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In vitro sperm characteristics and *in vivo* fertility of sex-sorted and conventional semen in suckled Nelore cows at a traditional schedule for timed-AI with estrus detection

[*Características espermáticas* in vitro *e fertilidade* in vivo *de sêmen convencional e sexado em vacas Nelore pós-parto submetidas à IATF tradicional com detecção de estro*]

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ABSTRACT

The aim of this study was to assess *in vitro* sperm characteristics and pregnancies/AI (P/AI) of conventional and sex-sorted semen at timed-AI of suckled, multiparous Nelore cows. All cows (n=348) were submitted to a traditional estradiol/progesterone(P4)-based protocol. At 48h after P4-device removal, the estrous behavior was recorded, and AI was performed with conventional or sex-sorted semen from two bulls. The following sperm assessments were performed: CASA, Hyposmotic Test, sperm morphometry and chromatin structure by TB staining. P/AI were reduced (P<0.001) for sex-sorted compared to conventional semen in cows expressing estrus (27*vs*47%) or not (11*vs*.37%). Membrane integrity (Bull1: 30.3±9.6 *vs*. 52.3±12.4%, P=0.01; Bull2: 24.5±3.0 *vs*. 48.7±1.6%, P=0.006) and sperm concentration (Bull1: 23.2±0.6 *vs.* 43.0±0.8x10^{6}sperm/mL, P<0.001; Bull2: 25.1±2.8 *vs*. 42.1±0.7x10^{6}sperm/mL; P<0.001) were reduced in sex-sorted compared to conventional semen, for both bulls. Total and progressive motility were reduced in sex-sorted semen for Bull1 (TM: 49.7±15.9 *vs.* 94.9±1.9%, P=0.007; PM: 16.7±3.4 *vs.* 44.1±13.2%, P=0.009) and no differences were detected for Bull2 (TM: 45.0±17.5 *vs*. 68.2±19.1%, P=0.098; PM: 12.8±4.7 *vs*. 30.0±13.0%, P=0.065). Sperm ellipticity from sex-sorted was lower than conventional semen for Bull2 (0.306±0.01 *vs*. 0.342±0.02, P=0.02) and no difference was detected for Bull1 (0.332±0.01 *vs*. 0.330±0.01, P=0.55). Reduced *in vivo* fertility was observed for sex-sorted semen, regardless of estrous behavior. *In vitro* sperm quality of sex-sorted semen was compromised for both bulls, but differently affected for each sire.

Keywords: bovine, estrous behavior, pregnancy, semen analysis, sexed sperm, timed-AI

RESUMO

O estudo teve como objetivo avaliar características espermáticas in vitro *e a taxa de concepção (TC) de sêmen convencional e sexado em um programa de IATF tradicional de vacas Nelore pós-parto. Todas a vacas (n=348) foram submetidas ao mesmo protocolo de IATF à base de estradiol e de progesterona. Após 48 horas da retirada do implante, foi determinada a expressão de estro dos animais e a IA foi realizada com sêmen convencional e sexado de dois touros Angus. As seguintes características espermáticas foram avaliadas: análise computadorizada do sêmen, teste hiposmótico, morfometria espermática e estrutura cromatínica por meio da coloração com azul de toluidina. A TC foi menor (P<0,001) para sêmen sexado comparado ao convencional, em vacas que expressaram estro (27* vs. *47%) e que não apresentaram estro (11* vs. *37%). A integridade da membrana plasmática (Touro 1: 30,3±9,6* vs. *52,3±12,4%, P=0,010; Touro 2: 24,5±3,0* vs. *48,7±1,6%, P=0,006) e a concentração espermática (Touro 1: 23,2±0,6* vs. *43,0±0,8x10^{6}sperm/mL, P<0,001; Touro 2: 25,1±2,8* vs. *42,1±0,7x10^{6}sperm/mL, P<0,001) foram menores no sêmen sexado comparado ao convencional, para ambos os touros. Motilidades total e progressiva foram menores no sêmen sexado comparado ao*

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convencional para o Touro 1 (MT: 49,7±15,9 vs. *94,9±1,9%, P=0,007; MP: 16,7±3,4* vs. *44,1±13,2%, P=0,009), enquanto diferenças não foram detectadas no Touro 2 (MT: 45,0±17,5* vs. *68,2±19,1%, P=0,098; MP: 12,8±4,7* vs. *30,0±13,0%, P=0,065). Elipticidade espermática do sêmen sexado foi menor do que do sêmen convencional no Touro 2 (0,306±0,01* vs. *0,342±0,02, P=0,020), mas não houve diferença no Touro 1 (0,332±0,01* vs. *0,330±0,01, P=0,552). Reduzida fertilidade* in vivo *foi observada para o sêmen sexado em relação ao convencional, independentemente da expressão de cio das vacas. A qualidade seminal* in vitro *do sêmen sexado foi comprometida para ambos os touros, mas diferentemente afetada para cada reprodutor.*

Palavras-chave: bovino, comportamento de estro, prenhez, análise seminal, sêmen sexado, inseminação artificial em tempo fixo

INTRODUCTION

Fixed-time artificial insemination (Timed-AI) programs with estradiol (E2) and progesterone (P4) have been widely used for *Bos indicus* cows as a reproductive management tool in commercial beef farms (Bó *et al*., 2003; Meneghetti *et al*., 2009; Sá Filho *et al*., 2009, 2012; Silva *et al*., 2018; Noronha *et al*., 2020) which has considerably increased the use of AI in *Bos indicus* cattle (Baruselli *et al*., 2017).

Many factors may account for the range in results of these reproductive programs as body condition score (BCS), animal category, suckling, size of ovulatory follicle and/or estrus expression at the time of AI (Meneghetti *et al*., 2009; Sá Filho *et al*., 2010a, 2011; Perry *et al*., 2014; Pugliesi *et al*., 2016; Rodrigues *et al*., 2018). Moreover, another important factor affecting the success of a timed-AI programs is the bull and/or type of semen used (Sá Filho *et al*., 2009; Oliveira *et al*., 2012a).

In beef cattle industry, pre-selection of a desired offspring sex may present significant economic advantages depending on the productive and/or management strategies according to each farm and/or market program. The sex of the offspring can be controlled with over 90% accuracy, based on small differences in chromosomal DNA content between the X and Y sperms (Seidel and Garner, 2002; Garner, 2006). However, fertility of sex-sorted is usually lower than non-sorted sperm (Seidel and Schenk, 2008; Schenk *et al*., 2009; DeJarnette *et al*., 2010; Seidel, 2014; Silva *et al*., 2018; Magata *et al*., 2021) mainly due to potential cellular damages (Moce *et al*., 2006; DeJarnette *et al*., 2008, 2011; Carvalho *et al*., 2013) and/or alterations in structural characteristics of sperm cells after the sex-sorting process (Carvalho *et al*., 2018).

Still, advances in sperm sex sorting procedures during flow cytometry and increased sperm dosage (Rath *et al*., 2013; Seidel, 2014; Gonzalez-Marin *et al*., 2016; Thomas *et al*., 2019) have enhanced the likelihood of successful pregnancy in females inseminated with sex-sorted semen (Gonzalez-Marin *et al*., 2016; Heuer *et al*., 2017; Gonzalez-Marin *et al*., 2018), mainly when longer intervals from the induction of ovulation to AI (Sá Filho *et al*., 2010b, 2012; Sales *et al*., 2011; Kurykin, 2017; Silva *et al*., 2018) and/or split-timed-AI are applied (Thomas *et al*., 2014a, 2014b, 2017, 2019).

Unfortunately, from a practical viewpoint, delaying the time for AI to perform inseminations closer to ovulation may be difficult, impractical, or even unfeasible for large scale timed-AI programs in extensive beef farms, due to increased animal handling and labor schedules, which may hamper the incorporation of sex-sorted semen into cattle reproductive management of commercial livestock programs. Therefore, few studies have recently applied a practical large-scale utilization of sex-sorted semen in *Bos indicus* beef cattle and simultaneously assessed field fertility and laboratory outcomes to evaluate *in vivo* and *in vitro* results.

Thus, the aim of the present study was to investigate pregnancy/AI (P/AI) as well as several *in vitro* sperm characteristics of conventional and sex-sorted semen from two Angus bulls, in a traditional Timed-AI program for first service of suckled multiparous Nelore cows expressing different intensity of estrous behavior.

MATERIALS AND METHODS

Animal handling protocols were approved by the Ethics and Animal Experimentation Committee from Universidade Federal de Minas Gerais (Process # CEUA UFMG 348/2018).

This study was conducted in a commercial beef farm located at Mato Grosso State, Center-West Region of Brazil. A total of 348 multiparous Nelore (*Bos indicus*) cows, ranging from 30 to 40 days postpartum, were used. All cows grazed on *Braquiaria brizantha* pastures and received free access to mineralized salt and water.

Cows were allocated into three breeding groups of approximately 120 cows each, according to calving date, according to the general management practices of the farm. All cows were submitted to the same timed-AI protocol (Noronha *et al*., 2020), for first service, as described below: On day 0 (D0), cows received 2mg (im) of estradiol benzoate (EB; Gonadiol®; Zoetis, São Paulo, SP, Brazil) and intravaginal P4 releasing device (CIDR®; Zoetis). On day 7 (D7), all animals received 12.5mg (im) of $PGF_{2\alpha}$ analogue, dinoprost tromethamine (Dinoprost; Lutalyse®; Zoetis). On day 9 (D9), CIDR® was removed and cows received 0.6mg (im) of estradiol cypionate (ECP; ECP®; Zoetis), 300IU (im) of eCG (Novormon®; Zoetis) and another dose of 12.5mg (im) of Dinoprost (Lutalyse®). Additionally, on D9, the cows received a heat detection patch (Estrotect®, IVP, Spring Valley, WI), which was placed between the hips and tail head. Forty-eight hours after P4 device removal (D11), the timed-AI was performed with conventional (frozen-thawed) or sex-sorted (x-sorted frozen-thawed) semen from two Angus bulls.

At the time of AI (D11), estrous behavior (estrus or no estrus) was determined by the activation of estrotect® device (Pohler *et al*., 2016). The cow was considered to have displayed estrus when a removal of >25 % of the rub-off coating from the patch was observed. Hence, cows with <25% of the rub-off coating from the patch were considered as "no estrus cows" and cows with >25 % of the rub-off coating from the patch were considered as "estrus cows". The BCS was also recorded at timed-AI by a single technician using a 1 to 5 scale (1 = severely emaciated, 5 = obese).

Regarding to inseminations on D11, cows were randomly inseminated by three technicians with sex-sorted (commercial frozen-thawed flow cytometry x-sorted semen) or conventional (commercial frozen-thawed non-sex-sorted semen) semen from two different sires: conventional semen from Bull 1, sex-sorted semen from Bull 1, conventional semen from Bull 2 and sex-sorted semen from Bull 2. Worth mentioning that only one semen batch from each sire was used (one batch of conventional semen from Bull 1, one batch of sex-sorted semen from Bull 1, one batch of conventional semen from Bull 2 and one batch of sex-sorted semen from Bull 2).

For each semen batch, five 0.25mL frozen straws were simultaneously thawed in a thermostatically controlled thawing bath, in a temperature of 37°C, for 30 sec (Oliveira *et al*., 2012b).

To assure a randomized experimental design per field variable, the two semen types (conventional or sex-sorted) from the two bulls were equally distributed across breeding groups and AI technicians, as follows: after loading a random cow in the chute, five 0.25mL frozen semen units from the same semen type and from the same bull were thawed. After five cows were inseminated, the type of semen, bull, or both were switched. Only one chute was used for AI, but each inseminator alternated every 20 mattings to ensure that each inseminator equally inseminated cows for each of the semen types and bulls.

For assessment of field fertility, P/AI was determined on D41 (30 days after timed AI) by detecting a viable conceptus using transrectal ultrasonography (5.0MHz transducer; 500 V, Aloka, Wallingford, USA).

Frozen semen samples from the same batches utilized in field trial were brought to the laboratory. Hence, for laboratory experiment, one batch from conventional semen of Bull 1, one batch from sex-sorted semen of Bull 1, one batch from conventional semen of Bull 2 and one batch from sex-sorted semen of Bull 2 were evaluated.

For each semen batch, two 0.25mL frozen straws were thawed in a thermostatically controlled

thawing bath, in a temperature of 37°C, for 30 sec. After thawing, the following *in vitro* sperm characteristics were assessed: sperm motility parameters, plasma membrane integrity, sperm morphology, concentration, chromatin structure and sperm morphometry. Three repetitions of each *in vitro* sperm analysis were performed for each semen batch evaluated.

Sperm motility parameters were assessed by computer assisted semen analysis (CASA) using the Sperm Class Analyzer (v.4.0.0, Microptic, Barcelona, Spain) and the CASA set-up was pre-adjusted for bovine sperm analysis: number of frames: 30; frames per sec: 60Hz; minimum contrast: 50; minimum cell size: 6 pixels; contrast with static cells: 30; straightness: 60%; average path velocity cutoff: 30μm/s; straight-line velocity cutoff: 20μm/s; static head size: 0.23 to 1.91; static elongation: 8 to 92%; magnification: 1.89X; temperature: 37°C.

The CASA parameters were evaluated by placing 5μL of thawed semen sample in a standard count analysis chamber (Makler counting chamber, SEFI Medical Instruments LTD, Haifa, Israel). Five fields were randomly selected for each analysis and the following variables were analyzed: Total Motility (TM), Progressive Motility (PM), Average Path Velocity (VAP), Straight-Line Velocity (VSL), Curvilinear Velocity (VCL), Amplitude of Lateral Head Displacement (ALH), Beat Cross Frequency (BCF), Straightness (STR), Linearity (LIN) and Percentage of Rapidly Moving Cells (RAPID).

Hyposmotic Swelling Test (HOST) was performed by incubating 20μL of semen with 1mL of a 100mOsm hyposmotic solution (Revell and Mrode, 1994) at 37°C for 60 min. After incubation, an aliquot of 20μL of semen diluted in hyposmotic solution was placed on a glass slide, covered with a coverslip, and evaluated by contrast phase microscopy. Two hundred sperm were evaluated under magnification 1000X. Sperm with swollen or coiled tails were considered viable. Percentage of viable sperm (HOST+cells) was calculated according to Revell and Mrode (1994).

To assess sperm morphology, the samples were diluted in pre-warmed (37°C) formaldehyde-phosphate buffered saline (Dulbecco's Phosphate Buffered Saline; DPBS, Biodux, Brazil; 4% Formaldehyde). Sperm cells (n=200) were counted under phase contrast microscopy at 1000X magnification and sperm morphological characteristics were classified as major defects (Maj Def), minor defects (Min Def) and total defects (Tot Def) according to Blom (1973). Sperm concentration analysis was performed in Neubauer chamber after 1:100 dilution in formaldehyde-phosphate buffered saline (DPBS; 4% Formaldehyde) under optical microscopy at 400X magnification.

For assessment of sperm chromatin structure and morphometry by Toluidine Blue staining, three smears were prepared for each sample. Sperm smear preparation was performed as previously described (Souza *et al*., 2018; Martins *et al*., 2021). Briefly, after thawing, sperm smears were fixed with ethanol acetic acid (3:1, V/V) for 1 min (Labsynth, A1084/A1019) and 70% ethanol for 3 min. Then, the smears were hydrolyzed for 25 min in 4M HCl (Labsynth, A1028), washed in distilled water and air-dried. One droplet of 0.025% Toluidine Blue (Labsynth, A1111) in Mcilvaine buffer (sodium citrate-phosphate; Labsynth A1026/F1034), pH 4.0, was placed over each smear and then covered with a coverslip. After 3 min, sperm head images were obtained by using a light microscope (objective 100X combined with a 10X eyepiece lens; Leica DM500) and image capture system (Leica LAS EZ software version 1.8.1, Wetzlar, Germany). At least 100 sperm heads were isolated for each smear.

The computational analyses of digital images were performed as described by Martins *et al*. (2021). Sperm head segmentation from digital images were subjected to histogram-based thresholding (second-order derivative), a binary image (mask) of each sperm head was obtained and a procedure of mathematical morphology (erosion operator) was applied. Then, the images were evaluated using algorithms developed in MATLAB language. Hence, sperm heads were analyzed to obtain the average pixel value that made up each head.

For chromatin condensation and heterogeneity quantitative analysis, the images of sperm head were transformed to grayscale (Lucio *et al*., 2016). The average intensity (pixel) from the 20 sperm heads (most compact and homogeneous) in each slide was used as the reference (standard head). Then, the difference between the average

value of the standard heads and the average value of each head analyzed was determined. This difference (Dif) was transformed as a percentage of the average value and used as a quantitative indicator of sperm chromatin decondensation. In addition, coefficient of variation (CV) of the gray level intensity for each head, which indicates sperm chromatin heterogeneity, was also calculated (Martins *et al*., 2021).

Regarding to sperm head morphometric analyses, standard morphometric measures (area, perimeter, width and length), and width:length ratio (W/L), ellipticity, shape factor (SF), Fourier harmonics (F0, F1, and F2), side symmetry (SS), and anterior-posterior symmetry (APS) measurements were determined using algorithms developed in the Scilab environment (Beletti *et al*., 2005; Oliveira *et al*., 2013; Lucio *et al*., 2016; Martins *et al*., 2021).

The statistical analyses for this experiment were divided in two different parts. In field experiment, to evaluate the predictive variables for P/AI of the cows, a mixed generalized linear model with binomial distribution (logistic regression) was used. The P/AI was analyzed as a binary response variable using logistic regression by the "glmer" function of package "lme4" from R software (R Core Team, 2019) fitted with a binary distribution. The variables considered in the initial model as fixed effects were BCS, AI technician, Bull (1 and 2), semen type (conventional and sex-sorted), estrous behavior (estrus and no estrus) and their interactions. A backward stepwise logistic regression model was used, and variables were continuously removed from the complete full model (according to Wald`s criterion) when $P > 0.10$.

Then, non-significant effects were excluded from the model and statistical differences in P/AI were analyzed by classical logistic regression (function "glm" from R software) using Estimated Marginal Means using "emmeans" package from R software.

In laboratory experiment, to assess the effects of bull (Bull 1 or Bull 2) and semen type (conventional or sex-sorted), ANOVA for two factors was performed. Separate tests were applied for each variable and the results obtained from all variables of laboratory analyses (mean from three repetitions of each semen batch) were tested for normality of residues and homogeneity of variance. Dependent variables that did not meet statistical premises were submitted to Log transformation by R software.

The main effects of each factor were globally tested (overall), as well as the effect of interaction between these factors (Bull *vs.* semen type). Then, mean values and their respective confidence intervals (95%) were calculated for each group. Pairwise comparison was applied using Tukey's correction to identify differences between the type of semen (conventional or sex-sorted) separately for each bull (Bull 1 or Bull 2).

Significant difference was considered when $P \leq 0.05$ and statistical tendency was considered when $P > 0.05$ and < 0.10.

RESULTS

The overall P/AI was 32.8% (114/348) and no effects of AI technician (P=0.223), BCS (P=0.155) or Bull (P=0.730) were detected. Additionally, no interactions among the field variables were detected (BCS*Bull: P=0.504; Estrous behavior*Bull: P=0.179; Bull*Semen type: P=0.116; Estrous behavior*Bull*Semen type: P=0.151; BCS*Bull*Semen type: P=0.502). However, estrous behavior (P=0.011) and semen type (P<0.001) were factors affecting pregnancy success.

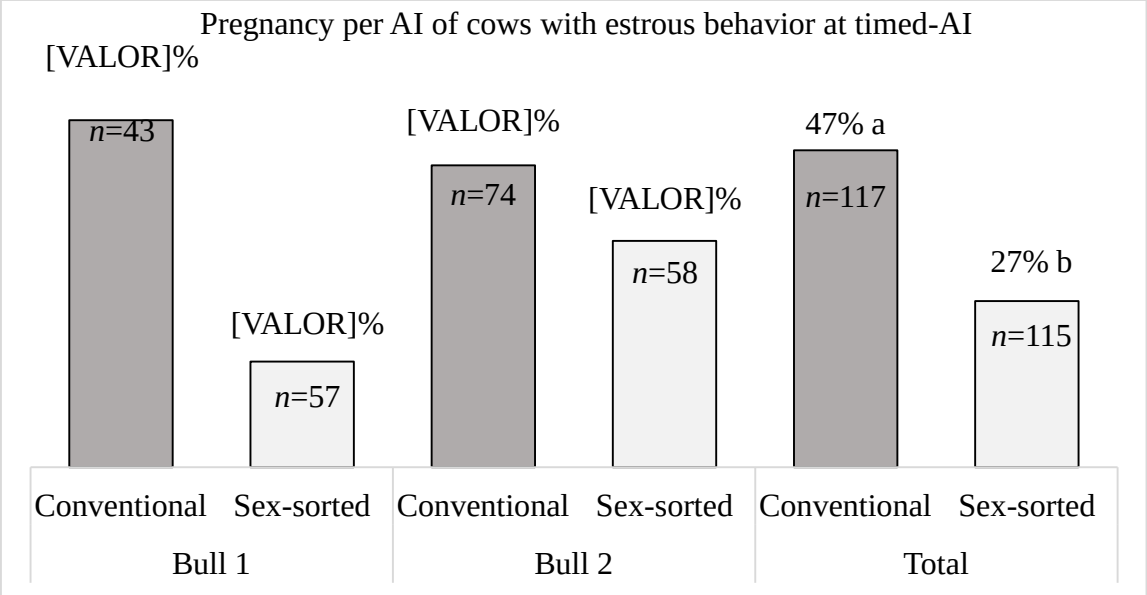
Overall P/AI from cows expressing estrus at AI (37%; n=232) was higher (P=0.011) than cows not expressing estrus (24%; n=116) and overall P/AI from cows inseminated with conventional semen (44%, n=176) was higher (P<0.001) than cows inseminated with sex-sorted semen (22%; n=172), as demonstrated in Table 1.

In the laboratory experiment, no differences were observed between bulls for TM (P=0.111) and PM (P=0.145), whereas effects of semen type were detected for both sperm parameters (TM: P=0.005; PM: P=0.004). Table 2 demonstrates that TM and PM of sex-sorted semen were reduced compared to conventional semen only for Bull 1 (TM: P=0.007; PM: P=0.009). For Bull 2, only statistical tendencies were detected (TM: P=0.098; PM: P=0.065).

Table 1. Pregnancy/AI (%) of suckled multiparous Nelore cows according to estrous behavior (estrus or no estrus) and semen type (conventional or sex-sorted) from two Angus bulls utilized in an estradiol/progesterone(P4)-based ovulation synchronization protocol with a traditional schedule for timed-AI (48 hours after P4 device removal)

	Bull 1		Bull 2		Total	
	Conventional	Sex-sorted	Conventional	Sex-sorted	Conventional	Sex-sorted
Estrus	51.2 (22/43)	19.3 (11/57)	44.6 (33/74)	34.5 (20/58)	47.0 a (55/117)	27.0 bc (31/115)
No estrus	43.4 (13/30)	16.0 (4/25)	31.0 (9/29)	6.3 (2/32)	37.3 ab (22/59)	10.5 c (6/57)
Total	47.9 (35/73)	18.2 (15/82)	40.8 (42/103)	24.4 (22/90)	43.8 a (77/176)	21.5 b (37/172)

Values with different lowercase letter differ (P<0.05).



a,b: different lowercase letters indicate P≤0.05.

Figure 1. demonstrates P/AI according to semen type in cows expressing estrus, for both bulls.

Statistical differences were detected for RAPID between bulls (P=0.042) and semen types (P=0.003). For Bull 1, higher (P=0.003) RAPID was observed in conventional compared to sex-sorted semen, while for Bull 2 no difference (P=0.132) was observed between sex-sorted and conventional semen, as demonstrated in Table 2.

Similarly, VAP was not different between bulls (P=0.149) but an effect of semen type was detected (P=0.042). Conventional semen demonstrated higher VAP compared to sex-sorted semen only for Bull 1 (P=0.043), whereas, for Bull 2, no difference (P=0.337) between conventional and sex-sorted semen was detected. The parameters VCL, VSL, BCF and STR were not different between bulls (VCL: P=0.111; VSL: P=0.250; BCF: P=0.593; STR: P=0.899) nor between semen types (VCL: P=0.095; VSL: P=0.089; BCF: P=0.083; STR: P=1.000; Table 2).

No difference was detected for LIN (P=0.876) between bulls. However, for Bull 2, lower LIN was detected in sex-sorted compared to conventional semen (P=0.050), while no difference was detected between conventional and sex-sorted semen for Bull 1 (P=0.764). Regarding to ALH, no difference was observed (P=0.165) between bulls, although an effect of semen type was detected (P=0.002). ALH of sex-sorted semen was higher than conventional

semen for both bulls (Bull 1: *P*=0.039; Bull 2: *P*=0.004), as also demonstrated in Table 2.
No interactions between bull and semen type were detected for any sperm variables assessed by CASA (TM: *P*=0.247; PM: *P*=0.384; RAPID: *P*=0.108; VAP: *P*=0.359; VCL: *P*=0.219; VSL: *P*=0.717; BCF: *P*=0.516; STR: *P*=0.105; LIN: *P*=0.195; ALH: *P*=0.307).

Table 2. Mean (± SD) of *in vitro* sperm motility parameters assessed by computer assisted sperm analysis (CASA) of conventional and sex-sorted semen, separated by bull

	Bull 1		Bull 2	
	Conventional	Sex-sorted	Conventional	Sex-sorted
TM (%)	94.87±1.86a	49.73±15.86b	68.17±19.05A	44.97±17.49B
PM (%)	44.07±13.16a	16.67±3.41b	30.00±13.00A	12.77±4.70B
RAPID (%)	55.90±14.58a	17.17±4.29b	28.57±15.54	13.23±5.36
VAP (μm/s)	40.56±9.06a	29.00±3.46b	31.80±5.46	26.86±0.80
VCL (μm/s)	59.16±9.93	45.90±3.90	46.33±9.40	44.03±0.41
VSL (μm/s)	30.03±8.77	22.53±3.95	24.80±5.72	19.73±0.70
BCF (Hz)	9.63±1.28	11.06±1.10	9.70±0.69	10.40±0.36
STR (%)	73.20±6.00	77.4±4.32	77.7±2.28	73.5±1.86
LIN (%)	50.00±7.10	48.86±4.27	53.23±3.00a	44.80±1.47b
ALH (μm)	2.63±0.05a	2.90±0.10b	2.43±0.23a	2.86±0.05b

For each bull, values with different lowercase letters (a,b) within a row indicate $P \leq 0.05$ and values with different capital letters (A,B) indicate $P > 0.05$ and < 0.10.
TM: Total Motility; PM: Progressive Motility; RAPID: Percentage of Rapidly Moving Cells; VAP: Average Path Velocity; VSL: Straight-Line Velocity; VCL: Curvilinear Velocity; BCF: Beat Cross Frequency: STR: Straightness; LIN: Linearity; ALH: Amplitude of Lateral Head Displacement.

According to HOST, no differences were detected between bulls (*P*=0.336) and no interaction (*P*=0.821) between bull and semen type was detected. However, an effect of semen type was observed (*P*=0.001). A reduction in the percentage of plasma membrane integrity was observed in sex-sorted compared to conventional semen, for both bulls (Bull 1: *P*=0.010; Bull 2: *P*=0.006), as demonstrated in Table 3.

Sperm concentration significantly differed between semen types (*P*<0.001), being reduced for sex-sorted compared to conventional semen, for both sires (Bull 1: *P*<0.001; Bull 2: *P*<0.001; Table 3).

No differences were observed for Major and Total sperm morphological defects between bulls (Maj Def: *P*=0.392; Tot Def: *P*=0.079) or semen types (Maj Def: *P*=0.771; Tot Def: *P*=0.079) and no interactions were detected for those sperm parameters (Maj Def: *P*=0.392; Tot Def: *P*=0.829), as also demonstrated in Table 3.

Table 3. Mean (± SD) of plasma membrane integrity, sperm concentration, motile sperm per straw and sperm morphology of conventional and sex-sorted semen, separated by bull

	Bull 1		Bull 2	
	Conventional	Sex-sorted	Conventional	Sex-sorted
HOST (%)	52.33±12.41a	30.33±9.60b	48.66±1.60a	24.50±3.04b
Concentration (x10^6/mL)	43.00±0.81a	23.15±0.59b	42.12±0.66a	25.08±2.77b
Major Defects (%)	1.33±1.15	1.00±1.00	1.33±0.58	2.00±1.00
Minor Defects (%)	5.33±0.58	3.67±1.53	6.67±1.15A	4.33±1.53B
Total defects (%)	7.00±1.53	5.00±2.08	8.00±1.00	6.00±2.08

For each bull, values with different lowercase letters (a,b) within a row indicate $P \leq 0.05$ and values with different capital letters (A,B) indicate $P > 0.05$ and < 0.10.
HOST: percentage of sperm cells with plasma membrane integrity in the Hyposmotic Swelling Test.

The results of sperm morphometry and chromatin integrity are demonstrated in Table 4. No differences were detected between bulls or semen types (nor interactions) for the following parameters: area of sperm head (bull: *P*=0.679; semen type: *P*=0.887; bull*semen type:

P=0.927), perimeter (bull: *P*=0.419; semen type: *P*=0.700; bull*semen type: *P*=0.789), width (bull: *P*=0.996; semen type: *P*=0.517; bull*semen type: *P*=0.754), length (bull: *P*=0.250; semen type: *P*=0.281; bull*semen type: *P*=0.526), Fourier 0 (bull: *P*=0.068; semen type: *P*=0.179; bull*semen type: *P*=0.610), Fourier 2 (bull: *P*=0.941; semen type: *P*=0.131; bull*semen type: *P*=0.791) and side symmetry (bull: *P*=0.227; semen type: *P*=0.663; bull*semen type: *P*=0.300). Furthermore, no effects of bull, semen type or interactions were detected for chromatin condensation (*P*=0.257, *P*=0.756 and *P*=0.506, respectively) nor for chromatin heterogeneity (*P*=0.574, *P*=0.302 and *P*=0.374, respectively).

No differences were detected between bulls for sperm ellipticity (*P*=0.849) or width:length ratio (W/L: *P*=0.184) and no interactions between bull and semen type were detected for both parameters (ellipticity: *P*=0.144; W/L: *P*=0.299). However, for sperm ellipticity, a significant global effect was detected for semen type (*P*=0.037) and the multiple comparisons identified that sperm heads from sex-sorted semen were significantly lower than conventional semen for Bull 2 (*P*=0.020), whereas no difference was observed between sex-sorted and conventional semen for Bull 1 (*P*=0.552). For W/L, statistical tendency was detected for semen type (*P*=0.069) and W/L of sex-sorted semen was only marginally higher (*P*=0.058) than conventional semen for Bull 2, but no difference in W/L was observed for Bull 1 (*P*=0.423). Regarding to Shape Factor, no effect of bull was observed (*P*=0.493) but interaction of bull and semen type was detected (*P*=0.023). For Bull 2, Shape Factor was reduced (*P*=0.028) in sex-sorted compared to conventional semen, while no difference (*P*=0.249) was detected for Bull 1 (Table 4).

In Fourier 1 parameter, effects of bull (*P*=0.034) and semen type (*P*=0.008) were observed but no interaction was detected (*P*=0.741). For Bull 1, conventional semen demonstrated higher (*P*=0.026) Fourier 1 compared to sex-sorted semen and, for Bull 2, only a marginally significant difference was detected (*P*=0.056). The antero-posterior symmetry (APS) was different between bulls (*P*=0.021) and semen types (*P*<0.001) but no interaction was detected (*P*=0.661). For Bull 1, reduced (*P*<0.001) APS was observed for conventional compared to sex-sorted semen, whereas, for Bull 2, conventional semen demonstrated higher (*P*=0.004) APS compared to sex-sorted semen, as also demonstrated in Table 4.

Table 4. Mean (± SD) of sperm chromatin structure and morphometry analysis of conventional and sex-sorted semen, separated by bull

	Bull 1		Bull 2	
	Conventional	Sex-sorted	Conventional	Sex-sorted
A (pixels)	10.090±0.945	10.173±0.306	9.974±0.145	9.992±0.655
P (pixels)	10.334±0.476	10.314±0.130	10.240±0.024	10.130±0.277
W (pixels)	2.007±0.100	2.022±0.047	1.993±0.035	2.035±0.089
L (pixels)	3.981±0.186	3.951±0.042	3.946±0.035	3.834±0.084
W/L	0.503±0.011	0.513±0.012	0.528±0.044A	0.531±0.013B
Ellipticity	0.330±0.010	0.332±0.010	0.342±0.024a	0.306±0.011b
SF	0.886±0.003	0.883±0.003	0.917±0.062a	0.889±0.002b
Fourier 0	945.134±84.879	916.191±23.448	896.568±52.752	835.011±28.358
Fourier 1	119.925±11.565a	103.777±1.568b	107.787±7.091A	94.513±5.009B
Fourier 2	81.770±7.798	87.313±9.855	80.995±4.533	88.694±2.629
SS	0.958±0.001	0.960±0.002	1.002±0.070	0.960±0.002
APS	0.920±0.001b	0.931±0.003a	0.966±0.072a	0.933±0.002b
Dif (%)	3.768±0.519	4.010±0.920	5.010±0.637	4.351±1.876
CV (%)	3.910±0.705	4.662±0.444	4.040±0.355	4.100±0.892

For each bull, values with different lowercase letters (a,b) within a row indicate *P*≤0.05 and values with different capital letters (A,B) indicate *P*>0.05 and <0.10.

A: area of sperm head; P: perimeter; W: width; L: length; W/L: width:length ratio, SF: Shape Factor; SS: side symmetry; APS: anterior-posterior symmetry; Dif: sperm chromatin decondensation measured by percentage of gray-level differences; CV: chromatin heterogeneity measured by coefficient of variation of the gray-level.

DISCUSSION

In the present study, it was demonstrated that the large-scale utilization of sex-sorted semen in an E2/P4-based ovulation synchronization protocol with the traditional schedule for timed-AI reduces P/AI of suckled multiparous Nelore cows compared to conventional semen, even in cows displaying estrus. The pregnancy success of sex-sorted semen was reduced for both bulls, even though the impairments of *in vitro* sperm characteristics from sex-sorted semen were different for each sire.

Estrous behavior at timed-AI is positively related to higher P/AI in beef cattle (Sá Filho *et al*., 2010a, 2011; Rodrigues *et al*., 2018). Females displaying estrus at the time of AI have larger ovulatory follicles and higher concentration of circulating E2 compared to cows not expressing estrus (Perry *et al*., 2014; Pugliesi *et al*., 2016), which improve ovulation rates, sperm transport, myometrium contractility, uterine fluid composition (Hawk, 1983) and uterine receptivity (Davoodi *et al*., 2016).

Similarly, in the present experiment, cows that displayed estrus achieved greater P/AI compared to cows that did not display estrus, independent of semen type. Since P/AI of sex-sorted semen was increased when utilized in cows expressing estrus, the P/AI of cows displaying estrus inseminated with sex-sorted semen was similar to cows not expressing estrus inseminated with conventional semen. Still, P/AI of sex-sorted semen was lower than conventional semen, in both, cows expressing estrus or not. Hence, cows expressing estrus inseminated with conventional semen had higher chances to became pregnant than cows expressing estrus inseminated with sex-sorted semen.

It has been constantly reported reduced pregnancy success in cows inseminated with sex-sorted semen after estrus detection (DeJarnette *et al*., 2008) or following Timed-AI protocols (Sá Filho *et al*., 2010b; DeJarnette *et al*., 2011; Sales *et al*., 2011; Sá Filho *et al*., 2012; Silva *et al*., 2018). Part of this fertility impairment is due to premature capacitation of flow cytometry sex-sorted sperm (Lu and Seidel, 2004; Moce *et al*., 2006; Carvalho *et al*., 2013) and diminished capacity to remain bound to oviductal cells (Carvalho *et al*., 2018). Thus, the reduced sperm longevity of sex-sorted semen in the female reproductive tract may alter the optimum time for AI conduction related to ovulation occurrence (Silva *et al*., 2018) also due to possible sperm DNA fragmentation and/or mitochondrial modifications (Gosalvez *et al*., 2011; Rath *et al*., 2013). Therefore, higher P/AI are commonly observed with sex-sorted sperm when AI occurs closer (0 to 12 h) to the time of ovulation (Sá Filho *et al*., 2010b; Kurykin, 2017; Silva *et al*., 2018).

Silva *et al*. (2018) also used an E2/P4-based protocol for Timed-AI with sex-sorted semen in suckled multiparous Nelore cows with ECP being administered simultaneously to P4 device removal, but the AI was performed 60 hours after P4 withdrawal. The authors obtained higher P/AI with sex-sorted semen than the current study. Nevertheless, greater pregnancy success was achieved with non-sex-sorted rather than with sex-sorted semen (Silva *et al*., 2018). From a practical viewpoint, the protocol of the present study (AI at 48 hours after P4 removal) has practical feasibility considering animal handling schedules for large-scale Timed-AI programs of extensive beef farms. However, even upon estrus detection at timed-AI, the present protocol shall not be recommended for the use of sex-sorted semen in *Bos indicus* suckled cows, due to the unsatisfactory P/AI obtained. Even though the sperm dosages > 4.0 $x10^6$ sperm/straw, the reduction in P/AI observed for sex-sorted semen may have been caused by several factors related to *in vitro* sperm characteristics (Moce *et al*., 2006; Seidel and Schenk, 2008; Schenk *et al*., 2009; DeJarnette *et al*., 2010; Carvalho *et al*., 2013, 2018; Magata *et al*., 2021).

Damages in sperm membranes resulting from oxidative stress and decreased motility in frozen-thawed sex-sorted commonly occurs during sorting, since interaction of these cells with biological, chemical, and/or mechanical stressors is particularly high (Suh *et al*., 2005; Garner, 2006; Schenk and Seidel, 2007, DeJarnette *et al*., 2008; Schenk *et al*., 2009). Furthermore, changes in pH/osmolarity, high semen dilution and incubation period may lead to reduced sperm linearity and decreased sperm motility of sex-sorted sperm (Schenk *et al*., 2009). Other cellular alterations may also be related to damages in chromatin stability due to sperm exposure to pressure, ultraviolet radiation and/or

Stain/DNA/laser interactions (Boe-Hansen *et al*., 2005; Garner, 2006; Blondin *et al*., 2009; Gosalvez *et al*., 2011; Kurykin, 2017).

Gonzalez-Marín *et al*. (2018) demonstrated that some issues related to sex-sorted sperm quality are mitigated by improvements in new sorting methods, which confers considerable benefits in preserving sperm integrity and motility parameters. Nonetheless, in the present study, both sires demonstrated reduced plasma membrane integrity in sex-sorted compared to conventional semen, as well as increased ALH.

The reduction in plasma membrane integrity observed for both bulls can explain, at least partially, the reduced field fertility observed for sex-sorted semen. Sperm membrane integrity significantly influences the bull fertilizing capacity (Januskauskas *et al*., 2003; Kasimanickam *et al*., 2007). Likewise, plasma membrane integrity and progressive motility were found to be negatively correlated with sperm lipid peroxidation, which negatively affects bull fertility (Kasimanickam *et al*., 2007). Additionally, harmful effects of dead sperm are consequence of extracellular release of free radicals that cause irreversible damages in sperm plasma membrane and motility, which would further display apoptosis-related markers and/or DNA-breaks (Roca *et al*., 2016). These harmful effects are especially evident during the storage of semen samples and particularly when they are frozen (Roca *et al*., 2016).

Oliveira *et al*. (2013) identified, among other sperm parameters, the variables TM, PM, RAPID_2h, plasma membrane integrity, Major Defects, Ellipticity, Fourier 0, Fourier 2 and chromatin heterogeneity as important predictors of conception rates in a timed-AI program of suckled Nelore cows. In the present work, reduced TM, PM, RAPID and VAP were observed for sex-sorted compared to conventional semen, only for Bull 1, which interestingly was the sire demonstrating higher numerical reduction in P/AI from conventional to sex-sorted semen in cows expressing estrus (see Fig. 1). Those *in vitro* sperm parameters were not statistically different between sex-sorted and conventional semen for Bull 2.

On the other hand, Bull 2 was the only sire demonstrating reduced LIN in sex-sorted compared to conventional semen and the only sire whose sperm ellipticity was reduced in sex-sorted sperm cells compared to non-sorted. Alterations in sperm elongation may affect sperm kinetics (Beletti *et al*., 2005) which could explain the LIN parameter modifications observed in sex-sorted semen from this bull.

The variables sperm head ellipticity and length/width ratio (L/W) are inversely correlated. Ellipticity can be described as a measure of elongation of the head contour and Shape Factor as the deviation of the head contour from smooth ellipse (Beletti *et al*., 2005). Hence, Shape Factor is a sperm characteristic related to sperm head ellipticity, which confirm the finding that, for Bull 2, sex-sorted sperm cells were found less elliptical than non-sorted cells. It also explains the reduction in APS observed for sex-sorted compared to conventional semen from Bull 2.

Relationship between chromatin condensation and sperm head morphometry have been widely reported (Ostermeier *et al*., 2001a, 2001b; Kipper *et al*., 2017; Souza *et al*., 2018; Martins *et al*., 2021). Sires with lower sperm ellipticity (or higher W/L) are more likely expected to demonstrate reduced field fertility (Oliveira *et al*., 2013; Kipper *et al*., 2017) and changes in sperm ellipticity were related to chromatin modifications (Kipper *et al*., 2017; Martins *et al*., 2021). Kipper *et al*. (2017) demonstrated that sperm cells with chromatin alterations had less elliptical shape to their heads. Even so, the present data do not allow confirmation of chromatin abnormalities in sex-sorted semen from Bull 2 since no differences in chromatin assessments (Dif and CV) were observed for this sire.

Fourier descriptors using harmonic amplitudes from 0 to 2 (Fourier 0, 1 and 2) were also considered. Quantifying changes in sperm head shape can be detected by Fourier parameters which characterize the curvilinear perimeter of sperm head (Ostermeier *et al*., 2001a) and are able to identify small differences in sperm nuclear shape from bulls with different fertility (Ostermeier *et al*., 2001b). In the present study, Fourier 1 was significantly reduced in sex-sorted compared to conventional semen from Bull 1. Because Fourier 1 measures the roundness of anterior head, reductions in this parameter indicates larger sperm head base (Ostermeier *et*

al., 2001a), which may explain the increase in APS from conventional to sex-sorted semen observed for this sire.

Sperm heads with chromatin alterations were reported to have lower Fourier 1 values (Martins *et al*., 2021) and subfertile bulls showed higher percentage of chromatin alteration in the base of sperm head (Souza *et al*., 2018). Chromatin changes in specific portions of sperm head (as alterations up to the basal third of sperm head) could justify the decrease in Fourier 1 parameter as well as the increase in APS observed for Bull 1. Still, since Dif and CV were not different in both semen types of this sire, and/or because the specific locations of sperm chromatin alterations were not measured, the origin of such isolated morphometric alteration (reduction in Fourier 1 parameter) in sex-sorted semen from Bull 1 is hard to be defined, considering the sperm chromatin assessment techniques utilized (Castro *et al*., 2018). It might also be worth mentioning, though, that DNA damage is a dynamic process with varying rates among individuals, and thus, sperm chromatin may exhibit some degree of sublethal damage and/or be affected at a subclinical level after X/Y sex-sorting, provoking detectable decreases in DNA integrity only after incubation (Gosalvez *et al*., 2011).

Sire effects related to *in vivo* and *in vitro* sperm fertility are commonly reported for conventional and sex-sorted semen (DeJarnette *et al*., 2008, 2010, 2011; Blondin *et al*., 2009; Sá Filho *et al*., 2009; Gosalvez *et al*., 2011; Sales *et al*., 2011; Oliveira *et al*., 2012a; Silva *et al*., 2018; Thomas *et al*., 2019; Magata *et al*., 2021). Our results demonstrated that overall P/AI of cows inseminated at timed-AI was reduced with sex-sorted semen from both bulls, albeit descriptive data seem to indicate more intense impairment of *in vivo* fertility in sex-sorted sperm from Bull 1. It was also interesting to note that, *in vitro* sperm quality of sex-sorted semen was differently affected in each bull, being identified further kinetic alterations and sperm damages in sex-sorted semen from Bull 1.

CONCLUSIONS

Even though that estrus expression at timed-AI improved fertility of suckled multiparous Nelore cows receiving sex-sorted semen, the type of semen affected pregnancy success, regardless of estrous behavior. For both bulls, reduced fertility was observed with sex-sorted semen compared to non-sorted semen, even in cows expressing estrus. Still, *in vitro* sperm quality of sex-sorted semen was compromised for both bulls, but differently affected for each sire.

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