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In vitro evaluation of cryopreserved bovine sperm and its relation to field fertility in fixed-time artificial insemination

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This study aimed to assess characteristics of bovine cryopreserved sperm and evaluate its relation to field fertility in fixed-time artificial insemination (FTAI). Semen samples of 16 bulls were used to inseminate 811 Nellore cows, and four of these bulls were also used to inseminate 101 Nellore heifers. Samples of the same ejaculate used for FTAI from each bull were analysed in the laboratory after thawing. Sperm motility and vigour were subjectively assessed by light microscope, and integrity of the plasma and acrosome membranes, and H_2O_2 production were evaluated by flow cytometer. Relation among sperm characteristics and pregnancy rate of cows and heifers were evaluated by univariate and multivariate logistic regression. Subjective sperm motility and vigour did not affect the probability of pregnancy in cows or heifers. In univariate analysis for pregnancy in cows, sperm traits related to acrosome injury positively affected probability of pregnancy mainly when associated with plasma membrane integrity; H_2O_2 production seems to be less important than plasma membrane integrity in affecting probability of pregnancy. In multivariate analysis, sperm traits related to injured acrosome positively affected probability of cow and heifer pregnancies while intact acrosome was negatively related to cow pregnancy. Intact plasma membrane and high H_2O_2 production were positively related to cow pregnancy but negatively related to heifer pregnancy. Results suggest that a capacitation-like status of the acrosome may benefit probability of pregnancy in cows.

KEYWORDS

cattle, flow cytometer, semen fertility

1 | INTRODUCTION

The sperm cell becomes infertile when one or more of its biochemical or morphological factors are affected (Siqueira et al., 2007), and by this, the evaluation of only one of these points does not guarantee the normal condition of the other. Therefore, a combination of several characteristics in a multifactorial analysis is more appropriate for the diagnosis of the functionality and integrity of the sperm cell (Tartaglione & Ritta, 2004).

In semen production centres, the main parameters that are usually used to assess bull seminal quality are sperm motility and morphology. Although being a minimum standard of semen quality, these analyses also attend for selection of semen donors.

Subjective sperm motility assessment has been statistically related to fertility, even for post-thawed semen in bulls (Rodríguez-Martínez, 2003). However, this parameter has shown to be an ineffective predictor for semen fertility in commercial artificial insemination (Siqueira et al., 2007); even kinematic analyses using CASA (computer-assisted

sperm analyser) have shown inconstant correlations between motility patterns and field fertility (Bailey, Robertson, & Buhr, 1994; Januskauskas, Johannisson, & Rodríguez-Martínez, 2001, 2003; Rodríguez-Martínez, 2013; Zhang, Larsson, Lundeheim, & Rodríguez-Martínez, 1998). Therefore, alternative methods for semen evaluation have been developed to enhance routine tests (Rodríguez-Martínez, 2013; Taş, Bacinoglu, Cirit, Ozdaş, & Ak, 2007; Verberckmoes, Soom, Depauw, Dewulf, & Kruif, 2002). Even though these methods have been proved to be useful for in vitro sperm evaluation, they still have limited ability to predict field fertility (Morado, Pereyra, Breininger, Sara, & Cetica, 2015). Thereby, simultaneous measurement of multiple sperm traits could improve the estimation of sperm fertility (Gadea, 2005; Gillan, Kroetsch, Maxwell, & Evans, 2008; Graham, 2001). In this context, flow cytometer analysis appears as a useful tool to evaluate several sperm characteristics and relate them to field fertility (Oliveira, Arruda, Andrade, Celeghini et al., 2012).

Therefore, this study aimed to evaluate the effects of sperm characteristics, such as subjective sperm motility and vigour, acrosome and plasma membrane integrity, and H_2O_2 production, on field fertility in a fixed-time artificial insemination (FTAI) programme of Nellore cows and heifers.

2 | MATERIALS AND METHODS

2.1 | Ethics

This study followed the Standards of Conduct for the Use of Animals in Research and Education approved by the Ethics Committee for Animal Use of the Universidade Federal de Viçosa, proc. n. 07/2015.

2.2 | Local and animals

Lactating Nellore cows (n = 811) and heifers (n = 101) were located in Carlos Chagas/MG, Brazil, 17.8511° South and 40.7171° West. Cows aged between 1,140 and 4,452 days, weighted between 400 and 450 kg with body condition score (BCS) ranging from 2 to 3 (scale of 1–5); heifers aged between 712 and 1,142 days, weighted between 350 and 400 kg with BCS ranging from 2.5 to 3.

Artificial inseminations were performed during the 2014/2015 breeding season (from November 2014 to February 2015). Females were kept in *Urochloa decumbens* pasture, with ad libitum mineral salt and water. Laboratorial evaluations were carried out in Laboratory of Animal Andrology of the Department of Veterinary and Microscopy and Microanalysis Sector, in the Universidade Federal de Viçosa, Brazil.

2.3 | Fixed-time artificial insemination

All females were synchronized for FTAI by following protocol (TECNOPEC 2008, available from https://www.abspecplan.com.br/upload/library/Manual_IATF_Bovinos.pdf): day 0: insertion of progesterone device (Primer®, Tecnopec) and administration, i.m., of 2 ml estradiol benzoate (RIC-BE®, Tecnopec); day 8: removal of progesterone device and administration, i.m., of 1 ml estradiol benzoate, 2 ml $PGF_{2\alpha}$ (Prolise®, Tecnopec) and 0.5 ml porcine follicle-stimulating hormone (Folltropin®, Tecnopec); and day 10: artificial insemination 60 hr after progesterone device removal. Pregnancy diagnosis was performed by ultrasound 30 days after artificial insemination.

2.4 | Semen samples

For semen evaluations, frozen semen samples (straws with 30×10^6 sperm) were used. Bulls that were used to inseminate at least 20 cows and at least 17 heifers were kept in data set. After data curation, 16 of the bulls (nine Nellore, four Tropical Montana composite, one Senepol, one Holstein and one Red Angus) that were used to inseminate the cows and four of the bulls (two Nellore, one Tropical Montana composite and one Senepol) that were used to inseminate heifers were included in analyses.

2.5 | Sperm motility and vigour

Immediately after thawing (37°C for 20–30 s), a droplet of semen (10 μl) was placed in a pre-heated (37°C) slide glass and covered by a coverslip and lead to an optical microscope at 400× magnificence (CBRA, 2013). Sperm motility (0%–100%) and sperm vigour (score from 0 to 5) were subjectively assessed by the same technician in at least four fields in each sample, and the results were expressed in average of the fields.

2.6 | Flow cytometer analyses

All analyses were performed in a BD FACSVerse™ (Becton-Dickinson®, Sunnyvale, CA, USA) flow cytometer. For flow cytometer calibration, semen samples from a fertile bull were diluted in PBS (phosphate-buffered saline) at 5×10^6 sperm/ml. The non-spermatic particles (debris) and particles with similar dispersion to sperm but without sufficient DNA were excluded.

Samples were first analysed without dyes, and then, the size × granularity scale was determined to specify where the sperm population was located. Sequentially, sperm and debris were sorted based on the sperm fluorescent characteristics (Ricci et al., 2002) through the use of the propidium iodide (PI) probe, which stains only structures with DNA. Tests were repeated until the appropriate selection of the expected population, and then, the best fit (compensation) obtained for each probe was determined as standard.

2.7 | Evaluation of acrosome and plasma membrane integrity

Acrosome and plasma membrane were evaluated according to a modified protocol described by Oliveira, Arruda, Andrade, Santos et al. (2012). A 150 μL aliquot of semen diluted in DPBS (Dulbecco's phosphate-buffered saline) (5×10^6 sperm/mL) was incubated with 10 μL of *Pisum sativum* agglutinin conjugated to fluorescein isothiocyanate (FITC-PSA; 100 μg/mL) and 3 μL of propidium iodide (PI;

TABLE 1 Pregnancy rates in Nellore cows and heifers submitted to FTAI (fixed-time artificial insemination) according to the bulls

Bull	Pregnancy rate in cows, % (n[a])	Pregnancy rate in heifers, % (n[a])
5	68.8 (32)	–
6	65.9 (41)	–
7	64.5 (31)	82.4 (17)
10	64.6 (130)	–
12	65.9 (41)	–
13	50.0 (46)	63.2 (19)
16	60.0 (30)	–
18	55.6 (63)	–
30	61.9 (97)	79.2 (24)
31	40.0 (20)	–
33	33.6 (107)	–
34	48.3 (29)	–
35	58.9 (56)	–
39	75.0 (20)	–
45	45.0 (20)	–
47	56.3 (48)	53.7 (41)

[a]Number of females.

0.5 mg/ml) at 37°C for 10 min. Then, 150 μL of DPBS was added and the sample was assessed. In each sample, 10,000 cells were analysed (approximately 200 sperm cells/second) in a 488 nm laser excitation. The purpose of these probes was to stain the cells with damaged plasma membranes (PI positive) and those that had injured acrosome (FITC-PSA positive).

Two-dimensional dot-plot graphics of FITC-PSA versus PI fluorescence were created. Each quadrant represented one of the following sperm subpopulations: (1) sperm with intact plasma and acrosome membranes (PI −, FITC-PSA −); (2) sperm with intact plasma membrane and damaged acrosome membrane (PI −, FITC-PSA +); (3) sperm with damaged plasma membrane and intact acrosome membrane (PI +, FITC-PSA −); and (4) sperm with damaged plasma and acrosome membranes (PI +, FITC-PSA +).

2.8 | Evaluation of H2O2 production (DCFDA/PI)

Production of H_2O_2 was assessed according to the protocol proposed by Macías-García et al. (2012). A 150 μL aliquot of semen was diluted in DPBS (5 x 10^6 sperm/mL) at 37°C and stained with 0.5 μL 2',7'-dichlorofluorescin diacetate (DCFDA; 1 mg/ml of 2 mM dimethyl sulphoxide) for 30 min; then, from this solution, 150 μL was collected, stained with 3 μL PI and incubated for 5 min. Finally, 150 μL DPBS was added and the sample was assessed.

Four different cell populations were observed: PI (+) and DCFDA (−), sperm cells with damaged plasma membrane and low intracellular H_2O_2 production; PI (+) and DCFDA (+), sperm cells with damaged plasma membrane and high intracellular H_2O_2 production; PI (−) and DCFDA (+), sperm cells with normal plasma membrane and high intracellular H_2O_2 production; and PI (−) and DCFDA (−), sperm cells with intact plasma membrane and low intracellular H_2O_2 production.

2.9 | Statistical analysis

Data analysis was performed in the Statistical Analysis System (SAS, 2002). For pregnancy rates, data were submitted to chi-square test (Freq Procedure) and rates were considered different when $p < 0.05$. Percentage data from flow cytometer analysis were submitted to angular transformation ($Y\prime = \arcsin(\sqrt{Y})$), and then, a univariate logistic regression (logistic procedure) was performed in order to evaluate the influence of each sperm trait on probability of pregnancy of cows and heifers; variables were considered significant when $p < 0.10$. Sperm traits and age of females were also evaluated by multivariate logistic regression, and only explanatory variables that were significant at $p < 0.25$ by stepwise selection were kept in final model. The association of predicted probabilities and observed responses was evaluated by the area under the receiver operator characteristic (ROC) curve (*c*) (Hosmer & Lemeshow, 2000).

3 | RESULTS

A total of 912 females were inseminated resulting in 525 pregnancies. The pregnancy rate was similar ($p = 0.059$) between heifers (66.3%) and cows (56.5%). The overall pregnancy rate per bull for all females ranged from 33.6% to 75.0%; there was significant effect of bull for cow ($p = 0.0003$) but not for heifer ($p = 0.080$) pregnancy (Table 1). Sperm parameters are shown in Table 2.

Results of analysis of maximum-likelihood estimates from the univariate logistic regression to predict probability of pregnancy in cows and heifers are in Tables 3 and 4, respectively. For both female categories, subjective sperm motility and vigour showed no significant effect on the probability of pregnancy. For pregnancy in cows, sperm traits related to acrosome damage positively affected the probability of pregnancy mainly when associated with plasma membrane integrity (higher β_1 value). Regarding the H_2O_2 production, it seems to have less importance than plasma membrane integrity in affecting the probability of cow pregnancy. For heifers, damaged plasma membrane and low H_2O_2 production were positively related to probability of pregnancy. High H_2O_2 production was negatively related to the pregnancy in heifers even when associated with intact plasma membrane.

For multivariate logistic regression, four variables were kept in the final model for pregnancy in cows and two variables for pregnancy in heifers (Table 5). For cows and heifers, sperm traits related to damaged acrosome positively affected the probability of pregnancy while intact acrosome was negatively related to cow pregnancy. High H_2O_2 production and intact plasma membrane were positively related to cow pregnancy but negatively related to heifer pregnancy. Age of female was positively related to cow pregnancy.

TABLE 2 Subjective sperm motility (%), sperm vigour (0–5) and flow cytometer parameters (%) from 16 bulls used for fixed-time artificial insemination of Nellore cows and heifers

Variables	Mean ± SEM	Range[a]
Sperm motility	58.44 ± 3.56	30.0–75.0
Sperm vigour (0–5)	2.63 ± 0.13	2.0–3.0
Damaged plasma membrane and intact acrosome membrane	8.25 ± 1.23	2.5–21.6
Damaged plasma and acrosome membranes	7.55 ± 1.50	0.03–17.3
Intact plasma membrane and damaged acrosome membrane	2.36 ± 0.51	0.003–6.4
Intact plasma and acrosome membranes	81.84 ± 2.18	67.4–95.1
Damaged plasma membrane and low H_2O_2 production	3.07 ± 0.99	0.2–16.3
Damaged plasma membrane and high H_2O_2 production	21.59 ± 4.97	0.6–75.4
Normal plasma membrane and high H_2O_2 production	5.52 ± 1.93	0.5–29.0
Normal plasma membrane and low H_2O_2 production	69.82 ± 5.49	18.7–94.8

[a]Minimum and maximum values.

TABLE 3 Analysis of maximum-likelihood estimates and area under receiver operator characteristic (ROC) curve from the univariate logistic regression used to predict probability of pregnancy in Nellore cows

Variable	β_0	β_1	*p*-Value	*c*
Sperm motility	0.4014	−0.1574	0.7836	0.508
Sperm vigour	0.3024	0.0822	0.3202	0.515
Damaged plasma membrane and intact acrosome membrane	0.7194	−1.8138	0.0632	0.548
Damaged plasma and acrosome membranes	−0.1515	1.8126	0.0005	0.570
Intact plasma membrane and damaged acrosome membrane	−0.1319	3.0823	0.0008	0.566
Intact plasma and acrosome membranes	1.2791	−0.8638	0.1177	0.519
Damaged plasma membrane and low H_2O_2 production	0.6036	−2.3845	0.0116	0.537
Damaged plasma membrane and high H_2O_2 production	0.5280	−0.6124	0.0316	0.547
Normal plasma membrane and high H_2O_2 production	0.0870	0.9647	0.0960	0.571
Normal plasma membrane and low H_2O_2 production	−0.3467	0.5878	0.0423	0.546
Age of female (days)	−0.2293	0.000213	0.0092	0.557

Note. β_0 (intercept) and β_1 are the parameter estimates of the logistic regression. *c*, area under ROC curve.

Based on estimates shown in Table 5, the probability of pregnancy for each bull was calculated and compared to the observed pregnancy rate of cows (Figure 1) and heifers (Figure 2). For cows, three bulls presented a difference between predicted and observed pregnancy rates higher than 20%. For heifer pregnancy, all bulls showed predicted rates similar to observed values.

4 | DISCUSSION

Several authors have showed a large variation in pregnancy rates for bulls that are used in FTAI. These variations have also been demonstrated in vitro for cleavage and blastocyst rates, in vitro capacitation and acrosome integrity (Marquant-Le Guienne, Humblot, Thibier, & Thibault, 1990; Meneghetti, Sá Filho, Peres, Lamb, & Vasconcelos, 2009; Rodríguez-Martínez, Larsson,

TABLE 4 Analysis of maximum-likelihood estimates and area under receiver operator characteristic (ROC) curve from the univariate logistic regression used to predict probability of pregnancy in Nellore heifers

Variable	β_0	β_1	*p*-Value	*c*
Sperm motility	0.4241	0.2953	0.9170	0.489
Sperm vigour	0.7455	0.2100	0.3531	0.547
Damaged plasma membrane and intact acrosome membrane	2.0189	−4.3338	0.1220	0.615
Damaged plasma and acrosome membranes	−0.0084	2.2814	0.2269	0.615
Intact plasma membrane and damaged acrosome membrane	0.5942	0.4042	0.8855	0.461
Intact plasma and acrosome membranes	0.9418	−0.2469	0.8811	0.505
Damaged plasma membrane and low H_2O_2 production	0.0063	6.5106	0.0812	0.616
Damaged plasma membrane and high H_2O_2 production	−0.1403	1.2770	0.2784	0.505
Normal plasma membrane and high H_2O_2 production	1.3242	−2.0763	0.0265	0.616
Normal plasma membrane and low H_2O_2 production	0.4963	0.2355	0.7941	0.495
Age of female (days)	−0.6224	0.00162	0.6557	0.518

Note. β_0 (intercept) and β_1 are the parameter estimates of the logistic regression. *c*, area under ROC curve.

TABLE 5 Analysis of maximum-likelihood estimates and area under receiver operator characteristic (ROC) curve of the multivariate logistic regression used to predict probability of pregnancy in Nellore cows and heifers

Parameter	Estimate	*p*-Value	*c*
In cows			
Intercept	−0.1876	0.5896	0.592
Damaged plasma membrane and intact acrosome membrane	−2.3689	0.0211	
Damaged plasma and acrosome membranes	1.7848	0.0009	
Normal plasma membrane and high H_2O_2 production	0.9137	0.1299	
Age of female (days)	0.000207	0.0126	
In heifers			
Intercept	0.5140	0.4366	0.648
Normal plasma membrane and high H_2O_2 production	−2.2243	0.0204	
Damaged plasma and acrosome membranes	2.8793	0.1576	

Note. *c*, area under ROC curve.

Zhang, & Söderquist, 1997; Sudano et al., 2011; Wei & Fukui, 1999).

Sperm motility is one of the most important characteristics associated with fertilizing ability being the main analysis used by the centres of artificial insemination (Crespilho et al., 2006; Olds-Clarke, 1996). Nevertheless, in this experiment, univariate and multivariate logistic regression showed that subjective sperm motility and vigour did not influence the probability of pregnancy. The absence of relation between CASA-analysed or subjective sperm motility and fertility was previously described (Bailey et al., 1994; Januskauskas et al., 1999; Oliveira, Arruda, Andrade, Celeghini et al., 2012; Stålhammar, Janson, & Philipsson, 1994), although positive correlation was also reported (Gillan et al., 2008; Li, Kalo, Zeron, & Roth, 2014). Therefore, findings of this study may suggest that sperm motility itself is not a suitable parameter to predict semen fertility, and it seems clear that other sperm characteristics should be considered to evaluate semen fertility potential. It is important to highlight that sperm motility evaluation should consider the sperm concentration (Mohanty et al., 2018). In this study, subjective sperm motility ranged from 30% to 75%, considering straws with 30 x 10^6 sperm, even the lowest sperm motility represented at least 9 x 10^6 of viable sperm cells which is considered as a satisfactory inseminating

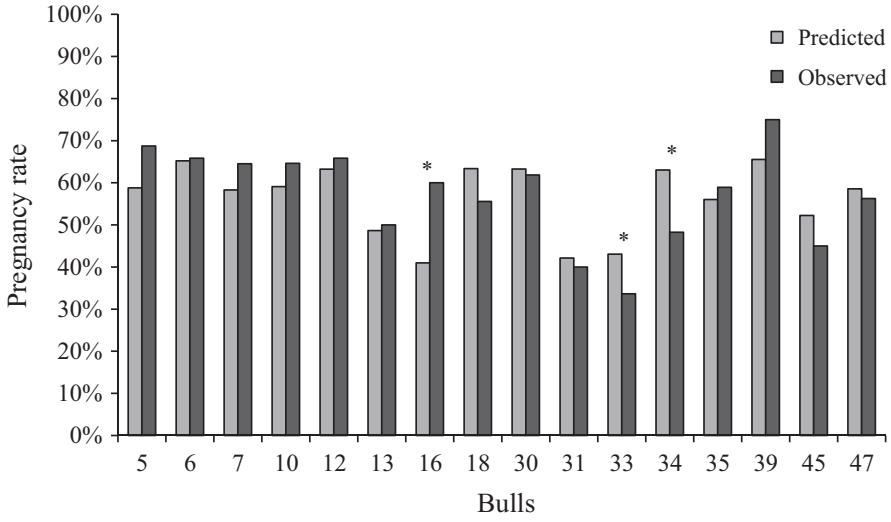

FIGURE 1 Observed and predicted pregnancy rates of bulls used in fixed-time artificial insemination of Nellore cows. Predicted values were estimated from the general formula $P = \frac{e^{(\beta_0+\ldots+\beta_p x_p)}}{1+e^{(\beta_0+\ldots+\beta_p x_p)}}$, where β_0, intercept; β_p, coefficients estimated by logistic regression for each parameter; and x_p, arcsine-transformed values of parameters obtained in flow cytometer approach and age of cows in days. *Bulls that presented a difference higher than 20% between predicted and observed pregnancy rates

dose (Den Daas, Jong, Lansbergen, & Wagtendonk-De Leeuw, 1998; Gérard & Humblot, 1991).

Results of univariate logistic regression of the evaluation of acrosome and plasma membrane integrity (FITC-PSA/PI) variables were conflicting. Proportion of damaged/reacted acrosome was positively while intact acrosome was negatively related to pregnancy. Multivariate logistic regression analysis also indicated that characteristics related to damaged/reacted acrosome showed positive influence on probability of pregnancy. It is noteworthy that cryopreservation process can lead acrosome undergoes to alterations similar to capacitation (Andrade et al., 2011; Cormier & Bailey, 2003; Green & Watson, 2001; Neil et al., 2003). These capacitation-like changes may have increased the chances of the sperm population with damaged/reacted acrosome to successfully fertilize the oocyte. It is important to highlight that FITC-PSA is a probe for acrosome reaction that recognizes both partial and complete acrosome-reacted sperm (Jaiswal, Cohen-Dayag, Tur-Kaspa, & Eisenbach, 1998; Köhn, Mack, Schill, & Zaneveld, 1997); therefore, we hypothesize that a high sperm population stained for FITC-PSA may increase the probability of pregnancy in cows. Moreover, results of heifer pregnancy were unexpected since damaged plasma and acrosome membranes were related to higher pregnancy rates; however, we highlight that the number of bulls used to analyse heifer pregnancy in this study was lower than for cow pregnancy, so the results of heifer pregnancy should be carefully taken.

Reactive oxygen species (ROS) have an important role on sperm physiology (Aitken, Ryan, Baker, & McLaughlin, 2004; De Lamirande & O'Flaherty, 2008; O'Flaherty, Lamirande, & Gagnon, 2006); however, excessive ROS production or failure in the sperm antioxidants systems may harm sperm functionality (Aitken & Baker, 2002; Agarwal, Saleh, & Bedaiwy, 2003). In this study, based on univariate analysis, a high level of H_2O_2 even when associated with intact plasma membrane seems to decrease probability of pregnancy in heifers; on the other hand, the level of H_2O_2 does not seem to affect probability of pregnancy in cows. Nevertheless, the multivariate analysis for cow pregnancy revealed that high H_2O_2 production associated with intact plasma membrane increased probability of

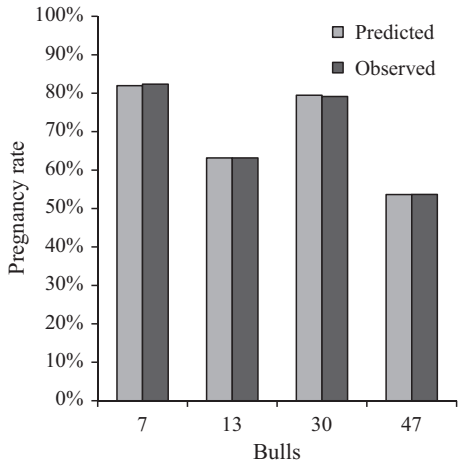

FIGURE 2 Observed and predicted pregnancy rates of bulls used in fixed-time artificial insemination of Nellore heifers. Predicted values were estimated from the general formula $P = \frac{e^{(\beta_0+\ldots+\beta_p x_p)}}{1+e^{(\beta_0+\ldots+\beta_p x_p)}}$, where β_0,intercept; β_p, coefficients estimated by logistic regression for each parameter; and x_p, arcsine-transformed values of parameters obtained in flow cytometer approach

pregnancy, which may be an unexpected result since it indicates that sperm population with high levels of H_2O_2 may increase the chances of pregnancy; however, a physiological increase in ROS occurs in the sperm cell during capacitation process (Aitken et al., 2004; De Lamirande & O'Flaherty, 2008; O'Flaherty et al., 2006). In fact, sperm with no superoxide (SO^-) or H_2O_2 production were negatively related to field fertility in cattle, which could be related to low metabolic activity of the sperm resulting in low pregnancy rates (Morrell, Valeanu, Lundeheim, & Johannisson, 2018).

This finding associated with results of the acrosome membrane may suggest that a capacitation-like status of sperm right after thawing may enhance the probability of pregnancy in Nellore cows. Nevertheless, capacitation status by CTC staining has showed no correlation with field fertility of Holstein bulls (Gillan et al., 2008), and Thundathil et al. (1999) have reported a positive correlation between proportion of uncapacitated sperm and field fertility

(non-return rate) in non-synchronized females. However, Gillan, Evans, and Maxwell (1997) pointed out that although the lifespan of capacitated ram sperm was limited, insemination directly into the uterus achieved pregnancy rates comparable to those of fresh semen.

Furthermore, the window of ovulation in synchronized females and the moment of insemination related to ovulation time may interfere in fertility results (Sales et al., 2011). It is known that estradiol benzoate promotes a LH surge 19.6 hr after administration with LH surge duration of 8.6 hr in Nellore heifers (Sales et al., 2012) and administration of estradiol benzoate on day 8 of FTAI protocol promotes ovulation 59.4 hr after P4 device removal in Nellore cows with 91% of ovulated cows having their ovulation in a 16 hr of window (48–64 hr) (Ayres et al., 2008). Therefore, we may hypothesize that an ovulation that occurs quite close to artificial insemination in a narrow window of ovulation may favour pre-capacitated sperm to fertilize oocyte.

In conclusion, further investigations regarding the capacitation status of the sperm cell using probes that detect both partial and complete acrosome reaction may clarify the findings reported in this study. Moreover, our findings suggest that the field fertility results of cryopreserved semen in a FTAI programme may be influenced by female fertility, so further analyses should consider the female status (heifer, primiparous or multiparous cows).

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

DSO was responsible for the conceptualization of the study, data collection, flow cytometer analyses and interpretation of results; JMPF performed the statistical analyses, interpretation of results and paper edition; VEGL participated in the FTAI and data collection; PPM and COS performed the flow cytometer analyses and paper edition; BW, EADM, EPC and SEFG were responsible for interpretation of results and paper edition; and JDG supervised all study and participated of interpretation of results and paper edition.

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