as synchronization of follicular wave emergence and follicle growth), the inclusion of intravaginal progesterone devices appears to be the most important. These devices block the gonadotropins secreted from the adenohypophysis. Such progesterone treatments are often coupled with the application of steroids or gonadotropin releasing factors (GnRH) at the time of device removal, allowing the luteolytic agents and ovulation inducers to act without inhibition, resulting in synchronized ovulation, which is one of the most relevant factors in increasing pregnancy per AI or conception rate (Bó et al., 2002; Ferreira et al., 2012).

Although significant progress has been made in improving hormonal protocols for increasing PR in TAI, such protocols yield only partially satisfactory results because of variations in animal fertility. Significant differences have been noted in the PR in TAI, which may be as high as 35% to 70% (Baruselli et al., 2002; Rocha et al., 2007; Oliveira et al., 2019).

One of the techniques for improving reproductive efficiency in TAI is estrus and ovulation resynchronization (RES), which involves performing two AIs within 42 d of each other. RES can result in a PR as high as 77%, when the percentages of the 1st and 2nd TAIs are summed (Freitas et al., 2007; Doroteu et al., 2015). Thus, RES is frequently employed in beef and dairy cattle breeding (Baruselli et al., 2017; Oliveira et al., 2019). Application of TAI with RES in *Bos taurus indicus* cows yielded a PR of 81.6% (cumulative rate), with optimal reproductive indicators (Oliveira et al., 2019).

Related to the follicle, previous studies have reported that the greater the preovulatory follicle, the greater the possibility of ovulation and the greater the area of the corpus luteum, increasing the pregnancy rate in TAI (Vianna et al., 1999; Ribeiro Filho et al., 2013).

In this context, and with the goal of increasing the reproductive efficiency of *Bos taurus indicus* cows, the present study hypothesized that two AIs performed at a 20 h interval could increase the PR in TAI protocols, because of the greater availability of viable semen in the female reproductive tract. Thus, AI performed closer to ovulation will increase reproductive indicators. Considering that oocytes have an average viability of 6-12 hours (STANFORD et al., 2002), in order to obtain high pregnancy rate in IATF programs, it is important that the AI be carried out close to ovulation, more specifically between 6 and 12 h before. (SAACKE, 2008). No specific literature was found regarding to the viability (in hours) of frozen bovine semen in the female's reproductive tract.

Therefore, the objectives of the present study were to (1) compare the reproductive efficiency indicators in *Bos taurus indicus* cows subjected to a TAI program involving a hormonal protocol with one AI, or two consecutive AIs (at 20 h interval) after the induction of ovulation with estradiol benzoate, and with traditional resynchronization and (2) to verify the influence of the preovulatory follicle diameter on the pregnancy rate.

MATERIALS AND METHODS

The use of animals was guided by international animal welfare standards.

Study site and animals

The study was conducted at a commercial farm in Brazil (26°10′12″S, 53°21′39″W), during the 2018/2019 breeding season. The climate of the region was characterized as subtropical, with defined climatic seasons and regular precipitation, and annual rainfall of 1200 mm. The average temperature from October to January ranged from 15.4 to 28.8°C. Suckling Nellore breed cows (*Bos taurus indicus*; *n* = 274), within an age range of 3.6 to 6.5 years, were included in this study. The mean body condition score (BCS) was 3.1

(range = 2.5 to 4.0) (1 = thin; 5 = obese (Bohnert et al., 2014)), and the average weight was 455 kg (range = 398 to 460 kg).

The cows were allowed to graze on pastures of *Brachiaria brizantha*, with free access to mineral salt (BellMais Fertilidade, Trouw Nutrition®, São Paulo; composition: Ca 120 g/kg, P 40 g/kg, Na 80 g/kg, S 18 g/kg, Mg 5 g/kg, Zn 2.5 g/kg, Cu 675 mg/kg, Fe 666 mg/kg, Mn 520 mg/kg, Co 40 mg/kg, I 50 mg/kg, and Se 13 mg/kg) and water. The cows were subjected to sanitary control with prophylactic vaccination against bovine infectious rhinotracheitis, bovine viral diarrhea, campylobacteriosis, leptospirosis, and clostridiosis, 30 d before the experiments.

Animal selection criteria

Prior to the study, the animals were subjected to transrectal ultrasound (US) examinations (Mindray DP 2200 Vet, China) for ovarian monitoring. Cows with follicles ≥ 8 mm in diameter ((larger diameter + smaller diameter)/2) (Gastal et al., 2008) or with a corpus luteum (CL) were considered as cycling animals (Souza et al., 2015) and included in the study. Cows with days open between 40 and 70 d and without endometritis (confirmed by visual detection), as well as animals with BCS > 2.5 were included.

Cows with follicles < 8 mm in diameter and without CL were considered in anoestrus, and those exhibiting mucous or turbid vaginal-vulvar discharge or other compromising signs were excluded from the study.

Study design

US examinations were performed 7 d before the administration protocol (D-7) for evaluating uterine and ovarian status, and eventual pathologies.

The cows were randomly distributed into three groups, i.e., group with one AI (1AIG; *n* = 88), group with two AIs (2AIG; *n* = 88), and group with RES (RESG; n= 98) (Figure1).

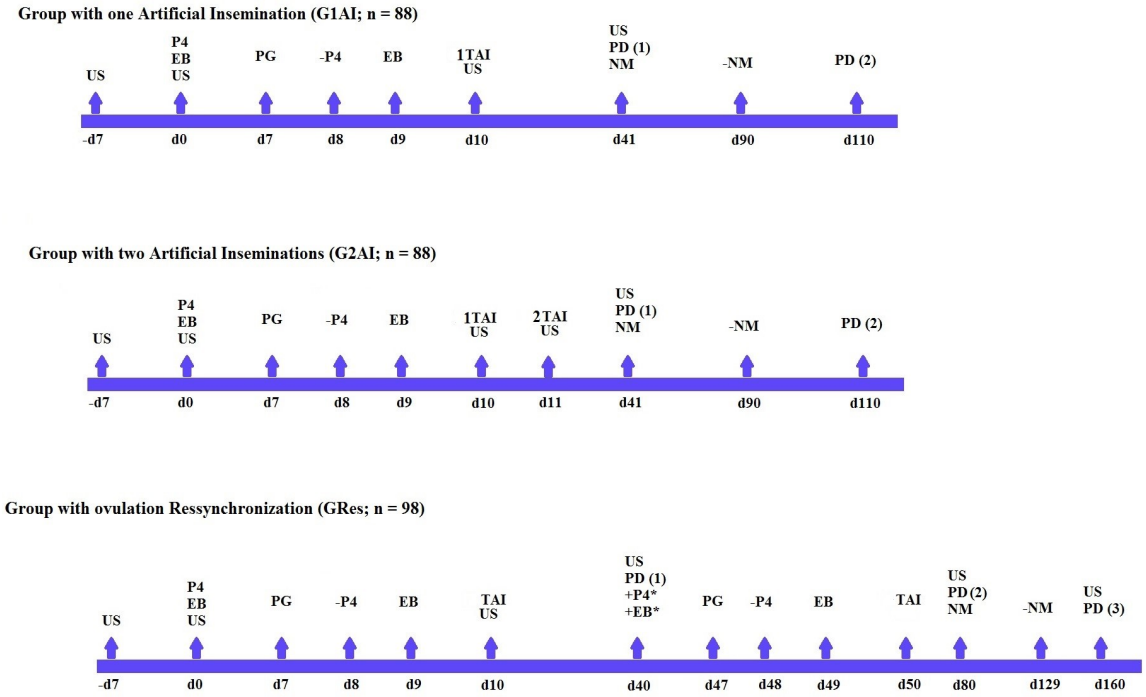

Figure 1 - Diagrammatic representation of protocols administered to multiparous *Bos taurus indicus* cows for timed artificial insemination with one or two inseminations and resynchronization.

Legend: *to non-pregnant cows. US: Ultrasonography; P4: Intravaginal progesterone device (1 g; Cronipress-Biogenesis Bago, Curitiba - Paraná – Brazil); EB: Estradiol benzoate (2 mg, intramuscular (IM); Bioestrogen - Biogenesis Bago); PG: Prostaglandin $F_{2\alpha}$ (500 µg, IM; Croniben Biogenesis Bago); TAI: Timed artificial insemination; NM: Natural mount; PD: Pregnancy diagnosis, D-7: ovarian monitoring 07 days before study; D: day.

On day zero (D0), cows in 1AIG were subjected to intravaginal device insertion with P4 (1 g), intramuscular (IM) injection with estradiol benzoate (EB; 2 mg) and US. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 500 µg, IM) was administered on D7, followed by the removal of intravaginal P4 device on D8. EB (1 mg, IM) was administered on D9, and ca. 24h after, the 1st AI was performed. The 2nd AI took place 20 hours after the 1st AI), along with US examination of the POF for diameter measurement. US pregnancy diagnosis (PD) was performed on D41 and clean-up bulls were introduced. The bulls were removed on D90, and the final PD was performed on D110.

Cows in 2A1G were subjected to the same protocol, except a second AI was performed on D11, 20 h after the first AI.

Cows in RESG were also subjected to the same protocol as in 1AIG, with the following modifications: PD was performed on D40 and non-pregnant cows received the P4 device and EB. $PGF_{2\alpha}$ was administered on D47, and the P4 device was removed on D48. EB was administered on D49, followed by TAI on D50. On D80, PD (US) was performed, and bulls were introduced. Bulls were separated from the cows on D129, and

the final PD was performed on D160. For insemination, the vulva was cleaned with water and paper towels, which was followed by intrauterine semen deposition.

Semen was thawed in an electronic defroster (WTA model, Cravinhos, São Paulo) at 35ºC for 30 s, and evaluated according to the criteria defined by the Brazilian College of Animal Reproduction (CBRA, 2013) prior to the study. Semen (20,000,000 sperm cells) from Red angus and Aberdeen angus breeds was used in this study. Proven fertile bulls were used for natural breeding, with one bull for every 30 cows.

Statistical analysis

The experiments were performed in a completely randomized design, with the hormonal treatment as the main variable (independent of other factors). PR in the three treatment groups was compared using the Chi-square test. Correlations between PR and the independent variables, including postpartum open days, BCS, age, parity, bull, POF diameter at 1st AI, and POF diameter at 2nd AI, were analyzed using Kendall's tau test. A binary logistic regression analysis was performed, considering the three groups simultaneously and including all independent variables and PR (dependent variable). To verify the incidence of POFs in the total number of animals, and to determine the influence of POF diameter on the PR, the follicles were categorized by dimension (mm) as: <10.0, 10.1–12.0, 12.1–14.0, 14.1–16.0, and >16.0 mm. Results were considered statistically significant at $P<0.05$.

RESULTS

The PR after TAI alone and after TAI with natural service (cumulative PR) for the three experimental groups is shown in Table 1.

Table 1 - Conception rates of suckling *Bos taurus indicus* cows in the group with one timed artificial insemination (1AIG), with two AIs (2AIG), and with resynchronization (RESG).

Groups	Conception rate after TAI (n; %)	Conception rate after TAI + natural service after breeding season (n; %)
1AIG	44/88 (50.0) [a]	75/88 (85.2)
2AIG	51/88 (58.0) [a]	78/88 (88.6)
RESG	72/98 (73.4) [b]	86/98 (87.7)

Different letters in the same column indicate significant difference at $P<0.05$

The PR after TAI was significantly higher in RESG than in the other two groups ($P<0.05$). However, no significant difference could be detected in the PR of the three groups at the end of the reproductive season (cumulative PR).

The effects of independent variables (open days, BCS, age, parity, and bulls) on the PR (dependent variable) of the three groups were analyzed by logistic regression. The POF diameter was observed to affect the PR in 1AIG and RESG (at the first AI) ($P<0.0001$), whereas no influence of the independent variables could be detected in 2AIG. In Kendall's tau correlation test, for the results of the first PD after TAI, the PFO diameter ($P<0.0001$) was observed to be correlated with the PR. An influence of the POF diameter at the second AI was also noted on the PR in 2AIG.

Table 2 - Preovulatory follicle diameter and ovulation before and after timed artificial insemination in suckling *Bos taurus indicus* cows.

Groups	∅ POF at 1st AI (mm;x±s)	∅ POF on RES (mm;x±s)	∅ POF at 2nd AI (mm;x±s)	Ovulation before 1st TAI (n;%)	Ovulation before 2nd TAI (n;%)
1AIG (n=88)	12.81±2.41			0/88 (0.0)	
2AIG (n=88)	12.16±2.43		13.64±0,29	3/88 (3.4)	24/88 (27.3)
RESG(n=98)	12.76±2.44	12.69±0.32		0/98 (0.0)	

Figure 2 shows the percentage of animals with the different POF diameter categories (i.e., <10.0, 10.1–12.0, 12.1–14.0, 14.1–16.0, and >16.0 mm).

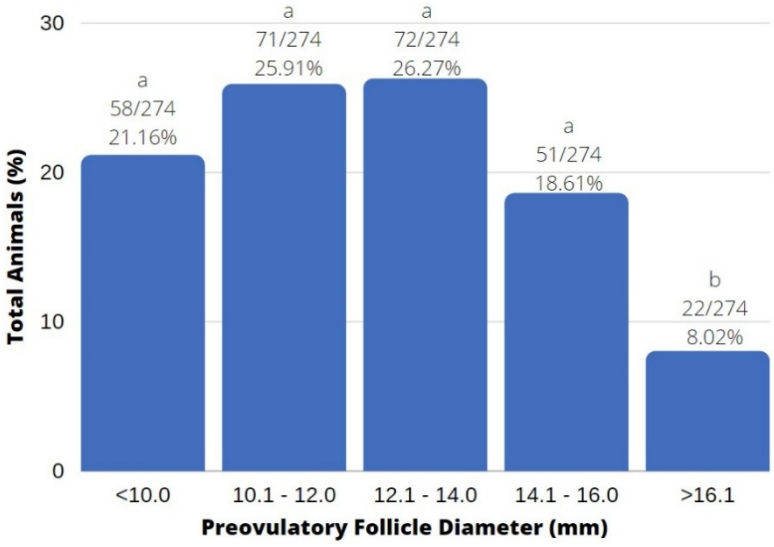

Figure 2 - Preovulatory follicle diameters (mm) measured on day 10 of the protocols in *Bos Taurus indicus* cows from all experimental groups (%). Different letters above the bars indicate significant difference at $P<0.05$.

The conception rate corresponding to the different POF diameter categories are shown in Figure 3.

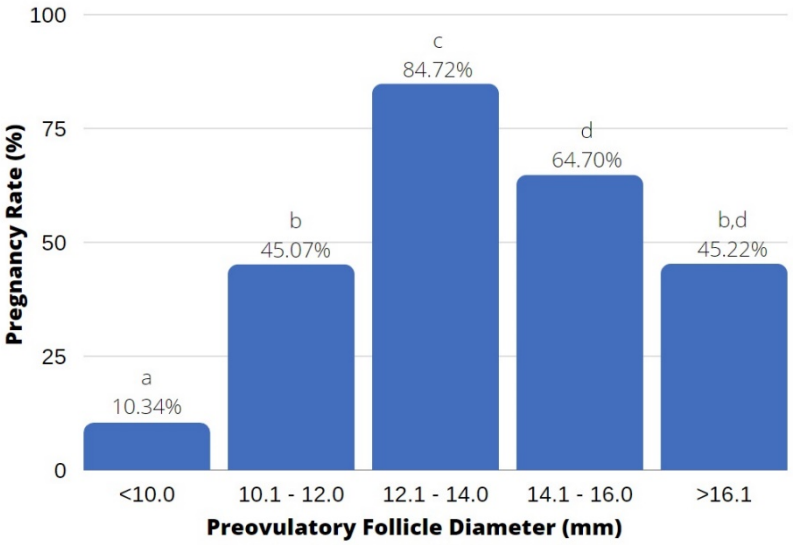

Figure 3 - Diameters of preovulatory follicles measured on day 10 of the protocols and the corresponding conception rate (%) in suckling *Bos taurus indicus* suckling cows subjected to one- or two-timed artificial inseminations or ovulation resynchronization. Different letters above the bars indicate significant difference at $P<0.05$.

DISCUSSION

Several studies have focused on the improvement of TAI protocols as the pregnancy indicators in these procedures are often unsatisfactory. One of the objectives of the present study was to compare the reproductive efficiency of TAI protocols with one AI, two AIs (at 20 h interval), and traditional resynchronization (PD 30 d after TAI).

The PR in 2AIG was 8.0% higher than that in 1AIG, although this difference was not significant. However, the PR in RESG was significantly higher than that of the other two groups ($P<0.05$) (Table 1). Previous studies have shown that RES can achieve higher PR when compared with conventional TAI. Pessoa et al. (2018) reported a PR of 81.5% and 83.7% in beef cows resynchronized 22 and 30 d after the first TAI, respectively.

The application of RES protocols can increase the PR by approximately 25% (Oliveira et al., 2019). In RES protocols, PRs as high as 75-81% can be achieved, when the PRs of the two TAIs are summed (Freitas et al., 2007; Doroteu et al., 2015; Oliveira et al., 2019). Our results were consistent with those of Doroteu et al. (2015), who investigated multiparous *Bos taurus indicus* cows under similar conditions and corroborated the advantages of such RES programs.

However, the costs of RES protocols must be taken into consideration. Two points are important to be noted here: (1) RES is only applied in non-pregnant animals after the first TAI, and (2) the non-pregnant animals after the first TAI would already be in better ovarian cyclicity conditions because of the hormonal action of the first administered protocol. Thus, the physiological status of the hypothalamic-pituitary-gonadal system would be more favorable to pregnancy, with a tendency to yield better results (PRs) in RES animals than in those not subjected to RES (Marques et al., 2015). This was also confirmed by Souza et al. (2016), who reported better cycling conditions in animals after the first TAI. This shows that pre-synchronization protocols produce better ovarian cyclicity when compared with those of animals not subjected to such protocols, supporting their use in animals with seasonal ovarian acyclicity (Souza et al., 2008; Gumen et al., 2012). Regarding the cost differences between protocols with 02 artificial inseminations versus resynchronization, it should be considering, that there was no significant difference in the pregnancy rate between these two groups, attributing a higher cost (P/AI) to RES protocol due hormones used and a greater number of animal handling. The lenght of the breeding season was similar in the groups, except in the RESG (traditional resynchronization group), which lasted about 6 weeks (Figure 1)

A 20 h interval between the AIs was adopted in the present study considering the management facilities available at such farms. We believe that reducing the time interval between the two inseminations (to less than 20 h) will result in a greater amount of viable semen available for a longer period of time within the female genital tract, serving ovulations over a longer period of time and subsequently increasing the conception rate.

The average POF diameter at the first TAI in the three groups ranged from 12.16 to 12.81 mm, which was consistent with the measurements reported by Coutinho et al. (2007) for *Bos taurus indicus* cows. The mean POF diameter at the second AI (in 2AIG) was observed to be 13.64 mm. The POF diameter increased by 1.48 mm in the 20 h between the first and second AIs (in 2AIG), which was comparable to the daily growth rate of POF reported by Borges et al. (2004) in *Bos taurus indicus* (1.40 mm/day), although Prata et al. (2018) reported lower rates (1.1 mm/day) (Table 2). Follicles measuring 12–14 mm in diameter were the most frequently observed (26.27% of all tested animals) (Figure 2). However, POFs with a diameter of 12.1–14.0 mm (considering all groups collectively) exhibited the greatest effect on PR (84.72%; $P<0.05$) (Figure 3).
This was consistent with previous observations that the larger the preovulatory follicles grow after ovulation, the larger the area of luteal tissue, and consequently, the higher the P4 production and PR percentage (Vianna et al., 1999; Ribeiro Filho et al., 2013).

Ovulation before TAI was only observed in 2AIG in 3.4% of the animals, which increased to 27.3% before the second TAI (Table 2).

The hypothesis of the present study could not be validated because the conception rate in TAI with RES was higher than that in the treatment with two AIs. Future studies should investigate TAI protocols with two AIs with a decreasing time interval between consecutive inseminations. We believe that such protocols can considerably improve reproductive indicators and may even replace RES protocols.

CONCLUSIONS

It was concluded that TAI with RES resulted in a higher PR when compared with TAI with one or two AIs; however, POFs ≥12.1 mm in diameter resulted in higher PR.

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