Livestock Science 146 (2012) 38–46

Contents lists available at SciVerse ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Assessment of field fertility and several in vitro sperm characteristics following the use of different Angus sires in a timed-AI program with suckled Nelore cows

Letícia Zoccolaro Oliveira [a,*], Rubens Paes de Arruda [b], André Furugen Cesar de Andrade [b], Eneiva Carla Carvalho Celeghini [b], Ricarda Maria dos Santos [c], Marcelo Emílio Beletti [c], Rogério Fonseca Guimarães Peres [d], Clara Slade Oliveira [a], Vera Fernanda Martins Hossepian de Lima [a]

[a] Department of Preventive Veterinary Medicine and Animal Reproduction, School of Agrarian Sciences and Veterinary Medicine, São Paulo State University (UNESP), Jaboticabal, SP, 14884-900, Brazil
[b] Department of Animal Reproduction, School of Veterinary Medicine and Animal Science, University of São Paulo (USP), Pirassununga, SP, 13630-000, Brazil
[c] School of Veterinary Medicine (FAMEV), Federal University of Uberlândia (UFU), Uberlândia, MG, 38400-902, Brazil
[d] Agropecuária Fazenda Brasil (AFB), Nova Xavantina, MT, 78690-000, Brazil

ARTICLE INFO

Article history:
Received 26 September 2011
Received in revised form 15 February 2012
Accepted 16 February 2012

Keywords:
Bull fertility
Conception rate
Timed-AI
Sperm characteristics

ABSTRACT

In order to provide information that may help researchers to understand the main cause(s) of differences in bull fertility frequently observed in field trials, this study aimed to investigate conception rates as well as several in vitro sperm characteristics of different sires of unknown fertility utilized in a Timed-AI (TAI) program. Suckled Nelore cows submitted to the same TAI protocol were allocated into eight breeding groups of approximately 120 animals each. Frozen semen doses from three Angus bulls and three different batches from each bull were utilized. Approximately 100 doses from each batch were used in TAI. Sires, batches and AI technicians were equally distributed across breeding groups. Cows were examined for pregnancy diagnosis 40 d after TAI. For in vitro sperm analyses, the same thawing procedure was repeated in the laboratory to mimic field conditions. The following in vitro sperm characteristics were assessed: computerized motility, thermal resistance, plasma and acrosomal membrane integrity, lipid peroxidation, morphology, morphometry and chromatin structure. No effect of breeding group, body condition score, AI technician and sire was observed. However, some significant differences among bulls were detected in laboratory analyses. Semen from sire presenting numerically lower ($P>0.05$) pregnancy/AI also presented lower ($P<0.05$) values in all sperm characteristics analyzed in thermal resistance test at 4 h (Total Motility, Progressive Motility, Average Path Velocity, Straight-Line Velocity, Curvilinear Velocity, Amplitude of Lateral Head Displacement, Beat Cross Frequency, Straightness, Linearity, and Percentage of Rapidly Moving Cells), higher ($P<0.05$) Major and Total Defects in sperm morphological test, lower ($P<0.05$) Length, Ellipticity and Fourier parameter (Fourier 0) in sperm morphometric analysis as well as higher ($P<0.05$) chromatin heterogeneity. It was concluded that, although no bull effect was observed in the field experiment, the sire that presented numerically lower pregnancy/AI also presented lower semen quality according to the laboratory analyses performed.

© 2012 Elsevier B.V. All rights reserved.

* Corresponding author at: Universidade Estadual Paulista (FCAV/UNESP Jaboticabal), Via de Acesso Professor Paulo Donato Castellane s/n (Departamento de Med. Vet. Preventiva e Reprodução Animal), Zip Code: 14884-900, Jaboticabal, SP, Brazil. Tel.: + 55 16 3209 2633; fax: + 55 16 3202 500.
E-mail address: leticiazoccolaro@yahoo.com.br (L.Z. Oliveira).

1871-1413/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.livsci.2012.02.018

1. Introduction

Timed artificial insemination (TAI) programs have been widely used as a reproductive management tool in beef and dairy farms (Bó et al., 2003; Meneghetti et al., 2009; Pursley et al., 1995; Sá Filho et al., 2009). These protocols allow simultaneous management of a large number of cows reducing labor and animal handling (Bó et al., 2003; Pursley et al., 1995). However, many causes may account for the range in results and/or any unsatisfactory indices of bovine TAI programs, highlighting several factors inherent to female physiology as body condition score (BCS), animal breed, animal category (heifers or cows, primiparous or multiparous, anoestrous or cycling animals), suckling, size of ovulatory follicle and timing of insemination relative to ovulation (Meneghetti et al., 2009; Perry et al., 2007; Sá Filho et al., 2009).

Another factor positively correlated with the success of TAI protocols, but that has not been receiving appropriate attention, correspond to quality of semen samples used in the programs. It has been established that individual bulls differ in their ability to fertilize oocytes and/or to develop to blastocyst stages following in vitro fertilization (IVF) procedures (Hillery et al., 1990; Sudano et al., 2011; Wei and Fukui, 1999; Zhang et al., 1997). In addition, a marked variability in field fertility among bulls taken individually has been reported (Andersson et al. 2004; Correa et al., 1997, Ward et al. 2001), the so called "bull effect".

Many in vitro methods have been used to determine bull fertility (Aitken et al. 2007; Beletti et al., 2005; Celeghini et al., 2007; Correa et al., 1997; Januskauskas et al., 2003; Ostermeier et al., 2001; Revell and Mrode, 1994; Tartaglione and Ritta, 2004; Verstegen et al., 2002), but the results of such assays do not always correlate with field outcomes (Sudano et al., 2011). Nevertheless, considering the complexity of events involved in the fertilization process, it is unlikely that a single sperm characteristic may reflect the real fertilization capacity of a semen sample. Hence, it is reasonable to consider that the fertility potential of a bull can be better estimated when a combination of in vitro sperm assays is performed.

Even though many factors inherent to semen quality can influence the results of a TAI (and AI) program, no studies were found in which a detailed in vitro assessment of sperm characteristics has been performed in order to specifically explore the quality of semen used in a TAI program. Thus, to provide information that may help researchers to understand the main cause(s) of differences in bull fertility commonly found in field trials, this study aimed to investigate conception rates as well as several in vitro sperm characteristics of different sires of unknown fertility utilized in the same TAI program.

2. Material and methods

2.1. Assessment of field fertility — field experiment

2.1.1. Animals and management

A total of 944 suckled multiparous Nelore cows from a commercial beef farm located in state of Mato Grosso, Brazil, were utilized in this study. All cows were maintained on *Brachiaria brizantha* or *Brachiaria decumbens* pasture and given mineralized salt and free access to water. Data were collected from November of 2010 to January of 2011 during Brazilian spring–summer breeding season. Cows presented BCS between 1.75 and 3.25 in a 1 to 5 scale (1 = emaciated, 5 = obese).

2.1.2. Reproductive management

After calving, suckled cows were allocated into 8 breeding groups of approximately 120 animals each, according to calving date. All cows receive the same TAI protocol beginning 30 to 40 d postpartum. The TAI protocol started with cows receiving a new or second use intravaginal releasing device containing 1.0 g progesterone (Sincrogest®, Ouro Fino Saúde Animal, Cravinhos, Brazil) and an i.m. injection of 2.0 mg of estradiol benzoate (EB; Sincrodiol®, Ouro Fino Saúde Animal, Cravinhos, Brazil). Progesterone device was removed 8 d later and animals received i.m. injections of: 500 μg of D-cloprostenol (PGF2α; Sincrocio®, Ouro Fino Saúde Animal, Cravinhos, Brazil), 300 UI of eCG (Novormon 5000®, Intervet Schering Plough Saúde Animal, São Paulo, Brazil) and 0.5 mg of estradiol cypionate (ECP®, Pfizer Saúde Animal, São Paulo, Brazil).

Cows were TAI by two experienced AI technicians 2 d after removal of progesterone device. Technicians inseminated the same number of cows and alternated inseminations between cows. Type of progesterone device utilized (new or second use), BCS (1 to 5) and AI technician were recorded for every cow.

Worth mentioning that, after progesterone device withdrawal (i.e., after device being used for 8 d in a random cow), the device was washed, disinfected, air dried and stored in a paper bag until to be used for a second time in another random cow.

The utilization of reused progesterone devices is a routine practice in Brazilian TAI programs. According to Maio et al. (2008) and Maturana Filho et al. (2010), Sincrogest® kept plasmatic progesterone concentrations over than 1 ng/mL during treatment (8 d) with 1st and 2nd use devices. This information support that second use Sincrogest® device can be utilized in synchronization of ovulation protocols for bovine TAI. For progesterone device disinfection, the following procedure was performed: after being removed from the cows, the progesterone device was washed with running water in order to remove vaginal mucus and any other debris on the device. Then, it was immersed in a bath solution of Biocid® (Pfizer Saúde Animal, São Paulo, Brazil) 1% (10 mL of Biocid® diluted in 1 L of water) during 5 min, rinsed again in running water and allowed to be air dried in a place protected from sun light.

2.1.3. Field experimental design

Frozen semen doses from three Angus bulls of same semen company and diluted in the same lactose based extender were utilized. Three batches from each bull and approximately 100 semen doses from each batch were used in the study.

Ten 0.5-mL frozen straws were thawed simultaneously in a thermostatically controlled thawing bath (Fertilize®, Fertilize, Uberaba, Brazil), in a temperature set on 36 °C, for 30 s, being utilized one straw for each cow at TAI. Sequence of

insemination (first, second, third, until tenth) and time (h and min) of semen removal from water bath during AI of each cow were recorded.

The Brazilian Association of Artificial Insemination recommends the thawing procedure of a single frozen semen straw in water bath at a temperature of 35 to 37 °C during 30 s for bovine AI. However, the large size of breeding herds using TAI protocols in Brazil have resulted in the routine practice of thawing simultaneously multiple straws of semen in the same water-bath unit to increase the convenience of semen handling and the number of inseminations in a short period. In the present study, a group of 10 straws was thawed together because we applied the exact same thawing and TAI procedures of the farm where the experiment was conducted. According to the field experiment, the mean time that all straws were put in the water bath until the removal of the last thawed straw from water bath was 6 min and 29 s, considering the minimum thawing period of 30 s as time 0. In order to guarantee a randomized experimental design and a balanced number of animals per field variable, the experiment was designed in a manner which semen from each of the 3 bulls was equally distributed in the breeding groups, AI technician, type of progesterone releasing device (new or second use) and straw sequence.

All cows were examined for pregnancy by transrectal ultrasonography 40 d after TAI. Detection of an embryonic vesicle with a viable embryo (presence of heartbeat) was used as an indicator of pregnancy.

2.2. Laboratory assessment of semen quality — laboratory experiment

2.2.1. Laboratory experimental design

Frozen semen samples from each bull (n = 3) and batch (n = 3) utilized in field trial were brought to the laboratory. For each semen batch, the 0.5-mL frozen straws were thawed in an identical thermostatically controlled thawing bath (Fertilize®), in a temperature of 36 °C, for 30 s. After 30 s, two semen straws were removed from water bath and semen from this two-straw pair was pooled in a microcentrifuge tube. The following in vitro sperm characteristics were assessed: sperm motility, sperm thermal resistance, plasma and acrosomal membrane integrity, lipid peroxidation, chromatin structure and sperm morphometry.

2.2.2. Computer-assisted semen analysis (CASA)

Sperm motility was assessed by CASA (Ivos-Ultimate®; Hamilton Thorne Biosciences, Beverly, USA). However, samples utilized in this experiment were all cryopreserved in lactose based extender. Thus, to make sperm more visible in such media, semen samples were stained with Hoechst 33342 (H342) dye (H-1399; Molecular Probes Inc, Eugene, USA).

CASA set-up was pre-adjusted for bovine sperm analysis (Celeghini et al., 2008) in IDENT option. For assessment of computer assisted sperm motility, an aliquot of 100 μL of frozen-thawed semen was put into a warmed microcentrifuge tube and 2 μL of H342 (40 μg/mL) was added. The semen-dye mixture was incubated for 20 min at 37 °C. After incubation, 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: 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).

2.2.3. Sperm thermal resistance test (TRT)

An aliquot of 250 μL of frozen-thawed semen was put into a warmed microcentrifuge tube which remained incubated at 37 °C. After 240 min of incubation (TRT 4 h), the same procedure described on Section 2.2.2 for CASA was applied. The following motility parameters were assessed: TM_4h, PM_4h, VAP_4h, VSL_4h, VCL_4h, ALH_4h, BCF_4h, STR_4h, LIN_4h, RAPID_4h.

2.2.4. Hypo-osmotic swelling test (HOST)

HOST was performed by incubating 20 μl of semen with 1 mL of a 100 mOsm hypoosmotic solution (Revell and Mrode, 1994) at 37 °C for 60 min. After incubation, an aliquot of 20 μl of semen diluted in hypoosmotic solution was placed on a glass slide, covered with a coverslip and evaluated by contrast phase microscopy.

Two hundred sperm were evaluated under magnification 1000×. Sperm with swollen or coiled tails were considered viable. Percentage of viable sperm (HOST + cells) was calculated according to Revell and Mrode (1994).

2.2.5. Flow cytometry analyses

Maintenance of sperm fertilizing potential depends on the integrity and functionality of different cellular structures (Õura and Toshimori 1990). Thus, the assessment of different aspects related to sperm physiology is needed for a superior semen evaluation (Arruda et al., 2011; Celeghini et al., 2007). In this sense, fluorescent probes have been widely used alone or in combination in order to evaluate, by epifluorescence microscopy, the integrity and functionality of specific compartments of sperm cells (Celeghini et al., 2007; Celeghini et al., 2008; Oliveira et al., 2012). However, in general, the number of examined sperm in each analysis does not exceed 500 sperm cells. Hence, the flow cytometry arises as an advantageous technique for sperm assessment, since this automated system has the ability to examine around 30,000 cells in a minute, allowing more accurate results in a short period (Arruda et al., 2011; De Andrade et al., 2011; Leite et al., 2010). In the present study, flow cytometry analysis was used for simultaneous assessment of plasma and acrosomal membranes and also for evaluation of lipid peroxidation of semen samples.

These analyses were carried out using FACSaria® (Becton-Dickinson, Sunnyvale, 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). Flow cytometer was calibrated as previously described (Celeghini et al., 2007; 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 $MgCl_2.6H_2O$, 0.4 mM $NaH_2PO_4.H_2O$, 5 mM glucose, 10 mM sodium lactate, 0.1 mM sodium pyruvate and 10,000 UI/100 mL sodium penicillin. The pH of medium was adjusted using 1 N NaOH until pH was 7.4. After addition of the dyes for each analysis, semen samples incubated in TALPm were analyzed in flow cytometer, which was controlled by BD FACSDiva 6.0 software (Becton Dickinson, San Jose, USA) as described by Leite et al. (2010).

2.2.5.1. Simultaneous assessment of plasma and acrosomal membranes. The plasma membrane is responsible for cellular osmotic equilibrium and homeostasis. Consequently, plasma membrane integrity is crucial to sperm survival inside the female reproductive tract, and to maintain fertilizing capability (Õura and Toshimori, 1990). Propidium iodide (PI) is a fluorescent probe with DNA specificity that binds to DNA of sperm cells possessing a damaged plasma membrane (Celeghini et al., 2007). In addition, acrosome integrity is essential to the occurrence of acrosome reaction, which is also a critical event to sperm fertilization (Õura and Toshimori 1990). The most commonly applied technique to evaluate acrosome integrity uses glycoprotein markers such as lectins (Celeghini et al., 2007). The Pisum sativum agglutinin (PSA) is a substance that binds specifically to the sugar a-mannoside found in the acrosomal matrix. This agglutinin, when bound to fluorescein isothiocyanate (FITC), marks damaged sperm acrosome in yellow-green (Celeghini et al., 2007; Cross et al. 1986).

To evaluate cells with intact plasma membranes as well as those that had undergone acrosome reaction, an aliquot was taken from the samples and added to TALPm. The resulting samples had a concentration of 5×10^6 spermatozoa/mL in a volume of 148 μL. Then, 2 μL of H342 (40 μg/mL) was added to stain the DNA of sperm cells so that particles with the same scatter properties as spermatozoa 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, St Louis, USA) and 10 μL of Pisum sativum agglutinin conjugated to fluorescein isothiocyanate (FITC-PSA, 100 μg/mL; L-0770; Sigma–Aldrich) were added to the samples (Celeghini et al. 2007). After 10 min of incubation at 37 °C, samples were diluted with the addition of 150 μL of TALPm to a concentration of 2.5×10^6 spermatozoa/mL and were analyzed by flow cytometer (De Andrade et al., 2011).

Two-dimensional dot-plots of FITC-PSA (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) 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). Percentage of sperm cells presenting intact plasma membranes (IPM: IPIA + IPDA) and percentage of sperm cells presenting intact acrosome (IA: IPIA + DPIA) were also calculated.

2.2.5.2. Evaluation of lipid peroxidation. Oxidative stress is a recognized contributor to defective sperm function (Aitken et al., 2007; Brouwers and Gadella, 2003; Kasimanickam et al., 2007; Neild et al., 2005). Spermatozoa is very susceptible to peroxidative damage because of their high cellular content of polyunsaturated fatty acids that are particularly vulnerable to this form of stress (Aitken et al., 2007). Recently, a fluorescence assay using the fluorophore 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY$^{581/591}$) has been successfully applied for detecting lipid peroxide formation in living bovine sperm cells (Brouwers and Gadella, 2003). This assay relies on the sensitivity of C11-BODIPY$^{581/591}$, a fluorescent fatty acid conjugate, which readily incorporates into biological membranes (Neild et al., 2005). Upon exposure to reactive oxygen species (ROS), the C11-BODIPY$^{581/591}$ responds to free radical attack with a irreversible shift in spectral emission from red to green that can be quantified by flow cytometry (Aitken et al., 2007; Neild et al., 2005).

In the present study, for assessment of lipid peroxidation, an aliquot was taken from the samples and added to TALPm to obtain samples with a concentration of 5×10^6 spermatozoa/mL and a final volume of 499.5 μL. Then, the fluorescent probe C11-BODIPY$^{581/591}$ (1 mg/mL, D-3861, Molecular Probes Inc., Eugene, USA) was added. The sample was incubated for 30 min at 37 °C. After this incubation period, 145 μL of this solution was transferred to another microtube and 2 μL of H342 (40 μg/mL) was added. This sample was incubated for 10 min at 37 °C. The H342 probe was used in order to avoid that particles presenting same size and/or granularity of sperm cells were included in the counting. After incubation with H342, 3 μL of PI (0.5 mg/mL) was added in order to identify cells with damaged plasma membrane. Then, the sample was incubated with PI for 5 min at 37 °C. Subsequently, the sample was diluted with TALPm to a concentration of 2.5×10^6 spermatozoa/mL and was analyzed by flow cytometry.

Two-dimensional dot-plots of C11-BODIPY$^{581/591}$ (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 lipid peroxidation; (2) IPNP: sperm with intact plasma membrane with no lipid peroxidation detected; (3) DPP: sperm with damaged plasma membrane and lipid peroxidation; and (4) DPNP: sperm with damaged plasma membrane and no lipid peroxidation detected. Positive controls were obtained after addition of 80 μM ferrous sulfate to additional sperm suspensions (Aitken et al. 2007).

2.2.6. Assessment of sperm morphology

To assess sperm morphology, semen samples were diluted and fixed in pre-warmed (37 °C) formaldehyde-PBS. Sperm cells (n = 200) were counted under differential interference-contrast microscopy (model 80i; Nikon, Tokyo, Japan) at a magnification of 1000×. Sperm Morphological characteristics were classified as major, minor and total defects according to Blom (1973).

2.2.7. Assessment of sperm chromatin structure and morphometry

For assessment of sperm chromatin structure and morphometry, two smears were prepared for each sample. Sperm smears were fixed with ethanol acetic acid (3:1, V/V) for 1 min and 70% ethanol for 3 min. Then, the smears were hydrolyzed for 25 min in 4 M HCl, washed in distilled water and air-dried. One droplet of 0.025% toluidine blue in McIlvaine buffer (sodium citrate-phosphate), pH 4.0, was placed over each smear and then 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, USA) with a ×100 objective lens (immersion) coupled to a Leica ICC50 camera (Leica Microsystems Inc., Buffalo Grove, USA) that was connected to a PC microcomputer. Using threshold-based image segmentation (Beletti and Costa, 2003), at least 100 sperm heads were isolated for each smear. After head segmentation, which was done using algorithms developed in SCILAB environment (SIP toolbox), sperm heads were analyzed to obtain the average pixel value that made up each head. Six heads with the smallest pixel values were selected automatically and defined as standard heads. These heads, theoretically, are the heads presenting most condensed chromatin. Subsequently, for each image, the difference between standard value of the smear and average value of each head analyzed was determined. This difference was transformed into a percentage (Dif;%) of the average pixel value for standard heads, which indicates sperm chromatin decondensation. Coefficient of variation (CV) of the gray level intensity for each head, which indicates sperm chromatin heterogeneity, was also calculated (Beletti et al., 2005; Kanayama and Beletti, 2011).

Area, Perimeter, Width, Length, Width:length ratio and Ellipticity of all sperm heads were determined using other algorithm developed in SCILAB (Beletti and Costa, 2003; Beletti et al., 2005). Fourier descriptors containing harmonic amplitudes from 0 to 2 (Fourier 0, 1 and 2) were also considered. Another estimated feature was sperm head Side Symmetry which 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).

2.2.8. Statistical analysis of field experiment

Pregnancy per AI 40 d after AI was analyzed using GLIMMIX procedure of SAS (version 9.2, SAS Inst. Inc., Cary, NC). Breeding group (1 to 8), BCS (1.75 to 3.25), progesterone intravaginal device (new or second use), AI technician (1 and 2) and Sire (Bulls 1, 2 and 3) were included in the model and $P<0.05$ was considered significant. Since all animals inseminated were multiparous Nelore cows with similar age, parity and age were not considered in the analysis.

2.2.9. Statistical analysis of laboratory experiment

Results obtained from all variables of laboratory analyses were tested for normality of residues and homogeneity of variance. Dependent variables that did not meet statistical premises were submitted to arcsine transformation. For each bull, data from the three batches were considered. These data were submitted for an analysis of variance (PROC GLM) and differences among Sires were separated using Tukey test. The results are presented as mean ± standard deviation, and $P<0.05$ was considered significant.

3. Results and discussion

The success of bovine AI programs depends on the use of good quality semen and better conception rates can be achieved using animals with high fertility during the breeding season (Sudano et al., 2011). In beef cows, suckling, negative energy balance, BCS and parity of cows are important factors affecting pregnancy rates (Meneghetti et al., 2009). In addition, breeding groups, hormonal treatments and AI technician are critically important to TAI outcomes (Sá Filho et al., 2009). According to pregnancy per AI on day 40, no effect of breeding group ($P=0.7658$), BCS ($P=0.4485$), AI technician ($P=0.4312$), progesterone device ($P=0.2078$) and sire ($P=0.4518$) was observed. However, some significant differences among bulls were observed in the laboratory analyses. Results regarding conception rates and in vitro motility characteristics of the three bulls utilized in the present TAI program are demonstrated in Table 1.

Female physiology factors influencing the success of bovine TAI have been broadly studied (Meneghetti et al., 2009; Perry et al., 2007; Sá Filho et al., 2009). In the present experiment, since no effects of BCS, breeding group, progesterone device and AI technician were observed, it can be stated that the TAI variables were very well distributed across the breeding groups.

Regarding the quality of semen used in AI programs, it has been reported that differences in fertility level could be attributed to variations in sperm qualitative characteristics (Correa et al., 1997). Ward et al. (2001) demonstrated that kinetics of embryo development post insemination may vary between bulls. Andersson et al. (2004) observed a high variability in fertility among bulls using different sperm concentrations per dose at AI. In the present field experiment, though, no significant effect of sire was observed. Since good conception rates were obtained and only numerical differences ($P>0.05$) were observed in pregnancy/AI among bulls, it can be implied that only sires with similar high fertility were utilized in this study. Nevertheless, significant differences among sires were observed in some in vitro sperm characteristics.

CASA provides an opportunity to assess multiple characteristics on a large sample of spermatozoa with high repeatability (Farrell et al., 1998; Verstegen et al. 2002). According to Farrell et al. (1998) multiple combinations of motility variables, as PM, BCF, LIN, VAP, VSL and VCL, presented high correlation with field fertility. In the present study, intriguing results were obtained in CASA evaluation at 0 h since lower ($P<0.05$) values for VSL, STR and LIN were observed in the bull that presented higher numeric ($P>0.05$) field fertility (Bull 3). However, interestingly, although only a numerically lower ($P>0.05$) conception rate was observed in Bull 2, a tendency for lower ($P=0.07$) TM was also observed for this sire. In this sense, Correa et al. (1997) observed that the total number of motile spermatozoa tended to be higher in high fertility bulls.

Table 1
Field fertility and in vitro motility parameters (obtained at 0 h and at 4 h of incubation after TRT) from semen of three sires utilized in a TAI program of Nelore suckled cows.

	Bull 1	Bull 2	Bull 3	P value
Conception rate (%) (n/n)	49.13 (142/289)	47.59 (148/311)	52.74 (183/347)	0.4518
TM(%)	44.53 ± 8.83	29.40 ± 8.91	43.59 ± 15.32	0.0669
PM (%)	36.87 ± 10.56	24.72 ± 7.04	30.28 ± 9.66	0.1213
VAP (μm/s)	62.75 ± 8.04	63.15 ± 4.59	56.56 ± 3.47	0.1144
VSL (μm/s)	56.71 ± 7.99[a]	56.54 ± 3.43[a]	48.60 ± 3.56[b]	0.0318
VCL (μm/s)	89.46 ± 9.69	93.24 ± 6.59	87.42 ± 7.42	0.4603
ALH (μm)	3.94 ± 0.22	4.12 ± 0.25	4.45 ± 0.63	0.1327
BCF (Hz)	25.04 ± 3.25	24.90 ± 3.44	24.80 ± 4.07	0.9936
STR (%)	90.49 ± 1.95[a]	89.99 ± 1.59[a]	86.56 ± 3.92[b]	0.0478
LIN (%)	66.17 ± 3.79[a]	63.71 ± 1.93[ab]	58.54 ± 6.15[b]	0.0236
RAPID (%)	38.28 ± 10.93	25.63 ± 7.35	33.24 ± 11.53	0.1384
TM_4h (%)	6.80 ± 4.74[a]	0.66 ± 0.62[b]	11.50 ± 4.64[a]	<0.0001
PM_4h (%)	4.36 ± 3.80[a]	0.26 ± 0.28[b]	6.78 ± 2.53[a]	0.0001
VAP_4h (μm/s)	47.00 ± 4.14[a]	24.20 ± 20.30[b]	50.51 ± 4.50[a]	0.0065
VSL_4h (μm/s)	42.18 ± 4.08[a]	20.34 ± 16.96[b]	40.82 ± 3.63[a]	0.0054
VCL_4h (μm/s)	71.32 ± 4.28[a]	42.68 ± 36.77[b]	87.90 ± 5.40[a]	0.0142
ALH_4h (μm)	3.28 ± 0.55[a]	1.05 ± 1.31[b]	5.04 ± 0.15[c]	<0.0001
BCF_4h (Hz)	19.26 ± 2.40[a]	10.12 ± 9.26[b]	20.84 ± 3.24[a]	0.0184
STR_4h (%)	89.72 ± 1.55[a]	45.98 ± 41.47[b]	81.78 ± 2.46[a]	0.0238
LIN_4h (%)	61.69 ± 4.18[a]	26.87 ± 24.41[b]	49.42 ± 2.25[a]	0.0117
RAPID_4h (%)	4.48 ± 3.86[a]	0.26 ± 0.28[b]	7.59 ± 2.86[a]	<0.0001

h: hour; TRT: sperm thermal resistance test; TAI: timed artificial insemination; TM: Total Motility; PM: Progressive Motility; VAP: Average Path Velocity; VSL: Straight-Line Velocity; VCL: Curvilinear Velocity, ALH: Amplitude of Lateral Head Displacement; BCF: Beat Cross Frequency; STR: Straightness; LIN: Linearity; RAPID: Percentage of Rapidly Moving Cells; TM_4h: TM after 4 h in TRT; PM_4h: PM after 4 h in TRT; VAP_4h: VAP after 4 h in TRT; VSL_4h: VSL after 4 h in TRT; VCL_4h: VCL after 4 h in TRT, ALH_4h: ALH after 4 h in TRT; BCF_4h: BCF after 4 h in TRT; STR_4h: STR after 4 h in TRT; LIN_4h: LIN after 4 h in TRT; RAPID_4h: RAPID after 4 h in TRT; [a,b] within a row, values with different superscript letters indicates ($P<0.05$).

The 4-h incubation period of TRT was intended to maximize the chance of occurrence of latent injury to spermatozoa and/or to evaluate possible differences in thermal resistance of the three sires utilized in TAI. It is interesting to note that all parameters assessed in TRT_4h (TM_4h, PM_4h, VAP_4h, VSL_4h, VCL_4h, ALH_4h, BCF_4h, STR_4h, LIN_4h, RAPID_4h) were lower ($P<0.05$) for Bull 2. These findings suggest that frozen semen from Bull 2 seems to be more susceptible to latent damage after in vitro thermal incubation, compared to Bulls 1 and 3. However, the assumption that this sire (Bull 2) would present lower capacity of sperm survival within the female reproductive tract was not confirmed, since there were no evident differences in the conception rate among the three bulls in the field trial of the present experiment.

Even though computer-based analysis provides high accuracy of in vitro motility evaluation (Farrell et al., 1998; Verstegen et al., 2002), the assessment of different aspects related to sperm physiology may guarantee better investigation of semen quality (Arruda et al., 2011; Celeghini et al., 2007). The laboratory results of sperm integrity, functionality, oxidative stress and morphology of the three bulls utilized in the present TAI program are demonstrated in Table 2.

Regarding plasma and acrosomal membrane evaluations, no significant differences were observed among bulls. Worth mentioning, though, that Bull 2 also presented lower numeric values ($P>0.05$) for HOST + cells, IPIA, IPM and IA; which may be related to the lower numeric ($P>0.05$) conception rate observed for this bull at TAI. Supporting this, Januskauskas et al. (2003) found significant correlations between field fertility and plasma membrane integrity assessed by PI. Tartaglione and Ritta (2004) demonstrated that combination of Eosin/Negrosin staining test with HOST presented high correlation coefficient with in vitro fertility. When sperm plasma and acrosomal membrane integrity results were included in the regression model, a higher correlation coefficient was obtained (Tartaglione and Ritta, 2004).

Another concern of semen fertility studies is the occurrence of sperm oxidative stress. Spermatozoa are susceptible to oxidation of their plasma membranes due to the presence of polyunsaturated fatty acids (Aitken et al., 2007). Reactive oxygen species (ROS) inhibit sperm motion and become cytotoxic through damage to proteins, nucleic acids and membrane lipids if ROS concentrations overcome the natural defense mechanisms of the cell and extending medium (Storey, 1997). Kasimanickam et al. (2007) observed that bulls with higher sperm lipid peroxidation were more likely to have a high DNA fragmentation and low plasma membrane integrity. Also, these bulls presented lower chances of siring calves (Kasimanickam et al., 2007). In the lipid peroxidation assessment of present study, although no significant difference was observed among bulls, a tendency for higher percentage of IPP ($P=0.0821$) and DPP ($P=0.0751$) was detected in Bull 2, which might suggest also a higher susceptibility for oxidative stress in the semen of this specific bull.

It has also been reported that low-fertility bulls generally had high seminal content of morphologically abnormal cells (Saacke, 2008). Sperm with classically misshapen heads did not access the egg following AI since 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

Table 2
Laboratory results of hypoosmotic swelling test (HOST), flow cytometry analyses and sperm morphology evaluation from semen of three sires utilized in a TAI program of Nelore suckled cows.

In vitro assay	Variable (%)	Bull 1	Bull 2	Bull 3	P value
HOST	HOST + cells	34.33 ± 5.32	30.50 ± 13.22	44.67 ± 17.31	0.1782
Plasma and acrosomal membrane integrity by PI/FITC-PSA fluorescent probes	IPIA	38.60 ± 6.39	35.10 ± 5.66	44.63 ± 12.08	0.1917
	IPM	38.88 ± 6.56	35.37 ± 5.78	44.78 ± 12.13	0.1904
	IA	67.27 ± 15.12	65.68 ± 10.67	78.40 ± 15.59	0.1689
Lipid peroxidation by C11-BODIPY$^{581/591}$ fluorescent probe	DPNP	68.35 ± 5.41	68.32 ± 6.31	58.97 ± 16.26	0.2975
	DPP	1.05 ± 0.60	2.75 ± 1.89	1.47 ± 1.16	0.0751
	IPNP	30.18 ± 5.11	27.62 ± 5.30	39.15 ± 15.59	0.1855
	IPP	0.43 ± 0.18	1.33 ± 1.40	0.43 ± 0.23	0.0821
Sperm morphology	Major defects	14.25 ± 3.09^{a}	25.33 ± 3.42^{b}	11.83 ± 3.04^{a}	<0.0001
	Minor defects	10.00 ± 3.86^{a}	9.67 ± 2.68^{a}	16.00 ± 4.52^{b}	0.0232
	Total defects	24.25 ± 3.60^{a}	35.00 ± 1.76^{b}	27.83 ± 6.93^{a}	0.0040

TAI: Timed artificial insemination; HOST + cells: percentage of viable sperm in the Hiposmotic swelling test; IPIA: sperm with intact plasma and acrosomal membranes; IPM: percentage of sperm cells presenting intact plasma membranes; IA: percentage of sperm cells presenting intact acrosome; DPNP: sperm with damaged plasma membrane and no lipid peroxidation detected; DPP: sperm with damaged plasma membrane and lipid peroxidation; IPNP: sperm with intact plasma membrane with no lipid peroxidation detected; IPP: sperm with intact plasma membrane suffering lipid peroxidation; ^{a,b} within a row, values with different superscript letters indicates ($P<0.05$).

hydrodynamics (Dresdner and Katz, 1981). Therefore, the higher ($P<0.05$) percentage of Major and Total Defects observed in Bull 2 may also have contributed to the numerically lower ($P>0.05$) conception rate of this bull at TAI.

According to Saacke (2008), abnormal-shaped heads should be of primary concern regarding male fertility. The recognition of uncompensable cells in the ejaculate is currently best based on abnormal levels of sperm with misshapen heads (Saacke, 2008). Quantifying changes in sperm head shape can be detected by Fourier parameters which characterize the curvilinear perimeter of sperm head using harmonic amplitudes to describe the sperm nuclear shape (Ostermeier et al., 2001; Saacke, 2008). The results regarding sperm morphometry and chromatin structure of the three sires utilized in the present TAI program are presented in Table 3.

Ostermeier et al. (2001) investigated the relationship between sire fertility and Fourier parameters and observed that Fourier descriptors were able to detect small differences in sperm nuclear shape from bulls with different fertility. Acevedo et al. (2002) reported that spermatogenic disturbance resulted in production of abnormal sperm and that sperm DNA vulnerability was positively associated with sperm having misshapen heads. This provided more support for the assertion that occurrence of sperm with misshapen heads can signal chromatin abnormalities and potential fertility incompetence of a semen sample (Saacke, 2008).

In view of that, Bull 2, which presented a numerically lower ($P>0.05$) pregnancy/AI, also presented lower values ($P<0.05$) for Length, Ellipticity and Fourier 0 compared with Bulls 1 and 3. Additionally, in Fourier 1 parameter, it can be observed a significantly lower value ($P<0.05$) for Bull 2 (lower numeric pregnancy/AI) compared with Bull 3 (higher numeric pregnancy/AI). Moreover, the results of sperm morphometry and chromatin structure analyses seemed to be related. A significantly higher ($P<0.05$) chromatin heterogeneity and a numerically higher ($P>0.05$) chromatin decondensation were observed in the sire presenting sperm morphometric alterations (Bull 2).

4. Conclusions

From the results it can be concluded that only high fertility sires were used in the present study, which contributed to the good conception rates obtained in the TAI program of suckled Nelore cows. Although no bull effect was observed

Table 3
Laboratory results of sperm chromatin structure and morphometry analysis from semen of three sires utilized in a TAI program of Nelore suckled cows.

	Bull 1	Bull 2	Bull 3	P value
Area (pixels)	5355.71 ± 419.82	5291.78 ± 134.28	5353.50 ± 152.44	0.8975
Perimeter (pixels)	23.96 ± 0.91	23.62 ± 0.25	23.96 ± 0.33	0.5126
Width (pixels)	4.68 ± 0.20	4.71 ± 0.09	4.66 ± 0.09	0.7855
Length (pixels)	8.98 ± 0.35^{ab}	8.77 ± 0.09^{a}	9.00 ± 0.12^{b}	0.0032
Width/length	0.521 ± 0.004^{a}	0.538 ± 0.007^{b}	0.518 ± 0.008^{a}	0.0003
Ellipticity	0.315 ± 0.004^{a}	0.301 ± 0.006^{b}	0.318 ± 0.007^{a}	0.0003
Fourier 0	3885.09 ± 282.82^{a}	3596.09 ± 60.59^{b}	3916.50 ± 118.64^{a}	0.0140
Fourier 1	433.54 ± 64.82^{ab}	434.03 ± 28.13^{a}	494.22 ± 35.97^{b}	0.0090
Fourier 2	325.03 ± 33.05	325.20 ± 24.47	329.89 ± 23.52	0.9409
Side Symmetry	0.972 ± 0.003	0.973 ± 0.001	0.974 ± 0.002	0.1558
Dif (%)	6.09 ± 2.89	7.92 ± 3.04	7.25 ± 4.65	0.6793
CV (%)	8.37 ± 3.33^{b}	10.86 ± 1.12^{a}	8.40 ± 1.88^{b}	0.0202

TAI: Timed artificial insemination; Dif: Chromatin decondensation is measured by percentage of gray-level differences; CV: Chromatin heterogeneity is measured by coefficient of variation of the gray-level; ^{a,b} within a row, values with different superscript letters indicates ($P<0.05$).

in the field experiment, the sire that presented numerically lower pregnancy/AI also presented lower semen quality according to the laboratory analyses performed. Although demonstrating similar field fertility, the bulls of the present experiment seem to differ in several in vitro sperm characteristics.

Yet, it is worth mentioning that only three bulls were used in the present experiment and additional experiments should be carried out using a larger number of bulls. Further studies contributing to the understanding of seminal differences that might be related to differences in sire fertility rates of TAI programs must be encouraged.

Conflict of interest

There is no conflict of interest for any of the authors.

Acknowledgments

The authors are grateful to Ouro Fino Saúde Animal for their support with the protocol and TAI hormones; and to Agropecuária Fazenda Brasil for allowing the use of their animals and facilities for this study. Also, the authors are grateful to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for financial support in form of grants and research assistance.

References

Acevedo, N.J., Bame, H., Kuehn, L.A., Hohenboken, W.D., Evenson, D.P., Saacke, R.G., 2002. Sperm chromatin structure assay (SCSA) and sperm morphology. In: Proc. 19th Tech. Conf. Artif. Insem. Reprod. 2002, 84–90.

Aitken, R., Wingate, J., De Iullis, G., Mclaughlin, E., 2007. Analysis of lipid peroxidation in human spermatozoa using BODIPY C11. Mol. Hum. Reprod. 13, 203–211.

Andersson, M., Taponena, J., Koskinena, E., Dahlbomb, M., 2004. Effect of insemination with doses of 2 or 15 million frozen–thawed spermatozoa and semen deposition site on pregnancy rate in dairy cows. Theriogenology 61, 1583–1588.

Arruda, R.P., Celeghini, E.C.C., Alonso, M.A., Carvalho, H.F., Oliveira, L.Z., Nascimento, J., Silva, D.F., Affonso, F.J., Lemes, K.M., Jaimes, J.D., 2011. Methods of the assessment of morphology and function of sperm: actual moment and future challenges. Rev. Bras. Reprod. Anim. 35, 145–151.

Beletti, M.E., Costa, L.F., 2003. A systematic approach tomulti-species sperm morphometrical characterization. Anal. Quant. Cytol. Histol. 25, 97–107.

Beletti, M.E., Costa, L.F., Guardieiro, M.M., 2005. Morphometric features and chromatin condensation abnormalities evaluated by toluidine blue staining in bull spermatozoa. Braz. J. Morphol. Sci. 22, 85–90.

Blom, E., 1973. The ultrastructure of some characteristic sperm defects and a proposal for a new classification of the bulls permiogram. Nord. Veterinar. Medicin. 25, 383–391.

Bó, G.A., Baruselli, P.S., Martinez, M.F., 2003. Pattern and manipulation of follicular development in *Bos indicus* cattle. Anim. Reprod. Sci. 78, 307–326.

Brouwers, J.F., Gadella, B.M., 2003. In situ detection and localization of lipid peroxidation in individual bovine sperm cells. Free Radic. Biol. Med. 35, 1382–1391.

Celeghini, E.C.C., Arruda, R.P., Andrade, A.F.C., Nascimento, J., Raphael, C.F., 2007. Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. Reprod. Domest. Anim. 42, 479–488.

Celeghini, E.C.C., Arruda, R.P., Andrade, A.F.C., Nascimento, J., Raphael, C.F., Rodrigues, P.H.M., 2008. Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin. Anim. Reprod. Sci. 104, 119–131.

Correa, J.R., Pace, M.M., Zavos, P.M., 1997. Relationships among frozen thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and the fertility of bulls in an artificial insemination program. Theriogenology 48, 721–731.

Cross, N.L., Morales, P., Overstreet, J.W., Hanson, F.W., 1986. Two simple methods for detecting acrosome-reacted human sperm. Gamete Res. 15, 213–226.

De Andrade, A.F.C., Zaffalon, F.G., Celeghini, E.C.C., Nascimento, J., Tarrago, O.F.B., Martins, S.M.M.K., Alonso, M.A., Arruda, R.P., 2011. Addition of seminal plasma to post-thawing equine semen: what is the effect on sperm cell viability? Reprod. Dom. Anim. 46, 682–686.

Dresdner, R.D., Katz, D.F., 1981. Relationship of mammalian sperm motility and morphology to hydrodynamic aspects of cell function. Biol. Reprod. 25, 920–930.

Farrell, P.B., Presicce, G.A., Brockett, C.C., Foote, R.H., 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology 49, 871–879.

Hillery, F.L., Parrish, J.J., First, N.L., 1990. Bull specific effect on fertilization and embryo development in vitro. Theriogenology 33, 249.

Januskauskas, A., Johannisson, A., Rodriguez-Martinez, H., 2003. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure and field fertility. Theriogenology 60, 743–758.

Kanayama, C.Y., Beletti, M.E., 2011. Computational evaluation of chromatin condensation and morphometric characteristics of rabbit (*Oryctolagus cuniculus*) sperm head. Arq. Bras. Med. Vet. Zootec. 63, 94–99.

Kasimanickam, R., Kasimanickam, V., Thatcher, C.D., Nebel, R.L., Cassell, B.G., 2007. Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters, and competitive index in dairy bulls. Theriogenology 67, 1004–1012.

Leite, T.G., Vale Filho, V.R., Arruda, R.P., de Andrade, A.F.C., Emerick, L.L., Zaffalon, F.G., Martins, J.A.M., Andrade, V.J., 2010. Effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved Gyr bull semen evaluated by CASA and flow cytometry. Anim. Reprod. Sci. 120, 31–38.

Maio, J.R.G., Souza, E.D.F., Carreiro, S.S., Sales, J.N.S., Crepaldi, G.A., Silva, K.C.F., Santos, G.M.G., Sá Filho, M.F., Carvalho, M.M., Seneda, M.M., Baruselli, P.S., 2008. Plamatic progesterone concentration and pregnancy rate of bovine females synchronized to FTAI with Sincrogest (intravaginal progesterone device). Anim. Reprod. 6, 306.

Maturana Filho, M., Gomes, G.C., Caetano, C.V.F., Kehrle, A., Miguez, P.H.P., Pimentel, J.R.V., Madureira, E.H., 2010. In vivo progesterone release kinetics: a comparative study of different intravaginal devices use in cattle. Reprod. Fertil. Develop. 23, 114.

Meneghetti, M., Sá Filho, O.J., Peres, R., Lamb, G., Vasconcelos, J.L.M., 2009. Fixed time artificial insemination with estradiol and progesterone for *Bos indicus* cows. I. Basis for development of protocols. Theriogenology 72, 179–189.

Neild, D.M., Brouwers, J.F., Colenbrander, B., Aguero, A., Gadella, B.M., 2005. Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. Mol. Reprod. Dev. 72, 230–238.

Oliveira, L.Z., Arruda, R.P., Celeghini, E.C.C., Andrade, A.F.C., Perini, A.P., Resende, M.V., Miguel, M.C.V., Lucio, A.C., Hossepian de Lima, V.F.M., 2012. Effects of discontinuous Percoll gradient centrifugation on the quality of bovine spermatozoa evaluated with CASA and fluorescent probes association. Andrologia 44, 9–15.

Ostermeier, G.C., Sargeant, G.A., Yandell, B.S., Parrish, J.J., 2001. The measurement of bovine sperm nuclear shape using the Fourier harmonic amplitudes. J. Androl. 22, 584–594.

Õura, C., Toshimori, K., 1990. Ultrastructural studies on the fertilization of mammalian gametes. Int. Rev. Cytol. 122, 105–151.

Perry, G.A., Smith, M.F., Roberts, A.J., MacNeil, M.D., Geary, T.W., 2007. Relationship between size of ovulatory follicle and pregnancy success in beef heifers. J. Anim. Sci. 85, 684–689.

Pursley, J.R., Mee, M.O., Wiltbank, M.C., 1995. Synchronization of ovulation in dairy cows using PGF2α and GnRH. Theriogenology 44, 915–923.

Revell, S.G., Mrode, R.A., 1994. An osmotic resistance test for bovine semen. Anim. Reprod. Sci. 36, 77–86.

Sá Filho, O.J., Meneghetti, M., Peres, R., Lamb, G., Vasconcelos, J.L.M., 2009. Fixed-time artificial insemination with estradiol and progesterone for *Bos indicus* cows. II. Strategies and factors affecting fertility. Theriogenology 72, 210–218.

Saacke, R.G., 2008. Sperm morphology: its relevance to compensable and uncompensable traits in semen. Theriogenology 70, 473–478.

Saacke, R.G., DeJarnette, J.M., Bame, J.H., Karabinus, D.S., Whitman, S., 1998. Can spermatozoa with abnormal heads gain access to the ovum in artificially inseminated super- and single-ovulating cattle? Theriogenology 51, 117–128.

Storey, B.T., 1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Mol. Hum. Reprod. 3, 203–213.

Sudano, M.J., Crespilho, A.M., Fernandes, C.B., Martins Junior, A., Papa, F.O., Rodrigues, J., Machado, R., Landim-Alvarenga, F.C., 2011. Use of Bayesian inference to correlate in vitro embryo production and in vivo fertility in

Zebu bulls. Vet. Med. Intern. 1–6. doi:10.4061/2011/436381 Article ID 436381.
Tartaglione, C.M., Ritta, M.N., 2004. Prognostic value of spermatological parameters as predictors of in vitro fertility of frozen–thawed bull semen. Theriogenology 62, 1245–1252.
Verstegen, J., Iguer-Ouada, M., Oclin, K., 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57, 149–179.
Ward, F., Rizos, D., Corridan, D., Quinn, K., Boland, M., Lonergan, P., 2001. Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development in vitro and fertility in vivo. Mol. Reprod. Develop. 60, 47–55.
Wei, H., Fukui, Y., 1999. Effects of bull, sperm type and sperm pretreatment on male pronuclear formation after intracytoplasmic sperm injection in cattle. Reprod. Fertil. Dev. 11, 59–65.
Zhang, B.R., Larsson, B., Lundeheim, N., Rodriguez-Martinez, H., 1997. Relationship between embryo development in vitro and 56-day nonreturn rates of cows inseminated with frozen–thawed semen from dairy bulls. Theriogenology 48, 221–231.