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Use of cooled buffalo semen as a strategy to increase conception rates in fixed-time artificial insemination programs during unfavorable reproductive periods

[*Uso de sêmen resfriado de búfalo como estratégia para aumentar as taxas de concepção em programas de inseminação artificial em tempo fixo, durante períodos reprodutivos desfavoráveis*]

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ABSTRACT

The objective of this study was to compare the reproductive efficiency of dairy buffaloes undergoing fixed-time artificial insemination (FTAI) protocols based on progesterone/estrogen (P_4/E_2) and eCG during unfavorable breeding season using cooled (CS) and frozen semen (FS). A total of 446 buffaloes (> 40 days postpartum) were randomly distributed into four blocks (years): B1-2014 (n = 143), B2-2015 (n = 34), B3-2016 (n = 90), and B4-2017 (n = 179). Each block was subdivided into two (AI with CS and FS using the same ejaculate of each bull). Thus, the block subdivision was as follows: B1 (CS = 71 and FS = 72); B2 (CS = 18 and FS = 16); B3 (CS = 47 and FS = 43); and B4 (CS = 90 and FS = 89). The ejaculates of eight Murrah bulls collected using an artificial vagina were divided into two aliquots: one aliquot was diluted in Botu-Bov® commercial extender and cooled (BB-CS), and the other was diluted in the same extender and frozen (BB-FS). BB-CS aliquots were cooled at 5 °C/24 h using a refrigerator. BB-FS group aliquots were also cooled, and after equilibrating at 5 °C for 4 h, were placed in a 21-L Styrofoam box, 5 cm above the surface of liquid nitrogen. In the afternoon (A) on D0 (2:00 p.m.) the animals received EB 2.0 mg IM (Estrogin®) and an ear implant (CRESTAR® 3.0 mg P_4). At D9 (A), the implant was removed, and the animals received eCG 400 IU IM (Folligon® 5000) + Cloprostenol $PGF_2\alpha$ 0.530 mg IM (Sincrocio®). At D10 (A), the animals received EB 1.0 mg IM (Estrogin®), and at D12 (8:00 a.m.), AI was performed. At D42, pregnancy was diagnosed via ultrasonography. Total CRs were 48.2% CS and 34.6% FS for years 2014 to 2017, with a significant difference of 13.7% ($P<0.05$). In conclusion, cooled semen resulted in higher CR than frozen semen in dairy buffaloes under the P4/E2 and eCG FTAI during the unfavorable reproductive season.

Keywords: fertility, estrus synchronization, cooled semen

RESUMO

O objetivo deste estudo foi comparar a eficiência reprodutiva de búfalas leiteiras submetidas a protocolos de inseminação artificial em tempo fixo (IATF) à base de progesterona/estrogênio (P4/E2) e eCG, durante a estação reprodutiva desfavorável, usando-se sêmen resfriado (SR) e congelado (SC) Um total de 446 búfalas (> 40 dias após o parto) foi distribuído aleatoriamente em quatro blocos (anos): B1-2014 (n = 143), B2-2015 (n = 34), B3-2016 (n = 90) e B4-2017 (n = 179). Cada bloco foi subdividido em dois (IA com SR e SC utilizando-se a mesma ejaculação de cada touro). Assim, a subdivisão do bloco foi a seguinte: B1 (SR = 71 e SC = 72); B2 (SR = 18 e SC = 16); B3 (SR = 47 e SC = 43); e B4 (SR = 90 e SC = 89). Os ejaculados de oito touros Murrah coletados com vagina artificial foram divididos em duas alíquotas: uma alíquota diluída em diluente comercial Botu-Bov® e resfriada (BB-SR), e a outra diluída no mesmo diluente e congelada (BB-SC). As alíquotas de BB-SR foram resfriados a 5°C/24h usando-se um refrigerador. As alíquotas do grupo BB-SC também foram resfriadas e, após equilíbrio a 5°C por 4h, foram colocadas em uma caixa de isopor de 21L, 5 cm acima da superfície do nitrogênio líquido. À tarde (A), no D0 (14h), os

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animais receberam BE 2,0 mg IM (Estrogin®) e um implante auricular (Crestar® 3,0 mg P4). No D9 (A), o implante foi retirado e os animais receberam eCG 400 UI IM (Folligon® 5000) + cloprostenol PGF$_2\alpha$ 0,530 mg IM (Sincrocio®). No D10 (A), os animais receberam BE 1,0mg IM (Estrogin®), e, no D12 (8h da manhã), foram realizadas as IAs. No D42, a gestação foi diagnosticada por ultrassonografia. As taxas de concepção (TC) totais foram 48,2% SR e 34,6% SC para os anos de 2014 a 2017, com uma diferença significativa de 13,7% (P<0,05). Em conclusão, o sêmen resfriado resultou em maior TC do que o sêmen congelado em bubalinos leiteiros sob P4/E2 e eCG FTAI durante a estação reprodutiva desfavorável.

Palavras-chave: fertilidade, sincronização de estro, sêmen resfriado

INTRODUCTION

The population count of Brazilian buffalo species is estimated to be 1.434.141 (IBGE, 2019); however, only 1% of females of reproductive age are inseminated (IBGE, 2016). This is mainly due to the small number of sires with high genetic quality and to the reduced number of buffaloes available for semen collection and freezing at business centers approved by the Ministry of Agriculture. Commercialization of frozen semen is only allowed by these centers (MAPA Normative Instruction nº 36, of October 27, 2015 - Brasil, 2015) leading to a low availability of frozen semen on the domestic market. The situation is worsened by the ban on importing animals and frozen semen from Asian countries with more advanced buffalo farming.

The low use of artificial insemination can also be attributed to a lack of well-organized artificial insemination programs in the country, the questionable efficiency to implement such programs due to the difficulty in detecting oestrus behavior (Baruselli, 1992) and the variable length of estrus 6-48h (Porto-Filho *et al*., 1999). All together resulting in high labor costs (Baruselli, 1996). Considering the aforementioned problems, some solutions have been proposed, such as the use of fixed-time artificial insemination (FTAI), which leads to the insemination of a large number of females on a predetermined day and time with no need for estrus observation and at the beginning of the breeding season, reducing the interval between calving and allowing the removal of females that have already undergone estrus at the end of the breeding season (Baruselli *et al*., 2004). This technique also allows to anticipate conception and calving within the respective reproductive seasons, besides increasing the probability of a new pregnancy in the next season and group calving (Gottschall *et al*., 2008). Some studies have reported that FTAI is a biotechnology that brings together the benefits of genetic improvement and reproductive management, as it allows a greater number of buffaloes to be artificially inseminated. Moreover, it makes insemination possible during periods (spring and summer) that are unfavorable for natural reproduction (Carvalho *et al*., 2011).

It is known that cryopreserved spermatozoa have a lower half-life in the female reproductive tract than fresh semen (Curry, 2000) and that reduced sperm quantity or quality results in an exponential fertility decrease in inseminated animals (Watson, 2000). The above stated problems can be solved by using cooled semen (CS) as an alternative to frozen semen (FS) to minimize the damage caused to the spermatozoa in the freezing process. The damages caused by cooling are potentially lower than those caused by freezing, with the disadvantage of preserving the spermatozoa outside the reproductive tract for a shorter time. However, CS preserves sperm viability, which in turn reduces the insemination dose, and has greater longevity in the female reproductive tract, ensuring higher fertilization rates (Bucher *et al*., 2009).

According to the author, this is particularly interesting in the case of asynchronous ovulation at the time of insemination. The use of cooling as an alternative to freezing can include and optimize the use of high genetic quality sires whose spermatozoa have low resistance to freezing in AI programs. Moreover, the absence of storage costs and the simple manipulation/use of CS in AI is an advantage when compared to FS (Vishwanath, 2003). In this context, the objective of this study was to compare the reproductive efficiency of dairy buffaloes undergoing FTAI protocols based on progesterone/estrogen (P_4/E_2) and eCG treatments during unfavorable reproductive season (URS - spring and summer), using cooled and frozen semen.

MATERIAL AND METHODS

All procedures used in this study were approved by the Animal Ethics Committee of the Veterinary School of the Federal University of Minas Gerais (EV/UFMG) under protocol 368/2015. The experiment was conducted at Bom Destino Farm, Morro do Ferro District, Oliveira, Minas Gerais, latitude 20º41'45" South and longitude 44º49'37" West, during the unfavorable reproductive period (URP - spring and summer), from 2014 to 2017. This study included eight Murrah bulls (*Bubalus bubalis*) from the above-mentioned farm, with the following average characteristics: 6.4 years of age; body condition score (BCS) = 3.6 (1-5); weight 763.8 kg; and scrotal perimeter = 36.9cm. The sires had a known fertility history, were managed separately from the females, and selected according to preconditioning for artificial vagina collection and quality of ejaculate that met the physical and morphological characteristics recommended in the Manual of Andrology (Manual..., 2013).

The collections were performed using an artificial vagina with an internal temperature between 42-45 °C (Sansone *et al*., 2000; Ohashi *et al*., 2011; Vale, 2011). Graduated sterile collecting cups (15 mL plastic tubes) were coupled to the artificial vagina. They were preheated and protected with an isothermal jacket to avoid contact with ultraviolet (UV) radiation and sudden temperature changes that could affect the semen. A false mating was induced (without semen collection, to increase sperm concentration in the ejaculate) before each collection. The ejaculate of the second mating was collected, immediately sent to the laboratory, and placed in a water bath at 37 °C for sperm motility and vigor analysis in an optical microscopy (Manual..., 2013). Sperm concentration was determined using a Neubauer chamber.

Final concentration was calculated based on previous seminal freezing and cooling tests done with each seminal donor. Total sperm number per doses was adjusted, at each collection and in function of each seminal donor, to obtain post 24 h cooling or post freezing, at insemination time, between 12 to 15 million viable sperm cells (Almeida *et al*., 2020). All samples were divided into two aliquots: one diluted in the Botu-Bov® commercial extender (Botupharma, Botucatu/SP, Brazil) and cooled (BB-CS) and the other diluted in the same extender and frozen (BB-FS). After being packed in 0.5 mL vials (IMV® Technologies, L'Aigle Cedex, France), the samples went through two types of processing. Initially, the semen samples diluted in BB-CS and BB-FS (vials) were packed in a plastic bag and then submerged in a glass vessel with water (260 mL) at 27 ºC. This vessel was placed in another glass vessel containing water (0.5 L) and stabilized in a refrigerator (vertical refrigerator measuring 47 cm wide × 59.5 cm high × 45 cm deep) at a constant temperature of 5 °C.

The samples were left in the refrigerator for 4 h for equilibration, obtaining a cooling curve of 0.25 ºC/min (from 27 to 5 ºC/mean period evaluated). After that, the semen to be cooled (BB-CS) was maintained for another 20 h at a refrigeration temperature of 5 °C. After the equilibration time (4 h), the freezing group vials (BB-FS) were placed 5 cm above a liquid nitrogen (N_2) surface and kept in a polystyrene box (39.0 cm long × 19 cm wide × 30.0 cm high). After 20 min, the vials were submerged in N_2. All samples were evaluated before AI for sperm quality characteristics. Requirements for the performance of the AI were: CS - total motility > 50% and total sperm defects ≤ 20%; FS - total motility > 30% and total sperm defects ≤ 20%.

In year "IV", due to unidentified factors, bulls that were supposed to be collected at the farm where AI would be performed, did not maintain ejaculates within minimal quality requirements for cooling and freezing. So, the semen was collected from healthy donors which were being kept under regular seminal collection schedule (every 7 days) at another farm, located 230 km from the site where AI would be performed. Seminal processing followed exactly the same procedures as previously described. The semen cooled at 5 ºC was packed in a polystyrene box suitable for transportation and maintained at constant temperature of 5 ºC until AI, 24 h after collection. Frozen semen was transported in a liquid nitrogen container.

This study included a total of 446 Murrah and crossbred females with the following mean characteristics: age = 5.7 years; BCS = 3.8 (1-5); weight = 654.3 kg; with multiparous and lactating dams (> 40 days postpartum) equally distributed in the groups and managed on Tifton pasture (*Cynodon* spp.) throughout the experiment, with

free access to mineral salt and water ad libitum. The animals were randomly distributed into four blocks according to the year: B1 = 143 (2014), B2 = 34 (2015), B3 = 90 (2016), and B4 = 179 (2017), each block being subdivided into two for AI with CS or FS using the same ejaculate of each bull, maintaining the balance between cyclic and acyclic animals in the groups. Thus, the subdivision of the blocks was structured as B1 (CS = 71 and FS = 72); B2 (CS = 18 and FS = 16); B3 (CS = 47 and FS = 43), and B4 (CS = 90 and FS = 89).

The females were submitted to evaluation of the ovarian dynamics during the unfavorable reproductive period (URP - spring and summer) with a 5.0 MHz linear transducer (Aloka-SSD 500, Tokyo, Japan) before estrus induction. Ultrasound corrections were performed by the same examiner, using the same image settings and resolutions. As stated, the females were assessed for cyclicity: cyclic when a corpus luteum was present on the day of implantation of the progesterone device and non-cyclic when without a corpus luteum. Another ultrasound evaluation was performed on the day of AI (D12) to verify the response to the protocol, and all animals were subsequently inseminated regardless of the response.

The following protocol was used to synchronize the estrous cycle: In the afternoon (A) on D0 (14h) the animals received estradiol benzoate 2.0 mg IM (BE, Estrogin®, Farmavet, SP, Brazil) and an ear implant (CRESTAR® 3.0 mg P4, MSD, Brazil). On D9 (A) the implant was removed, and the animals received eCG 400 IU IM (Folligon® 5000, MSD, Brazil) + Cloprostenol $PGF_2\alpha$ 0.530 mg IM (Sincrocio®, Ourofino, SP, Brazil). On D10 (A) the animals received BE 1.0 mg IM (Estrogin®), and on D12 in the morning (M) (8:00 am), AI was performed.

A positive diagnose was based on the visualization of an embryonic vesicle and confirmed by the detection of the embryonic heartbeat. To avoid any misinterpretation of the pregnancy diagnosis, bulls were placed in female lots only 15 days after AI for subsequent fertilization of non-pregnant females. Diagnosis of pregnancy was used as a dependent variable for the statistical analysis. The following variables: year of insemination, age, weight, postpartum days, and BCS, were used as independent variables, seeking an association between dependent and independent variables. Sometimes the semen preservation method was used as a second source of variation. The presence of association was analyzed through contingency tables using the chi-square test (Sampaio, 2007). Logistic regression was used to verify the association between pregnancy diagnosis and some variables with more than two categories (nominal) in order to evaluate which of the categories were associated. The odds ratio was used to verify the strength of association (Dohoo, 2010).

RESULTS

The ovarian activity status of buffalo cows at the beginning of estrus induction (average of the four years) is summarized in Table 1. In addition, more CL were found in the right ovary than in the left ($P<0.05$), but the dominant follicles, when present, were uniformly distributed in the ovaries. In all groups, during the four years, the percentage of cows with low ovarian activity exceeded the number of cyclic cows added to cows with dominant follicles.

Table 1. Ovarian structures of buffaloes undergoing gynecological examination prior to estrus induction and synchronization for FTAI, sum of the years I to IV

Ovarian structures					
Absence n(%)	CLRO n(%)	CLLO n(%)	DFRO n(%)	DFLO n(%)	Total n(%)
257(57.6^{a})	121(27.1^{b})	55(12.3^{c})	6 (1.3^{d})	7(1.6^{d})	446 (100.0)

Means with different letters differ from each other ($P<0.05$); n = number and % = percentage. CLRO = corpus luteum in the right ovary; CLLO = corpus luteum in the left ovary; DFRO = dominant follicle in the right ovary; and DFLO = dominant follicle in the left ovary.

Buffalo cows did not show a similar follicular growth pattern among years (Table 2). In year I, ovaries had follicles in the two larger diameter categories (0.50 - to 10.0 mm or >10.0 mm), while in years II and III all cows had follicles in the middle category. In contrast, in year IV, all buffaloes had follicles in the lowest follicular size category. The total gestation rates (CS + FS) for

the four years of evaluation are similar to one another, being always below 50% (Table 3).

The conception rates obtained during each of the four years of study with cooled semen were superior (P<0.05) to those of frozen semen. There was a difference in CR between CS and FS of 11.8, 12.5, 25.1 and 9.5 favoring CS for the years I to IV, respectively (Table 4). Table 5 shows the effect of BCS on the CR using different types of semen. There was no significant difference among the three BCS categories for CS, as opposed to FS.

The variable bull had no effect on the CR of buffaloes undergoing FTAI in the unfavorable reproductive period. Conception rates obtained for bulls 1 to 8 were respectively 40.0(36/90), 40.4(19/40), 47.6(20/42), 39.2(29/74), 36.7(11/30), 43.0(34/79), 43.9(25/57) and 40.7(11/27). The values for the eight bulls having their semen used cooled or frozen in the synchronization protocols varied between 36.7 and 47.6%. (P>0.05). Once all variables associated to FTAI were standardized, the present evaluation attributed the best CR results after insemination using CS (final mixed logistics). The *odds ratio* indicated that the CS used was 4.6 times more likely to be successful, resulting in a larger number of pregnant buffaloes than the FS (Table 6).

Table 2. Mean values of follicular diameter of buffaloes undergoing gynecological examination before estrus induction and synchronization for FTAI during the years I to IV

	Follicular diameters		
Year	< 0.50 mm %(n/N)	0.50-10.0 mm %(n/N)	>10.0 mm %(n/N)
I	0.0(0/143)	45.5(65/143)	54.5(78/143)*
II	0.0(0/34)	100.0(34/34)	0.0(0/34)
III	0.0(0/90)	100.0(90/90)	0.0(0/90)
IV	100.0(179/179)	0.0(0/179)	0.0(0/179)
Total	40.1^{a}(179/446)	42.4^{a}(189/446)	17.5^{b}(78/446)

Means with different letters in the same rows differ from one another (P<0.05); % = percentage; n = number of females by follicular diameter category; and N = total number of females evaluated per group. *Females with corpus luteum are included.

Table 3. Mean total CR by year for buffaloes undergoing FTAI (cooled plus frozen semen) during the unfavorable reproductive period (October to December) between the years I and IV

Year	Pregnant %(n/N)*
I	39.2(56/143)
II	44.1(15/34)
III	43.3(39/90)
IV	41.9(75/179)
Total	41.5(185/446)

* = p>0.05; % = percentage; n = number of females per group; and N = total number of females evaluated per group.

Table 4. Mean CR per year in buffaloes undergoing FTAI during the unfavorable reproductive period (October to December) between the years I and IV

	Type of semen	
Year	Cooled %(n/N)	Frozen %(n/N)
I	45.1^{a}(32/71)	33.3^{b}(24/72)
II	50.0^{a}(09/18)	37.5^{b}(06/16)
III	55.3^{a}(26/47)	30.2^{b}(13/43)
IV	46.6^{a}(42/90)	37.1^{b}(33/89)
Total	48.2^{a}(109/226)	34.6^{b}(76/220)

Means with distinct letters in the same rows differ from one another (P<0.05); % = percentage; n = number of pregnant females; and N = total number of females evaluated per group.

Table 5. Mean CR by BCS in buffaloes undergoing FTAI in the unfavorable reproductive period (October to December) from I to IV

CR	BCS			Total
	≤ 3.0 (n/N)	> 3.0 e ≤ 4.0 %(n/N)	> 4.0 e ≤ 5.0 %(n/N)	
CS	48.2^{Aa}(27/56)	48.1^{Aa}(64/133)	48.6^{Aa}(18/37)	48.2^{A}(109/226)
FS	45.8^{Aa}(33/72)	32.5^{Bb}(37/114)	17.6^{Bc}(6/34)	34.5^{B}(76/220)
Total	46.9^{a}(128)	40.9^{b}(247)	33.8^{c}(71)	41.5(185/446)

Means with different letters (upper case in the column and lower case in the row) differ from one another (P<0.05); % = conception rate; n = number of pregnant females; and N = number of females evaluated per group.

Table 6**.** Risk factor (*odds ratio*) for buffalo CR using cooled and frozen semen in synchronization protocols containing progesterone, estrogen and eCG during the unfavorable reproductive period (October to December)

Variable	OD (%)	SE
Cooled semen	48.2^{a}	33.2
Frozen semen	34.5^{b}	32.1
Semen variation	13.7	04.6

Means with different letters in the columns differ from each other (P<0.05); % = Percentage; OR = Odds Ratio; SE = Standard error.

The probability of pregnancy with CS in the IATF protocol in buffaloes was higher than with FS, with 2.9 times more chances of a female becoming pregnant with CS compared to the use of FS as indicated by the proportion comparison test. The use of CS can be a determinant for greater efficiency of the FTAI program, since the analysis of CS showed a lower failure rate in semen conservation, but, above all, a higher proportion of high-quality sperm used in females submitted to synchronization and FTAI protocols.

When the CR was evaluated according to the number of postpartum days, the best results obtained for cooled and frozen semen were within the interval between 60 and 90 days postpartum (Table 7). Conception rate was not influenced by follicular growth activity at the time of estrus induction. CRs in buffalo cows with follicles of < 0.5mm, 0.50 to 10.0 mm or ≥ 10 mm were respectively: 41.9% (75/179), 39.2% (74/189) and 46.2% (36/78) (p>0.05).

Table 7. Average CR according to number of days postpartum in buffaloes undergoing FTAI during the unfavorable reproductive period (October IV)

Type of semen	CR in days postpartum		
	30-60 %(n/N)	60-90 %(n/N)	> 90 %(n/N)
Cooled	48.5^{Aa}(16/33)	56.9^{Aa}(33/58)	44.4^{Aa}(60/135)
Frozen	30.6^{Ba}(11/36)	38.6^{Ba}(22/57)	33.9^{Ba}(43/127)
Total	39.1^{b}(27/69)	47.8^{a}(55/115)	39.3^{b}(103/262)

Means with different letters (upper case in the column and lower case in the row) differ from one another (P<0.05); % = percentage; n = number of pregnant females; and N = total number of females evaluated per group.

DISCUSSION

The use of breeding biotechnology, including FTAI, has been widely supported by buffalo technicians and producers in recent years because this technique can anticipate conception and calving within the respective reproductive seasons, besides increasing the probability of a new pregnancy in the subsequent season and concentrate births (Baruselli *et al*., 2004). It can be observed from data presented in Table 1 that most of the buffalo cows in the beginning of estrus induction were acyclic with few showing relevant follicular growth. This was even more pronounced in year IV. In all four years, the schedule for ovulation induction began in October until December, while the physiological breeding season for the site of the experiment was foreseen from late March to July. Also, in year IV, due to management adjustments, the proportion of primiparous cows and cows recently introduced into the herd was higher as compared to previous

years. In addition, the newly introduced animals had slightly lower body score conditions (≤3) than residents (≥3.5).

All those adjustments made in the fourth year are likely to be the reason for more discrete ovarian activity (less follicular growth) for cows managed that year. According to Rhodes et al (Rhodes *et al*., 2003) low body reserves associated to increased energy intake requirements for milk production are factors related to low ovarian activity. However, despite the fact that cows of year IV were not as homogeneous as were the groups of cows from previous years, it did not change the conception rate, which remained similar to previous years. Barusselli and Carvalho (2005) reported that the lower body score condition favored a decrease in the conception rate. It may well be that the difference observed in the BSC in year IV compared to previous years has not been pronounced enough to cause a decrease in the conception rate.

It is worth to highlight that by the time buffalo cows were induced, the right ovary presented a higher proportion of CL which was in disagreement with the finding by Danell (1987), who reported in a group of cycling buffalos a slightly higher proportion of CL in the left ovary (51,2%) than in the right (48,8%). The general conception rate (CS+FS) obtained from present study the transitional period to the physiological reproductive season was 41.5% and there was no significant difference between the years (P>0.05). Similar values, 43.1 (Porto-Filho *et al*., 2004) and 37.0 (Frares *et al*., 2013) have been reported in studies carried out during the unfavorable reproductive period. In all three studies, the eCG dosage used was 400 IU. In contrast, a 62.7% conception rate was obtained in acyclic buffaloes induced during the unfavorable reproductive period (Carvalho *et al*., 2016). In addition, within type of semen, chilled or frozen, conception rates were similar between years (Table 4).

On the other hand, in the four years separately and for the general conception rate (4 years combined) the values obtained were significantly higher with chilled semen (48.2%) than with frozen semen (34.6) (P<0.05). Additionally, *Odds Ratio* data showed a higher probability of conception for CS in dairy buffaloes (Table 5). This information agrees with the literature (Januskauskas e Zilinskas, 2002). Also, our data provided the indication that the use of CS had a 4.6 point higher probability to result in pregnancy as compared to FS strengthening even more the efficacy of the chilled semen.

The artificial insemination schedule of the present study followed the guidelines proposed by Baruselli (1994). The author demonstrated, using frozen semen that a better conception rate was obtained when the insemination was performed in the morning (72.2%) as compared to the afternoon (46.3). As in the present study inseminations, either with cooled or frozen semen, were done in the morning, as suggested above, the time of the insemination would not be the cause of a lower conception rate with frozen semen compared to chilled semen. Another effect which could explain the difference of conception rate between cooled or frozen semen is the number of viable spermatozoa per dose at the time of insemination. Although it has been reported that the number of spermatozoa reaching the fertilization site is reduced compared to the number of spermatozoa used in the insemination dose (Breen *et al*., 2005), a higher number of viable sperm cells at insemination could *per se* increase the number of sperm cells on the fertilization site and hence increase the chance for fertilization.

Seminal donors in the present study, for other purposes, were maintained for more than 6 months under a weekly schedule of seminal freezing. Thus, the average post thaw sperm motility of each of them was well known, and the motility drop curve of their sperm cells submitted to cooling to 5 °C for more than 72 hours was studied in advance (part of another study). This information was used to calculate at each collection, the required dilution factor for cooling or freezing to obtain, at insemination time, a similar number of viable spermatozoa (motile sperm cells). In the present experiment, the number of viable sperm cells fluctuated between 12 to 17 million per dose for both frozen and cooled semen. Consequently, the number of viable cells per insemination dose is not likely to explain the difference in conception rate between type of semen.

Cryopreservation is a technique used to preserve sperm cells. However, depending on the cryopreservation process used (chilled or frozen), damage to the cells can be more or less pronounced, and damage is expressed by the

decrease in sperm motility. In the present study, the decrease in sperm motility for all seminal donors used was higher in frozen/thaw cells than after 24-hour incubation at 5 °C. This finding was also observed in other studies of our lab (unpublished data). This clearly indicates that sperm cells chilled for 24 hours are less injured than frozen/thawed cells. Other reports support this reasoning (Curry, 2000; Watson, 2000; Sá Filho *et al*., 2009), it is likely that less injured sperm cells (chilled semen) had a better fertilizing capacity. The present findings show that buffalo females inseminated with CS are 4.6-fold more likely to become pregnant presenting better quality and a less injured cells when chilling was used. This trend has been reported in artificial insemination programs in bovine (Watson, 2000; Rolim Filho *et al*., 2011; Borchardth *et al*., 2018). The higher fertilizing capacity may in part result from a longer lifespan in the female reproductive tract of CS compared to FS. A longer longevity would allow a larger period for fertilization of ovulations which occur outside the expected time.

The repetition of inseminations for four consecutive years provided the opportunity to analyze several aspects which could result in a variation in the conception rates. Although in all postpartum intervals studied the difference in conception rate in favor of CS semen was maintained, a difference in conception rate between post-partum periods (Table 6) was observed only when data of cooled and frozen semen were combined. A higher conception rate was observed in the period of 60 to 90 days postpartum as compared to 30 to 60 days or > 90 days. The puerperium period in buffalos lasts on average for 27,5±7,77 days (Borchardth *et al*., 2018), and recovering of cycling ovarian activity during the reproductive season and with cows showing good body score condition occurs around days 15 and 60 (Shah, 1990), 24 to 100 days postpartum (Baruselli, 1992).

Thus, lower conception rates found in the cow group of 30 to 60 days postpartum are certainly due to the inclusion of animals in this group with no fully recovered genital tracts. On the other hand, it is not clear why the conception rate was lower in the group of >90 days postpartum. In this group of cows, if induction would have started during the favorable breeding season, an even better conception rate would be expected in this group due to a longer time for recovery of the uterus and ovarian activity. In fact, in the present study we were in the unfavorable breeding season, when most animals were still acyclic, confounding a plausible explanation for this slight decrease in conception rate.

Evaluating the conception rate of CS and FS combined, it could be observed that there was a decrease in conception rate as BCS increased. This was not detectable while using CS but was remarkable when FS was used (Table 7). At the farm where the experiment was carried out, all buffalo cows were well fed and the predominance of upper grade of BCS could easily be noticed (exception for the year IV). The accumulation of fat is a factor which may hamper warmth dissipation and buffaloes are known as a specie which may suffer more easily in hot weather when shade or water are not adequately available. The farm was located in a tropical region with day temperature reached up to 32 °C. Thermal stress triggers changes in plasma concentrations of hormones and metabolites in milk cows, such as increased production and secretion of glucocorticoids, especially cortisol. High levels of cortisol caused by stress can inhibit GnRH secretion by the hypothalamus and LH by the pituitary, essential hormones in the follicular and preovulatory phase (Breen *et al*., 2005).

Following the same line, Borchardth *et al.* (2018), hypothesize that a high BCS has a negative influence by delaying ovulation time. It could then be conjectured that GnRH and LH release could have been impaired in cows with high BCS due to thermal stress, and, in consequence, ovulation rates dropped. However, if this argument was fully true, the decrease in CR, particularly in the two upper BCS groups, would have occurred in the same proportion for both groups of semen, however, herein cooled semen showed better results. This opens up the possibility to consider that frozen semen was more susceptible to any increase in body temperature than cooled semen. This proposition is in part supported by the fact that in the lower BCS group no difference in CR was detected between CS and FS. The higher cell damage during the freezing/thawing process as compared to cooling (Watson, 2000; Rolim Filho *et al*., 2011; Borchardth *et al.*, 2018) may have contributed to raise this susceptibility.

Monteiro *et al*. (2016) in a study done in buffaloes submitted to FTAI during the FRP and using the same amount of eCG (400 IU), as used in the present study, found a 61.5% pregnancy rate in cows with a mean follicular diameter of 14.2 mm. Based on those findings they concluded that the presence of large follicles in the ovaries at the time of AI increases the chance of fertilization. Our results did not show such tendency, conception rates were similar in buffalo with ovaries with follicles < 0.5 mm, 0.50 to 10.0 mm or ≥ 10 mm at AI. A finding like the present one was reported by Porto-Filho *et al.* (2004) for buffaloes submitted to FTAI in the URP.

An effect of ejaculate or of seminal donor could have contributed to a variation in CR between groups of cows or groups of semen. In the present study eight seminal donors were used, one per insemination session (group of cows), and, for each session the ejaculate was split for either chilling or freezing simultaneously. Therefore, effect of ejaculate in favor of chilled or in detriment of frozen semen can be excluded. Additionally, quite a large number of seminal donors was used and CR among seminal donor was similar, showing how homogeneous the group of males was as far as fertility is concerned, reducing the possibility of an effect of seminal donor in favor of one or of another technique of seminal preservation (the low number of females per group of type of semen per bull did not allow a more robust statistical analysis).

CONCLUSION

The present study demonstrated that cooled semen for 24 h resulted in higher conception rate than frozen semen in a FTAI program and that high body score condition is detrimental to conception rate when frozen semen is used. Thus, cooled buffalo semen represents a promising alternative to frozen semen. It is important to highlight that sometimes it was difficult to maintain a regular and continuous success rate of collection of some buffalo seminal donors; this may be a restrictive factor for the use of cooled or fresh semen. The solution to surpass this difficulty needs to be addressed.

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REFERENCES

ALMEIDA, J.; BRITO, M.F.; BECERRA, V.A.B. *et al*. Effectiveness of tes-tris or tris association with low density lipoprotein on in vitro longevity of refrigerated buffalo semen. *Braz. Arch. Vet. Med. Anim. Sci.*, v.72, p.729-736, 2020.

BARUSELLI, B.P. Ovarian activity and reproductive behavior in the postpartum period in buffaloes *(Bubalus bubalis).* 1992. 99p. Dissertation (Master) - Faculty of Veterinary Medicine and Animal Science, University of São Paulo, SP.

BARUSELLI, B.P. Reproduction of buffaloes. In: BRAZILIAN SYMPOSIUM ON BUBALINOCULTURE, 1996, Cruz das Almas. *Anais...* Cruz das Almas, BA: UFBA, 1996. p.117-153.

BARUSELLI, P.S. Sexual behaviour in buffaloes. In: WORLD BUFFALO CONGRESS, 4., 1994, Cairo. *Proceedings…* Cairo: Faculty of Veterinary Medicine, 1994. v.1, p.158-173.

BARUSELLI, P.S.; CARVALHO, N.A.T. Biotecnologias da reprodução em bubalinos (*Bubalus bubalis*). *Rev. Bras. Reprod. Anim*., v.29, p.417-425, 2005.

BARUSELLI, P.S.; REIS, E.L.; CARVALHO, N.A.T. *et al*. eCG increase ovulation rate and plasmatic progesterone concentration in Nelore (*Bos indicus*) heifers treated with progesterone releasing device. *Resumo*, 2004.

BORCHARDTH, S.; SCHULLER, L.; WOLF, L. *et al*. Comparison of pregnancy outcomes using either an Ovsynch or a Cosynch protocol for the first timed AI with liquid or frozen semen in lactating dairy cows. *Theriogenology*, v.107, p.21-26, 2018.

BRASIL - Ministry of Agriculture, Livestock and Supply Normative Instruction nº. 36, of October 27, 2015. Establish minimum sanitary requirements for the production and commercialization of bovine and buffalo semen in the country. *Official Diary*, 2015.

BREEN, K.M.; BILLINGS, H.J.; WAGENMAKER, E.R. *et al*. Endocrine basis for disruptive effects of cortisol on preovulatory events, *Endocrinology*, v.146, p.2107-2115, 2005.

BUCHER, A.; KASIMANICKAM, R.; HALL, J.B. *et al*. Fixed-time AI pregnancy rate following insemination with frozen-thawed or fresh-extended semen in progesterone supplemented CO-Synch protocol in beef cows. *Theriogenology*. v.71, p.1180-1185, 2009.

CARVALHO, N.A.T.; BERNARDES, O.; BARUSELLI, P.S. Seasonality of calving for the production of buffalo milk to pasture in central southern Brazil. *Res. Technol*., v.8, p.1-5, 2011.

CARVALHO, N.A.T.; SOARES, J.G.; BARUSELLI, P.S. Strategies to overcome seasonal anestrus in water buffalo. *Theriogenology*, v.86, p.200-206, 2016.

CURRY, M.R. Cryopreservation of domestic livestock. *Rev. Reprod*. v.5, p.46-52, 2000.

DANELL, B. *Oestrus behavior, ovary morphology and cyclical variation in follicular system and endocrine pattern in water buffalo heifers*. 1987. Thesis (PhD) - Department of Obstetrician Gynecology, Faculty of Veterinary Medical, Swedish.

DOHOO, I.R.; MARTIN, W.; STRYHN, H. *Veterinary epidemiologic research*. 2.ed. Charlottetown, Canadá: University of Prince Edward Island, 2010. 865p.

FRARES, L.F.; WEISS, R.R.; KOZICKI, L.E. *et al*. Estrus Synchronization and Fixed-Time Artificial Insemination (FTAI) in dairy buffaloes during seasonal anestrus. *Braz. Arch. Biol. Technol*., v.56, p.575-580, 2013.

GOTTSCHALL, C.S.; MARQUES, P.R.; CANELLAS, L.C. *et. al*. Aspects related to estrus synchronization and ovulation in beef cattle. *Hora Vet*., v.164, p.43-48, 2008.

IBGE. INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA. Available in: https://agenciadenoticias.ibge.gov.br/agencia-sala-de-imprensa/2013-agencia-de noticias/releases/29163-ppm-2019-apos-dois-anos-de-queda-rebanho-bovino-cresce-0-4; 2019. Accessed in: 10 Nov. 2020.

IBGE. INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA. Available in: http://www.ibge.gov.br/home. Accessed in: 21 Feb. 2016

JANUSKAUSKAS, A.; ZILINSKAS, H. Bull semen evaluation post-thaw and relation of semen characteristics to bull's fertility. *Vet. Zootec.*, v.39, p.1-8, 2002.

MANUAL for andrological examination and evaluation of animal semen. 3.ed. Belo Horizonte: Brazilian College of Animal Reproduction, 2013. 104p.

MONTEIRO, B.M.; SOUSA, D.C.; VASCONCELLOS, G.S. *et al*. Ovarian responses of dairy buffalo cows to timed artificial insemination protocol, using new or used progesterone devices, during the breeding season (autumn-winter). *Anim. Sci. J*., v.87, p.13-20, 2016.

OHASHI, O.M.; SANTOS, S.S.D.; MIRANDA, M.S. *et al*. Morphology of genital system, reproductive disorders and management of male buffalo (*Bubalus bubalis*). *Rev. Bras. Reprod. Anim*., v.35, p.88-94, 2011.

PORTO-FILHO, R, M.; CARVALHO, N.A.T.; VIEL JÚNIOR, J.O. *et al*. eCG dosage reduction in a protocol for synchronization of ovulation for timed artificial insemination during the off breeding season in buffalo. 2., 2004, Corrientes. In: BUFFALO SYMPOSIUM OF AMERICAS. *Proceedings…* Corrientes: CBRA, 2004. CD-room.

PORTO-FILHO, R.M.; BARUSELLI, O.S.; MADUREIRA, E.H. *et al*. Detection of cio in buffaloes through the radiotelemetry system. *Rev. Bras. Reprod. Anim*., v.23, p.356-358, 1999.

RHODES, F.M.; MCDOUGALL, S.; BURKE, C.R. *et al*. Treatment of cows with extended postpartum anestrous interval. *J. Dair. Sci.*, v.86, p.1876-1894, 2003.

ROLIM FILHO, S.T.; RIBEIRO, H.F.L.; VALE, W.G. *et al*. Uterine involution, ovarian activity, first postpartum heat and reproductive disorders in buffaloes. *Cienc. Anim. Bras*., v.12, p.221-227, 2011.

SÁ FILHO, O.J.; MENEGHETTI, M.; PERES, R. *et al*. Fixed time artificial insemination with estradiol and progesterone for *Bos indicus* cows. II. Strategies and factors affecting fertility. *Theriogenology*, v.72, p.210-218, 2009.

SAMPAIO, I.B.M. *Statistics applied to animal experimentation*. 3.ed. Belo Horizonte: Foundation of Study and Research in Veterinary Medicine and Animal Husbandry, 2007. 264p.

SANSONE, G.; NASTRI, M.J.F.; FABBROCINI, A. Storage of buffalo (*Bubalus bubalis*) semen. *Anim. Reprod. Sci*., v.62, p.55-76, 2000.

SHAH, S.N.H. *Prolonged calving intervals in Nili-Ravi buffalo*. 1990. 162p. Thesis (Doctorate) - Utrecht: Rijksuniversiteitte Utrecht.

VALE, W.G. Biotechnological advances in buffalo reproduction. *Technol. Marcha* v.24, p.89-90, 2011.

VISHWANATH, R. Artificial insemination: the state of the art. *Theriogenology*, v.59, p.571-584, 2003.

WATSON, P.F. The causes of reduced fertility with cryopreserved semen. *Anim. Reprod. Sci*., v.60-6, p.481-492, 2000.