Assessment of in vitro sperm characteristics and their importance in the prediction of conception rate in a bovine timed-AI program

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ABSTRACT

The aims of this study were to assess in vivo fertility and in vitro sperm characteristics of different sires and to identify sperm variables important for the prediction of conception rate. Multiparous Nelore cows (n = 191) from a commercial farm underwent the same timed artificial insemination (timed-AI) protocol. Three batches of frozen semen from three Angus bulls were used (n = 9). A routine semen thawing protocol was performed in the laboratory to mimic field conditions. The following in vitro sperm analyses were performed: Computer Assisted Semen Analysis (CASA), Thermal Resistance Test (TRT), Hyposmotic Swelling Test (HOST), assessment of plasma and acrosomal membrane integrity, assessment of sperm plasma membrane stability and of lipid peroxidation by flow cytometry and assessment of sperm morphometry and chromatin structure by Toluidine Blue staining. For statistical analyses, Partial Least Squares (PLS) regression was used to explore the importance of various sperm variables in the prediction of conception rate. The following in vitro sperm variables were determined to be important predictors of conception rate: total motility (TM), progressive motility (PM), TM after 2 h of thermal incubation (TM 2h), PM after 2 h of thermal incubation (PM 2h), Beat Cross Frequency after 2 h of thermal incubation (BCF 2h), percentage of rapidly moving cells after 2 h of thermal incubation (RAP 2h), intact plasma membrane evaluated by HOST, intact plasma and acrosomal membranes evaluated by flow cytometry, intact plasma membrane suffering lipid peroxidation, major defects, total defects, morphometric width/length ratio, Fourier 0 and Fourier 2 and Chromatin Heterogeneity. We concluded that PLS regression is a suitable statistical method to identify in vitro sperm characteristics that have an important relationship with in vivo bull fertility.

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1. Introduction

Many classical and modern methods have been used for laboratory assessment of bull sperm quality following cryopreservation of semen (Revell and Mrode, 1994; Verstegen et al., 2002; Brouwers and Gadella, 2003; Beletti et al., 2005; Celeghini et al., 2007; Kasimanickam et al., 2007). However, the results of such assays do not always correlate with the actual fertility of a semen sample (Zhang et al., 1999; Sudano et al., 2011).

The relationship between in vitro semen quality and field fertility has been the subject of considerable study (Amann, 1989; Farrell et al., 1998; Zhang et al., 1999; Larsson and Rodriguez-Martinez, 2000; Rodriguez-Martinez, 2003; Sudano et al., 2011); even so, to date, the best way to estimate the fertility of a particular bull has been to use a field fertility test, which is expensive and time-consuming (Zhang et al., 1999; Larsson and Rodriguez-Martinez, 2000).

Embryo culture techniques have enabled the exploration of in vitro bull fertility, and positive correlations between in vitro embryo production and in vivo bull fertility have been reported (Marquant-Le Guenene et al., 1990; Lonergan, 1994; Zhang et al., 1997; Larsson and Rodriguez-Martinez, 2000; Ward et al., 2001; Sudano et al., 2011). Sudano et al. (2011) demonstrated that it is possible to estimate bull fertility with in vitro production (IVP) outcomes using a Bayesian statistical inference model. Although such a model may provide interesting results, it is still unclear whether the ability of a bull to fertilize oocytes in vitro is a useful predictor of in vivo fertility following AI. Additionally, practical and/or easy handling techniques for assessing semen quality would be more useful for the semen industry than IVP procedures.

While a practical in vitro semen assay that could replace field fertility trials for determining bull fertility would be of great benefit to AI programs (Ward et al., 2001), it is unlikely, given the complexity of the reproductive process, that a single sperm characteristic will reflect the true fertilization capacity of a semen sample. Hence, a combination of diverse laboratory assays might more accurately reflect semen fertility than a single test. Moreover, identifying a set of sperm characteristics considered important for predicting conception rate would be a useful method of monitoring and/or predicting bull fertility.

Thus, with the goal of providing information that may help researchers identify semen parameters that are highly correlated with field fertility, this study aimed to assess the in vitro sperm characteristics and in vivo fertility of different sires and to identify sperm variables that are important predictors of conception rate in a timed artificial insemination (timed-AI) program.

2. Material and methods

2.1. Assessment of field fertility

2.1.1. Animals and management

For this experiment, data from a subset (n = 191) of 944 suckled multiparous Nelore cows enrolled in a timed-AI program were used. All animals were from a commercial beef farm in the state of Mato Grosso, Brazil; animals were maintained on Brachiaria brizantha or Brachiaria decumbens pasture and provided mineralized salt and free access to water. Cows with body condition scores (BCS) between 1.75 and 3.25 on a 1–5 scale (1 = emaciated, 5 = obese) were used. All cows received the same timed-AI protocol for their first service.

After calving, suckled cows were allocated into eight different breeding groups (approximately 120 animals each) according to calving date. From 30 to 40 d postpartum, all cows (n = 944) received the same timed-AI protocol. This protocol started (Day 0), with cows receiving an intravaginal progesterone releasing device (Sincrogest®, Ouro Fino Saúde Animal, Cravinhos, SP, Brazil) and 2.0 mg of estradiol benzoate intramuscularly (IM) (EB; Sincrodiol®, Ouro Fino Saúde Animal, Cravinhos, SP, Brazil). The progesterone device was removed 8 d later, and cows were given 500 μg of d-cloprostenol IM (PGF2α; Sincroci®, Ouro Fino Saúde Animal, Cravinhos, SP, Brazil), 300 IU of eCG IM (Novormon 5 000®, Intervet Schering Plough Saúde Animal, São Paulo, SP, Brazil), and 0.5 mg of estradiol cipionate IM (ECP®, Pfizer Saúde Animal, São Paulo, SP, Brazil). Cows underwent timed inseminations (with no detection of estrus), performed by two experienced AI technicians, 2 days after removal of the progesterone device (Day 10). The day of timed-AI was different for each breeding group. Each AI technician inseminated the same number of cows.

2.1.2. Field experimental design

One hundred frozen semen doses from each of three batches from three Angus bulls from the same company were used. Semen thawing and semen handling protocols were performed according to routine procedures of the farm where the experiment was conducted.

For timed-AI, the following procedure was performed for each breeding group: after loading a random cow into the chute, ten 0.5 mL frozen semen straws from the same batch were thawed simultaneously in a thermostatically controlled thawing bath (electrical water-bath unit containing 400 mL of water; Fertilize®, Fertilize, Uberaba, MG, Brazil) adjusted to 36 °C. Thirty seconds after placement of these 10 straws into the thawing bath, one straw was removed (first straw), immediately loaded in the AI gun and used for insemination of the first cow. During insemination with the first straw, a second straw was removed from the thawing bath, and a second AI gun was prepared. After the first AI was finished, another cow was randomly loaded in the chute, and the AI gun with the second straw was used. In a similar manner, the other straws were all loaded into AI guns and used in sequence.

The thawing and loading of AI guns and the timed-AI procedures were carried out in a covered and protected area, free of wind and direct sun light. Approximately twelve groups of ten frozen straws were thawed for each day of timed-AI. The sequence of insemination (1st, 2nd, 3rd until 10th) and the time (h and min) of semen removal from the thawing bath were recorded for each cow.

To assure a randomized experimental design and a balanced number of animals per field variable, semen from the three bulls was equally distributed across breeding groups, AI technician and straw sequence. The mean time each
semen straw remained in the thawing bath (first to tenth) was calculated after the end of the field experiment using records from the 944 inseminations. These data were later used to mimic the field conditions of semen thawing for the laboratory analyses.

All cows were examined for pregnancy by transrectal ultrasonography 40 days after timed–AI.

For the calculation of conception rate (pregnancy per AI), cows were separated into groups corresponding to sequence of insemination (1st Straw–AI, 2nd Straw–AI, 3rd Straw–AI until 10th Straw–AI) to avoid erroneous results due to a possible influence of the sequence of insemination on conception rate (Oliveira et al., 2012).

2.2. Laboratory assessment of semen quality—laboratory experiment

All chemicals used in the laboratory experiment were from Sigma–Aldrich (St. Louis, MO, USA), with exceptions being noted where applicable.

2.2.1. Laboratory experimental design

Frozen semen samples from each bull and batch \((n = 9)\) used in the field trial were brought to the laboratory.

According to the field experiment, the mean time that all straws remained in the water bath until the removal of the last thawed straw of the water bath was 6 min and 29 s (i.e., it took 6 min and 29 s for the 10 straws to be removed from the thawing bath, assuming time 0 to be 30 s).

To mimic the field procedures, ten 0.5–ml frozen straws (four frozen semen straws from the same batch and six frozen mock straws containing only milk-based diluents) were thawed simultaneously at a temperature of 36 °C for a minimum of 30 s for each of the nine batches utilized. The same thermostatically controlled thawing bath of the field experiment was used.

Two semen straws from the same batch were removed from the water bath after 30 s, while the remaining straws remained in the thawing bath. The laboratory analyses of these two first straws (1st Straw–Lab) were later used to evaluate the relationship between 1st Straw–Lab characteristics and data from the 1st Straw–AI group of the field experiment. To evaluate the semen quality of the 1st straw (1st Straw–Lab), the semen from the two-straw pair was pooled in a microcentrifuge tube, and several in vitro sperm characteristics were analyzed.

Six minutes and 29 s after the removal of the two initial straws (1st Straw–Lab), two others semen straws from the same batch were removed from the thawing bath. The laboratory analyses of these two last straws (10th Straw–Lab) were later used to evaluate the relationship between 10th Straw–Lab characteristics and 10th Straw–AI data of the field experiment. To evaluate the quality of the 10th straw (10th Straw–Lab), semen from this two-straw pair was pooled in a microcentrifuge tube, and the same in vitro sperm characteristics assessed for the 1st straw were evaluated. The six other simultaneously thawed mock straws containing milk-based diluents were discarded. The following in vitro semen analyses were performed for the 1st and 10th Straw–Lab samples.

2.2.2. Computer-assisted semen analysis (CASA)

Sperm motility was assessed by CASA (Ivos-Ultimate®; Hamilton Thorne Biosciences, Beverly, MA, USA). However, samples used in this experiment were all cryopreserved in the same milk extender, and evaluation of bovine semen in lactose based diluents has always presented a problem because the presence of large numbers of fat globules causes the media to become opaque (Van Damark et al., 1959). Thus, in an attempt to make the sperm more visible in this media, sperm was stained with Hoechst 33342 (H342) dye (H–1399; Molecular Probes Inc., Eugene, OR, USA), which causes sperm to fluoresce in ultraviolet light.

In the present experiment, Hoechst 33342 staining was prepared in a concentration of 5 mg/mL. The frozen semen straws presented a sperm concentration between 25 and 35 × 10⁹ sperm/mL. The CASA set-up was pre-adjusted for bovine sperm analysis in IDENT® option with the following parameters: number of frames: 30; frames per s: 60 Hz; minimum contrast: 50; minimum cell size: 6 pixels; contrast to static cells: 30; strictness: 60%; average path velocity cutoff: 30 μm/s; minimum average path velocity: 40 μm/s; straight-line velocity cutoff: 20 μm/s; cell size: 6 pixels; cell intensity: 80; static head size: 0.23–1.91; static head intensity: 0.56–1.20; static elongation: 8–92; magnification: X 1.89; video frequency: 60; illumination intensity: 2203; and temperature: 37 °C.

For CASA assessment of sperm motility, an aliquot of 100 μL of frozen-thawed semen was warmed in a microcentrifuge tube and 2 μL of H342 (40 μg/mL) was added. It was incubated for 20 min at 37 °C. After incubation, the post-thawing sperm motility was evaluated, which involved placing 6 μL of H342 stained semen sample in a standard count analysis chamber (Makler counting chamber, SEFI Medical Instruments LTD, Haifa, Israel). Six fields were randomly selected for each analysis. The following variables were analyzed by CASA: 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 the percentage of rapidly moving cells (RAP). Approximately 100.000 cells were evaluated by CASA for each semen sample.

2.2.3. Sperm thermal resistance test (TRT)

The thermal–resistance test (TRT) was carried out to verify post-thaw longevity of semen samples. Immediately after removal from the thawing bath, an aliquot of 250 μL of frozen-thawed semen was put into a warmed microcentrifuge tube, which remained incubated at 37 °C. After 120 min of incubation (2 h), the same procedure described on Section 2.2.2 for CASA was applied and the following motility parameters were assessed for TRT_2 h: TM_2 h, PM_2 h, VAP_2 h, VSL_2 h, VCL_2 h, ALH_2 h, BCF_2 h, STR_2 h, LIN_2 h, RAP_2 h.

2.2.4. Hyposmotic swelling test (HOST)

The HOST was performed by incubating 20 μL of semen with 1 mL of a 100 mOsm hyposmotic solution (Revell and Mrode, 1994) at 37 °C for 60 min. After incubation, 20 μL of the solution was coverslipped and evaluated by
contrast phase microscopy. Two hundred sperm were evaluated under magnification 1000×. Sperm with swollen or coiled tails were considered viable. The percentage of viable sperm in HOST (HOST + cells) was calculated according to Revell and Mrode (1994).

2.2.5. Flow cytometry analyses

These analyses were carried out using the FACsarria® (Becton-Dickinson, San Jose, CA, USA) flow cytometer equipped with a 405 nm Near UV laser and 488 nm Sapphire blue laser and filters B (Band Pass 450/20), C (Long Pass 595 nm/Band Pass 610/20 nm), D (LP 556 nm/BP 575/26 nm) and E (LP 502 nm/BP 530/30 nm). The flow cytometer was calibrated, using two aliquots of a single fresh semen sample (motility > 80%), with one aliquot submitted to flash frozen in liquid nitrogen (to damage sperm membranes, resulting in a great proportion of damaged sperm) as described previously (Celeghini et al., 2007) and the other used as a control (great proportion of membrane-intact sperm). The cells were simultaneously excited by an argon laser at 488 nm and by a Near UV laser at 405 nm (De Andrade et al., 2011).

Samples for staining and flow cytometry analysis were diluted in a modified Tyrode’s medium (TALPm) with 114 mM NaCl, 3.2 mM KCl, 0.5 mM MgCl2·6H2O, 0.4 mM NaH2PO4·H2O, 5 mM glucose, 10 mM sodium lactate, 0.1 mM sodium pyruvate and 10,000 UI/100 mL sodium penicillin. The pH of the medium was adjusted with the use of 1 N NaOH until the pH was 7.4. After the addition of the dyes for each analysis, the semen samples incubated in the TALPm were analyzed in the flow cytometer, which was controlled by the BD FACSDiva 6.0 software (Becton-Dickinson). The samples were processed through the instrument at an acquisition rate of approximately 600 to 1000 events/s, acquiring 10,000 cells per analysis (Leite et al., 2010).

2.2.5.1. Simultaneous assessment of plasma and acrosomal membranes. To evaluate which cells had intact plasma membranes and which cells had undergone the acrosome reaction, an aliquot was taken from the samples and added to the TALPm. The resulting samples had a concentration of 5 × 10⁶ sperm/ml in a volume of 148 μL. Two microliters of H342 (40 μg/mL) were added to stain the DNA of the cells such that the cells with the same scatter properties as sperm were not counted. After 10 min of incubation at 37 °C, 3 μL of propidium iodide (PI, 0.5 mg/mL; 28.707-5; Sigma–Aldrich) and 10 μL of Pism sativum agglutinin conjugated to fluorescein isothiocyanate (FITC-PSA, 100 μg/mL; L-0770; Sigma–Aldrich) were added to the samples. The purpose of the use of these probes was to stain the cells with damaged plasma membranes (PI positive) and the cells that had undergone the acrosome reaction (FITC-PSA positive) (Celeghini et al., 2007). After 10 min of incubation at 37 °C, the samples were diluted with the addition of 150 μL of the TALPm to a concentration of 2.5 × 10⁶ sperm/mL and analyzed by flow cytometry (De Andrade et al., 2011).

Two-dimensional dot-plots of FITC-PSA (Filter E) vs. PI fluorescence (Filter C) were generated from a total of 10,000 events. Each quadrant represents one of the following sperm subpopulations: (1) IPIA: sperm with intact plasma and acrosomal membranes; (2) IPDA: sperm with an intact plasma membrane and a damaged acrosomal membrane; (3) DPIA: sperm with a damaged plasma membrane and an intact acrosomal membrane; and (4) DPDA: sperm with damaged plasma and acrosomal membranes (Leite et al., 2010). The percentage of sperm presenting with intact plasma membranes (IPM: IPIA+IPDA), the percentage of sperm presenting with intact acrosomes (IA: IPIA+DPIA) and the IPIA subpopulation were considered for the relationship analysis.

2.2.5.2. Assessment of sperm plasma membrane stability. For assessment of plasma membrane stability by flow cytometry (Rathi et al., 2001), the samples were incubated in 147 μL of the TALPm solution at a concentration of 5 × 10⁵ sperm/mL with 2 μL of H342 (40 μg/mL). Subsequently, 0.5 μL of fluorescent probe Yo-Pro-1 (Y3603, Molecular Probes Inc., Eugene, OR, USA) was added to the sample (7.5 μM), resulting in a final concentration of 25 nM. Yo-Pro-1 cannot penetrate an intact plasma membrane and therefore only binds to the DNA of cells with a damaged plasma membrane. Samples were incubated for 20 min, after which merocyanine 540 (M540) fluorescent probe (M 24571, Molecular Probes Inc., Eugene, OR, USA) was added (810 μM) to obtain a concentration of 2.7 μM (in 150 μL); samples were later incubated for 70 s, diluted in 150 μL of TALPm and analyzed by flow cytometry. The M540 staining showed two distinct populations of viable cells (Yo-Pro-1 negative): one population characterized by cells with low fluorescence emission (considered non-capacitated, with a low level of lipid bilayer disorganization; LBD) and another population characterized by high fluorescence (capacitated, with a high level of lipid bilayer disorganization; HBD) captured in the long pass-595 and band-pass-610/20 nm. The positive Yo-Pro cells (cells with a damaged plasma membrane; YoPro + cells), and the LBD and HBD sperm populations were considered for relationship analysis.

2.2.5.3. Assessment of lipid peroxidation. The fluorescent fatty acid conjugate, 4, 4-difluoro-5-(4-phenyl-1,3-butaediyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl acid (C11-BODIPY581/591), is a fluorescent membrane probe that changes irreversibly from red to green upon exposure to reactive oxygen species (ROS); this probe has been used to assess lipid peroxidation in bovine sperm (Brouwers and Gadella, 2003).

For the assessment of lipid peroxidation, an aliquot was taken from the samples and adjusted to a concentration of 5 × 10⁶ sperm/mL with TALPm in a final volume of 499.5 μL. The C11-BODIPY581/591 (1 mg/mL, D-3861, Molecular Probes Inc., Eugene, OR, USA) was subsequently added, and the sample was incubated for 30 min at 37 °C. After incubation, 145 μL of this solution was transferred to another microtube, and 2 μL of H342 (40 μg/mL) were added. This sample was incubated for 10 min at 37 °C. The H342 probe was used to prevent particles with the same size and/or granularity as the sperm from being included in the count. After the incubation period with the H342 probe,
3 μL of PI (0.5 mg/mL) were added to the sample to mark cells with damaged plasma membranes. The sample was then incubated with PI for 5 min at 37 °C. Finally, 150 μL of TALPm were added to dilute the sample to a concentration of 2.5 × 10⁶ sperm/mL, and samples were analyzed by flow cytometry.

Two-dimensional dot-plots of C11-BODIPY581/591 (Filter E) vs. PI fluorescence (Filter C) from a total of 10,000 events were generated. Each quadrant represented one of the following sperm subpopulations: (1) IPP: sperm with intact plasma membrane suffering from lipid peroxidation (PI [-], C11-BODIPY581/591 [+]); (2) IPNP: sperm with intact plasma membrane with no lipid peroxidation detected (PI [-], C11-BODIPY581/591 [-]); (3) DPP: sperm with damaged plasma membrane and lipid peroxidation (PI [+], C11-BODIPY581/591 [+]); and (4) DPNP: sperm with damaged plasma membrane and no lipid peroxidation detected (PI [+], C11-BODIPY581/591 [-]). Random events were isolated with HCl, Microsystems and numerical analysis (Beletti et al., 1973). Fifty smears were prepared for subsequent assessment of sperm chromatin structure and morphology. The sperm smears were fixed with ethanol acetic acid (3:1, v/v) for 1 min and with 70% ethanol for 3 min. The smears were later hydrolyzed for 25 min in 4 M HCl, washed in distilled water and air-dried. One droplet of 0.025% toluidine blue in Mcllvaine buffer (sodium citrate–phosphate; LabChem Inc., Pittsburg, PA, USA), pH 4.0, was placed over each smear, and smears were covered with a coverslip.

Fifty gray-level digital images of each slide were obtained randomly using a Leica DM500 microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) with a 100× objective lens (immersion) coupled to a Leica ICC50 camera (Leica Microsystems Inc.) connected to a computer with an image analysis system (Leica Image Processing and Analysis System; Leica LAS EZ Software, Leica Microsystems Inc.). Images typically consisted of shades of gray that varied from black at the weakest intensity to white at the strongest. The image analysis that evaluates the distribution and the values of pixels is referred to as texture analysis. Using threshold-based image segmentation (Beletti and Costa, 2003), at least 100 sperm heads were isolated for each smear. The sperm head segmentation analysis was performed using algorithms developed in numerical computation software (Scilab™ software; Scilab Enterprises, Versailles, France) through the Scilab Image Processing toolbox (SIP toolbox). The sperm heads were analyzed to obtain the average values of pixels making up each head. Six heads with the smallest pixel values were selected automatically and defined as standard heads; these heads, theoretically, are the heads with the most condensed chromatin. For each image, the difference between the standard value of the smear and the average value of each head was determined; this difference was transformed into a percentage (Diff %) of the average pixel value for the standard heads and used as an indicator of the level of sperm chromatin decondensation (DIF). The coefficient of variation (CV) of the grey level intensity for each head, indicating sperm chromatin heterogeneity, was also calculated (Beletti et al., 2005; Kanayama and Beletti, 2011).

Area, perimeter, width, length, width:length ratio (WLR) and ellipticity of all sperm heads were determined using algorithms developed in Scilab (Beletti and Costa, 2003; Beletti et al., 2005). Fourier descriptors containing harmonic amplitudes from 0 to 2 (Fourier zero: Fourier_0, Fourier one: Fourier_1 and Fourier two: Fourier_2) were considered. Sperm head symmetry was also assessed. Side Symmetry (SS), in particular, is a measurement that identifies asymmetries along the principal sperm axis. All of these symmetries were calculated using the procedure described by Beletti and Costa (2003), which involves flipping the object along its major (or minor) axis and identifying the area of overlap between the original and flipped areas (Beletti et al., 2005).

### 2.3. Statistical analysis

As stated above, the sequence of insemination may affect conception rate, depending on which bull is used in a reproductive program (Oliveira et al., 2012). Hence, for the present field trial, only data from cows inseminated with the 1st straws (1stStraw-AI; n = 97) or the 10th straws of the sequence (10thStraw-AI; n = 94) were included in this study. Each batch was considered to be an experimental group (n = 9).

Partial Least Squares (PLS) regression was the statistical procedure used to explore the significance of laboratory variables in predicting conception rate. The PLS analysis was performed separately for each straw using the SAS (version 9.2, SAS Inst. Inc., Cary, NC, USA). The results of sperm variables from 1stStraw-Lab were compared with the conception rates of 1stStraw-AI, and the results of sperm variables from 10thStraw-Lab were compared with the conception rates of 10thStraw-AI.

In each statistical trial, a regression coefficient and a variable importance plot was obtained for each sperm variable. The regression coefficients represent the significance of each variable in the prediction of the response (conception rate). The variable importance plot, conversely, represents the contribution of each predictor toward the fitting of the PLS model for both predictors and response; it is based on Wold’s (1994) Variable Importance for Projection (VIP) statistic, which summarizes the contribution of a variable to the model. If a predictor has a relatively small coefficient (in terms of absolute value) and a small VIP value, then it is a prime candidate for deletion. Wold’s
3. Results

In this study, PLS regression was used to explore the significance of sperm variables in the prediction of conception rate. Because the analyses were performed separately by straw, results from the 1st and 10th straw are shown separately.

3.1. 1st Straw results

The mean conception rate of all cows inseminated with the 1st straw at timed-AI (1st Straw-AI) was 57.7% (56/97).

The PLS factors were assessed for every in vitro sperm characteristic and significant variables were selected according to Wold’s criterion (SAS, 2001). The first run of the PLS procedure for the 1st straw resulted in the exclusion of the following sperm variables (VIP < 0.8): VAP, VSL, ALH, STR, LIN, ALH.2 h, STR.2 h, LIN.2 h, HBD, Min_Def, Area, Perimeter, Width, Fourier.1, SS and DIP.

The second run of the PLS procedure was performed with the remaining variables. This run resulted in exclusion of the following parameters: VCL, VSL.2 h, VCI.2 h, IA, LBD and length. Ellipticity was also excluded because it is essentially equivalent to WLR; YoPro + cells were excluded because they represent the inverse of the IPM parameter.

The third run of the PLS procedure was performed with the remaining variables.

Fig. 1 shows the Variable Importance Plot obtained in the second and third PLS procedures for the 1st straw.

The following variables were found to be significant for prediction of conception rate after the third run of the PLS procedure of the 1st straw: TM, PM, BCF, RAP, TM.2 h, PM.2 h, VAP.2 h, BCF.2 h, RAP.2 h, HOST, IPIA, IPM, IPNP, IPP, Maj_Def, Tot_Def, WLR, Fourier.2, Fourier.2 and Chromatin Heterogeneity (CV).

3.2. 10th Straw results

The mean conception rate of all timed-AI cows inseminated with the 10th straw (10th Straw-AI) was 43.6% (41/94).

The first run of the PLS procedure of the 10th straw resulted in exclusion of the following variables (VIP < 0.8): PM, BCF, RAP, VAP.2 h, VSL.2 h, VCL.2 h, STR.2 h, Area, Perimeter, Length, Fourier.1, Fourier.2 and SS.

The second run of the PLS procedure was performed with the remaining variables. This run resulted in exclusion of the following variables: LIN, Fourier.2 and DIF. Ellipticity was also excluded because it is essentially equivalent to WLR; YoPro + cells were excluded because they represent the inverse of the IPM parameter.

The third run of the PLS procedure was performed with the remaining variables.

Fig. 2 shows the Variable Importance Plot obtained in the second and third PLS procedures of the 10th straw.

The following variables were found to be significant for prediction of conception rate after the third run of the PLS procedure for the 10th straw: TM, VAP, VSL, VCL, ALH, STR, TM.2 h, PM.2 h, ALH.2 h, BCF.2 h, LIN.2 h, RAP.2 h, HOST, IPIA, IPM, IA, LBD, HBD, IPNP, IPP, Maj_Def, Min_Def, Tot_Def, Width, WLR and Chromatin Heterogeneity (CV).

3.3. Sperm variables selected as important predictors of conception rate considering the results of the 1st and 10th Straws

The PLS procedures for the 1st and 10th straws resulted in the identification of important in vitro variables for the prediction of field fertility. The cutoff value for VIP was based on Wold’s criterion (SAS, 2001) which considers a value less than 0.8 to be “small”. Variables with VIP values lower than 0.8 were therefore excluded after the first and second repetition of the PLS procedure for each straw trial.

The following sperm variables were found to be significant for prediction of conception rate in both trials (i.e., for both the 1st and 10th straw trial): TM, TM.2 h, PM.2 h, BCF.2 h, RAP.2 h, HOST, IPIA, IPM, IPNP, IPP, Maj_Def, Tot_Def, WLR, Fourier.2, Fourier.2 and Chromatin Heterogeneity.

Several of these variables represented the same and/or notably similar sperm characteristics. Thus, of the predictors found to be significant by PLS, we selected only the variables found significant in both trials and excluded IPM (which is essentially equivalent to the HOST characteristic) and IPNP (which represents the inverse of the IPP characteristic). In addition, based on the scientific literature, the sperm variables PM, Fourier.2 and Fourier.2 (which were identified only in the 1st Straw trial) were included in the group of 15 variables considered important predictors of field fertility: TM, PM, TM.2 h, PM.2 h, BCF.2 h, RAP.2 h, HOST, IPIA, IPP, Maj_Def, Tot_Def, WLR, Fourier.2, Fourier.2 and Chromatin Heterogeneity.

The data obtained for the 1st Straw and for the 10th Straw (in field and laboratory experiments) of the 15 important variables are presented in Table 1.

4. Discussion

In this study, semen quality was analyzed using classical and modern laboratory tests. To explore the relationship between in vitro sperm characteristics and conception rate, PLS analysis was performed, and a group of variables found to be significant for the prediction of conception rate was
Fig. 1. Variable Importance Pilot obtained in the second (A) and third (B) Partial Least Square (PLS) procedures of 1st straw trial. The cutoff of the Variable Importance for Projection (VIP) was determined according to Wold in Umetrics (SAS, 2001) which considers a value less than 0.8 to be "small" for the VIP: TM: total motility; PM: progressive motility; VCL: curvilinear velocity; BCF: beat cross frequency; RAP: percentage of rapidly moving cells; TM$_{2\, h}$: TM after 2 h of thermal incubation; PM$_{2\, h}$: PM after 2 h of thermal incubation; VAP$_{2\, h}$: VAP after 2 h of thermal incubation; VSL$_{2\, h}$: VSL after 2 h of thermal incubation; VCL$_{2\, h}$: VCL after 2 h of thermal incubation; BCF$_{2\, h}$: BCF after 2 h of thermal incubation; RAP$_{2\, h}$: RAP after 2 h of thermal incubation; HOST: intact plasma membrane evaluated by Hiposmotic Swelling Test; IPIA: intact plasma and acrosomal membranes evaluated by flow cytometry; IPM: total percentage of sperm cells presenting intact plasma membranes; IA: total percentage of sperm cells presenting intact acrosome; YoPro: cells with damaged plasma membrane evaluated by YoPro; LBD: low level of lipid bilayer disorganization; IPP: intact plasma membrane suffering lipid peroxidation detected; IPP: intact plasma membrane with no lipid peroxidation detected; Maj_Def: major defects; Tot_Def: total of defects; WLR: width:length ratio; Fourier$_0$: mathematic parameter Fourier 0; Fourier$_2$: mathematic parameter Fourier 2; CV: chromatin heterogeneity.

identified. To the best of our knowledge, this study is the first to use PLS analysis to assess the relationship between a detailed in vitro assessment of sperm characteristics and field fertility rates of different bulls used in the same timed-AI program.

The ability of a specific laboratory test to predict bull fertility can be directly related with the statistical analysis performed (Sudano et al., 2011). The PLS analysis was chosen because of its ability to solve complex problems in many fields, including those involving
Fig. 2. Variable Importance Pilot obtained in the second (A) and third (B) Partial Least Square (PLS) procedures of 10th straw trial. The cutoff of the Variable Importance for Projection (VIP) was determined according to Wold in Umetrics (SAS, 2001) which considers a value less than 0.8 to be “small” for the VIP; TM: total motility; VAP: average path velocity; VSL: straight-line velocity; VCL: curvilinear velocity; ALH: amplitude of lateral head displacement; STR: straightness; LIN: linearity; TM$_{2}$h: TM after 2 h of thermal incubation; PM$_{2}$h: PM after 2 h of thermal incubation; ALH$_{2}$h: ALH after 2 h of thermal incubation; BCF$_{2}$h: BCF after 2 h of thermal incubation; LIN$_{2}$h: Lin after 2 h of thermal incubation; RAP$_{2}$h: RAP after 2 h of thermal incubation; HOST: intact plasma membrane evaluated by Hiposmotic Swelling Test; IPIA: intact plasma and acrosomal membranes evaluated by flow cytometry; IPM: total percentage of sperm cells presenting intact plasma membranes; IA: total percentage of sperm cells presenting intact acrosome; YoPro: cells with damaged plasma membrane evaluated by YoPro; LBD: low level of lipid bilayer disorganization; HBD: high level of lipid bilayer disorganization; IPNP: intact plasma membrane with no lipid peroxidation detected; IPP: intact plasma membrane suffering lipid peroxidation; Maj_Def: major defects; Min_Def: minor defects; Tot_Def: total of defects; WLR: width:length ratio; Fourier_Z: mathematic parameter Fourier 0; DIF: chromatin decondensation; CV: chromatin heterogeneity.

only a limited dataset, a situation frequently encountered in biological experiments. The PLS method can also be used to analyze data from multiple modalities collected on the same observations (Krishnan et al., 2011). Hence, with this statistical method, we were able to investigate the relationships between laboratory sperm characteristics and field fertility outcomes, even though we had few repetitions and a small number of bulls and/or batches. The use of PLS methods has been facilitated by the development of more sophisticated and efficient computer algorithms. For instance, PLS methods have been used in the analysis of relationships between measures of brain activity and behavior (Krishnan et al., 2011).
Several parameters of semen quality can influence the success of AI programs (Sá Filho et al., 2009); it is therefore interesting to note that cows inseminated with the 10th straws had overall lower conception rates (44%) than cows inseminated with the 1st straws (58%). This result demonstrates that the sequence of insemination may affect conception rate, which justifies the separate PLS analyses of 1st and 10th straw trials.

It is also interesting to note that several sperm parameters were found to be important predictors of fertility (TM, PM, TM₂ h, PM₂ h, BCF, 2 h, RAP, 2 h) in both sperm motility tests (CASA evaluation at 0 h and TRT 2 h). Farrell et al. (1998) similarly demonstrated that multiple combinations of CASA variables had higher correlations with bull fertility than single CASA parameters; for instance, the single sperm parameter Total Motility was weakly correlated with field fertility ($r^2 = 0.34$), while the combination of Progressive Motility, ALH and BCF was strongly correlated ($r^2 = 0.83$) and the combination of Progressive Motility, ALH, BCF and VSL was even more strongly correlated ($r^2 = 0.89$). In this study, we confirmed that sperm motility evaluations are important for the assessment of semen quality, especially because CASA allows for the highly repeatable assessment of multiple sperm motility characteristics (Farrell et al., 1998; Verstegen et al., 2002). Interestingly, the post-thaw sperm longevity CASA after TRT 2 h assay seemed to identify more sperm variables as good predictors of in vivo semen fertility than did the CASA assessment of frozen-thawed semen performed before the thermal incubation.

Interesting results were also obtained for the assessment of sperm membrane integrity. Plasma membrane functionality measured by the hypotonic swelling test (HOST + cells), plasma membrane integrity measured by PI and acrosomal membrane integrity measured by FITC-PSA (IPIA cells) were considered good predictors of conception rate. In contrast, Brito et al. (2003) found no significant correlation between bovine IVF and plasma membrane integrity measured by Eosin/Negrosin staining, CFDA/PI, SYBR-14/PI and HOST. However, in agreement with our results, Januskauskas et al. (2003) detected significant correlations between field fertility and plasma membrane integrity assessed by PI. Tartaglione and Ritta (2004) also demonstrated that the combination of Eosin/Negrosin staining test and HOST was highly correlated with in vitro fertility; these authors further showed that when sperm plasma and acrosomal membrane integrity (assessed by Trypan/Blue Giemsa staining) were included in the regression model, a higher correlation coefficient was obtained. Overall, therefore, investigators should realize that the capacity for predicting semen fertility is higher when a higher number of sperm evaluations are performed.

In an elegant study by Kasimanickam et al. (2007), the competitive index of bull fertility was found to be positively correlated with plasma membrane integrity and total progressive motility. According to the authors, plasma membrane integrity significantly influenced the fertilizing capacity of a sire; however, plasma membrane integrity and progressive motility were found to be negatively correlated with sperm lipid peroxidation, and lipid peroxidation was found to be negatively correlated with bull fertility (Kasimanickam et al., 2007). In this study, the variable...
related to sperm lipid peroxidation (IPP) was identified as an important predictor of conception rate. In agreement with our results, negative correlations between lipid peroxidation and IVF fertilization rates have been reported in humans (Zabloudovsky et al., 1999). The elevated concentration of polyunsaturated fatty acids on sperm membranes contributes to the high susceptibility of sperm to oxidative damage (Kasimanickam et al., 2007). In addition, ROS have been reported to damage plasma membrane structures, thus impairing sperm function and motility (Aiitten et al., 2007). A high degree of sperm plasma membrane lipid destabilization (phospholipids disorder) may lead to functional capacitation, thus reducing sperm lifespan and fertilizing capacity (Mortimer and Maxwell, 2004; Lydka et al., 2011).

The morphological variables identified as important predictors of fertilization were sperm major and total defects, width/length ratio (WLR) and Fourier 0 and 2. It has been reported that low-fertility bulls generally have a high seminal content of morphologically abnormal cells (Saacke, 2008). Sperm with classically misshapen heads cannot access the egg following AI because they do not traverse the female reproductive tract and/or participate in fertilization (Saacke et al., 1998). Even small geometrical differences in head morphology can cause large differences in sperm hydrodynamics (Dresdner and Katz, 1981). The WLR, which also represents sperm ellipticity, is a factor derived from the basic morphometric measurements of sperm head (Beletti et al., 2005). The Fourier parameters, which were also selected as important predictors of conception rate, characterize the curvilinear perimeter of sperm head using harmonic amplitudes to describe the sperm nuclear shape (Ostermeier et al., 2001). Ostermeier et al. (2001) investigated the relationship between sire fertility and Fourier parameters of sperm morphometric analysis and observed that Fourier descriptors were able to detect small differences in sperm nuclear shape. Therefore, according to Saacke (2008), Fourier harmonic amplitude analysis is the most promising method of quantifying changes in sperm head shape.

For the assessment of chromatin structure, the coefficient of variation (CV) of gray level intensity for each sperm head (which indicates sperm chromatin heterogeneity) was identified as an important predictor of conception rate. Acevedo et al. (2002) reported that the vulnerability of sperm DNA to acid denaturation was positively associated with the occurrence of misshapen sperm heads, suggesting that the occurrence of misshapen heads can signal the presence of sperm chromatin abnormalities and a potential inability to fertilize. Kasimanickam et al. (2007) reported that certain deleterious effects of sperm lipid peroxidation are also related to impairment of sperm DNA, which may reduce bull fertility. Sires with high sperm DNA fragmentation indices were found to have low sperm fertilization potential, while sires with low DNA fragmentation indices were determined to have higher chances of siring calves (Kasimanickam et al., 2007).

While we were able to explore important relationships between in vivo bull fertility and in vitro sperm characteristics in this study, it is worth mentioning that timed-AI programs have varied outcomes and that only nine batches of semen and a small number of cows were used. Therefore, the sperm variables identified as important predictors of conception rate should be re-evaluated with a larger number of cows, sires and batches, preferably using bulls with particularly high and low fertility rates to confirm and/or improve the accuracy of our results.

5. Conclusions

We concluded that PLS is a suitable statistical method for exploring and summarizing the relationship between in vitro sperm characteristics and field fertility results. Using this statistical method, it was possible to identify the sperm variables most highly related with bull fertility. The variables TM, PM, TM_2 h, PM_2 h, BCF_2 h, RAP_2 h, HOST, IPIA, IPP, Maj_Def, Tot_Def, WLR, Fourier_2, Fourier_2 and Chromatin Heterogeneity were selected as important predictors of conception rate based on the field results of a bovine timed-AI program.

It is important to note that, despite the intense efforts of researchers around the world, no single laboratory test has accurately predicted the true fertilizing capacity of a semen sample. Our results highlight the fact that the evaluation of a combination of in vitro sperm characteristics can more accurately predict semen quality than the evaluation of a single parameter. Nevertheless, further studies are needed to contribute to the understanding of how differences in bull semen are related to differences in fertility rates obtained in timed-AI programs.

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