2012

Effect of sequence of insemination after simultaneous thawing of multiple semen straws on conception rate to timed AI in suckled multiparous Nelore cows

THERIOGENOLOGY, NEW YORK, v. 78, n. 8,pp. 1800-1813, NOV, 2012
http://www.producao.usp.br/handle/BDPI/42599

Available online at www.sciencedirect.com

SciVerse ScienceDirect

Theriogenology 78 (2012) 1800–1813

Theriogenology

www.theriojournal.com

Effect of sequence of insemination after simultaneous thawing of multiple semen straws on conception rate to timed AI in suckled multiparous Nelore cows

L.Z. Oliveira[a,*], R.P. Arruda[b], A.F.C. de Andrade[b], R.M. Santos[c], M.E. Beletti[c], R.F.G. Peres[d], J.P.N. Martins[e], V.F.M. Hossepian de Lima[a]

[a] *Department of Veterinary Medicine and Animal Reproduction, Faculdade de Ciências Agrárias e Veterinárias, UNESP, Univ Estadual Paulista, Jaboticabal, SP, Brazil*

[b] *Laboratory of Semen Biotechnology and Andrology, Department of Animal Reproduction, School of Veterinary Medicine and Animal Science, University of Sao Paulo, Pirassununga, SP, Brazil*

[c] *School of Veterinary Medicine, Federal University of Uberlândia, Uberlândia, MG, Brazil*

[d] *Agropecuária Fazenda Brasil (AFB), Nova Xavantina, MT, Brazil*

[e] *Department of Animal Science, Michigan State University, East Lansing, Michigan, USA*

Received 19 August 2011; received in revised form 8 July 2012; accepted 8 July 2012

Abstract

The objective was to determine the effect of sequence of insemination after simultaneous thawing of multiple 0.5 mL semen straws on conception rate in suckled multiparous Nelore cows. The effect of this thawing procedure on in vitro sperm characteristics was also evaluated. All cows (N = 944) received the same timed AI protocol. Ten straws (0.5 mL) of frozen semen from the same batch were simultaneously thawed at 36 °C, for a minimum of 30 sec. One straw per cow was used for timed AI. Frozen semen from three Angus bulls was used. Timed AI records included sequence of insemination (first to tenth) and time of semen removal from thawing bath. For laboratory analyses, the same semen batches used in the field experiment were evaluated. Ten frozen straws from the same batch were thawed simultaneously in a thawing unit identical to that used in the field experiment. The following sperm characteristics were analyzed: sperm motility parameters, sperm thermal resistance, plasma and acrosomal membrane integrity, lipid peroxidation, chromatin structure, and sperm morphometry. Based on logistic regression, there were no significant effects of breeding group, body condition score, AI technician, and sire on conception rate, but there was an interaction between sire and straw group ($P = 0.002$). Semen from only one bull had decreased ($P < 0.05$) field fertility for the group of straws associated with the longest interval from thawing to AI. However, the results of the laboratory experiment were unable to explain the findings of the field experiment. Sperm width:length ratio of morphometric analysis was the single sperm characteristic with a significant interaction between sire and straw group ($P = 0.02$). It was concluded that sequence of insemination after simultaneous thawing of 10 semen straws can differently affect conception rates at timed AI, depending on the sire used. Nevertheless, the effects of this thawing environment on in vitro sperm characteristics, remain to be further investigated.

Keywords: Conception rate; Semen characteristics; Simultaneous semen thawing; Timed AI

1. Introduction

Timed artificial insemination (timed AI) programs provide an organized approach to enhance the use of AI and to improve reproductive efficiency in cattle [1]. Hence, in the past two decades, numerous timed AI

* Corresponding author. Tel.: +55 16 3209 2633; fax: +55 16 3202 4275.

E-mail address: leticiazoccolaro@yahoo.com.br (L.Z. Oliveira).

0093-691X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2012.07.007

protocols have been used worldwide as reproductive management tools for beef and dairy farms [1–5].

In South America, timed AI protocols with progesterone and estradiol are most commonly used on commercial beef farms [3–5]. These protocols allow simultaneous management of a large number of cows and reduce labor needs and handling of cattle. However, because size of breeding herds and the use of timed AI protocols continue to increase in Brazil, the number of cows that need to be bred in a short interval also increases. To facilitate timed AI of large numbers of cows, it is routine practice to simultaneously thaw multiple semen straws in a water bath unit. Under these conditions, some straws remain in the thawing bath while insemination occurs. Consequently, the thermal environment of the water bath could influence sperm viability and thus fertility [6].

Although female physiology factors influencing the reproductive success of bovine timed AI programs have been studied broadly [5,7–9], factors corresponding to the quality of semen used in reproductive programs have not been studied extensively. Thus, in order to evaluate the effect of simultaneous thawing of multiple semen straws on conception rate (CR) and sperm characteristics, we conducted two experiments. The first experiment was a field study designed to determine the effect of sequence of insemination after simultaneous thawing of 10 semen straws (0.5 mL) on CR at timed AI in suckled multiparous Nelore cows. The second experiment was a laboratory study assessing several in vitro sperm characteristics of semen used in this thawing process.

2. Materials 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 the state of Mato Grosso, Brazil) were used in this study. All cows were maintained on *Brachiaria brizantha* or *Brachiaria decumbens* pasture, with ad libitum access to water and mineralized salt. Data were collected from November 2010 to January 2011 (Brazilian spring to summer breeding season). Cows had a body condition score (BCS) between 1.75 and 3.25 on a 1 to 5 scale (1 = emaciated, 5 = obese [10]).

2.1.2. Reproductive management

After calving, suckled cows were allocated into eight breeding groups (approximately 120 animals each), according to calving date. Beginning 30 to 40 days postpartum, all cows (N = 944) received the same timed AI protocol for first service. This protocol started (Day 0) with cows receiving a new or second-use intravaginal progesterone releasing device (Sincrogest; Ouro Fino Saúde Animal, Cravinhos, SP, Brazil) and 2.0 mg of estradiol benzoate im (Sincrodiol; Ouro Fino Saúde Animal). The progesterone device was removed 8 days later and cows were given 500 μg of d-cloprostenol im (Sincrocio; Ouro Fino Saúde Animal), 300 IU of eCG im (Novormon 5000; Intervet Schering Plough Saúde Animal, São Paulo, SP, Brazil) and 0.5 mg of estradiol cipionate im (Pfizer Saúde Animal, São Paulo, SP, Brazil). Cows were timed-inseminated by two experienced AI technicians 2 days after removal of progesterone device (Day 10). Day of timed AI was different for each breeding group. Type of progesterone device (new or second use), BCS (1 to 5) and AI technician were recorded for each cow.

The first four breeding groups were synchronized with only new progesterone devices (N = 477), whereas the last four breeding groups received only used progesterone devices (N = 467). Hence, the number of cows receiving new or used progesterone devices was similar.

2.1.3. Field experimental design

Frozen semen doses from three batches of each of three Angus bulls from the same semen company and diluted in the same milk based extender were used. Approximately 100 semen doses from each batch were used. Semen thawing and semen handling protocols were performed according to routine timed AI procedures of the farm where the experiment was conducted.

For timed AI, an identical procedure was performed for each breeding group: after loading a random cow in 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 s after insertion of 10 straws into the thawing bath, one straw was removed (first straw) and immediately loaded in the AI gun. A skilled person was designated only for removing semen from thawing bath and loading AI guns. Thus, the two AI technicians received a prepared AI gun and immediately used it for AI. One chute was used for breeding cows, with inseminators alternating (breeding one cow and allowing the other inseminator to breed the next cow).

During insemination using the first straw, a second straw was removed from thawing bath and a second AI gun was prepared. After the first AI was finished, an-

other cow was randomly loaded in the chute and the AI gun with a second straw was used. Similarly, the other straws were loaded in AI guns and used in sequence until the tenth straw, in the same matter as the second straw. Sequence of insemination (1st, 2nd, 3rd until 10th) and time (h and min) of semen removal from thawing bath of each cow were recorded. Approximately 12 groups of 10 frozen straws were thawed for each day of timed AI. Semen handling and thawing procedures, AI gun preparation, and inseminations were done in a covered area (to avoid direct sunlight). During the thawing process, the water-bath was agitated slightly to avoid direct contact among semen straws.

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, type of progesterone releasing device (new or second use), and straw sequence. Semen cane and canister of the nitrogen tank used were organized and identified with a batch and bull number, respectively. Therefore, each group of 10 semen straws simultaneously thawed was assured to be from the same batch. In addition, the subsequent group of straws was guaranteed to be different from the batch thawed before.

The mean time of each semen straw remaining in thawing bath (first to tenth) was calculated after the end of field experiment, using records from 944 inseminations. The mean interval from insertion of 10 straws in the thawing bath to removal of the last straw was 6 min and 29 sec, considering a minimum of 30 sec as Time 0. The mean time (± SD) and range of each straw remaining in the thawing bath was 1st straw (0 ± 0; 0 to 0 sec), 2nd straw (49 ± 43; 30 to 180 sec), 3rd (92 ± 46; 60 to 210 sec), 4th straw (141 ± 56; 90 to 240 sec), 5th straw (180 ± 59; 120 to 300 sec), 6th straw (228 ± 65; 150 to 360 sec); 7th straw (266 ± 79; 180 to 390 sec); 8th straw (306 ± 86; 210 to 450 sec); 9th (346 ± 90; 240 to 510 sec); and 10th straw (389 ± 97; 270 to 630 sec).

For analysis of effect of sequential insemination on CR, cows were separated into three groups, according to the distribution of incubation time by straw (Fig. 1). Because most of straws 1, 2, and 3 were removed from thawing bath between 0 and 2.5 min (Fig. 1), cows inseminated with 1st, 2nd, and 3rd semen straws were included in Straw Group 1. Because most of straws 4, 5, and 6 were removed from thawing bath between 2.5 and 5.0 min (Fig. 1), cows inseminated with 4th, 5th, and 6th semen straws were included in Straw Group 2. In the same way, because most of straws 7, 8, 9, and 10 were removed from thawing bath between 5.0 and 7.5 min (Fig. 1), cows inseminated with 7th, 8th, 9th, and 10th semen straws were included in Straw Group 3.

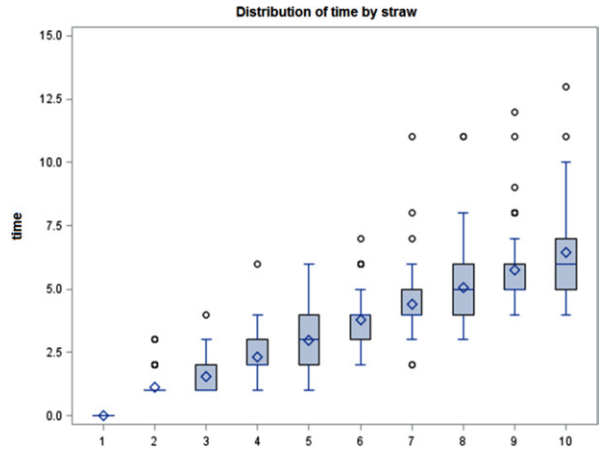

Fig. 1. Distribution of incubation time by straw (box plot graph) according to data of 944 inseminations, regarding the interval semen straws remained in a water bath after simultaneous thawing of 10 semen straws (minimum thawing period of 30 sec defined as Time 0).

All cows were examined by transrectal ultrasonography 40 days after timed AI. Detection of an embryonic vesicle with 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) used in the field trial were evaluated in a laboratory study. To mimic the field procedure, 10 frozen straws (0.5 mL) of the same batch were thawed simultaneously in an identical water bath unit used for field experiment (Fertilize), set at 36 °C, for a minimum of 30 sec. Two semen straws were removed from thawing bath after 30 sec (First Straw Group), whereas the remaining straws stayed in the thawing bath. In order to evaluate semen quality of First Straw Group, semen from each two-straw pair was pooled in a microcentrifuge tube and the following variables were analyzed: sperm motility, sperm thermal resistance, plasma and acrosomal membrane integrity, lipid peroxidation, chromatin structure, and sperm morphometry. Six min and 29 sec after removal of two initial straws (First Straw Group), two other straws were removed from the thawing bath (Last Straw Group). In order to evaluate semen quality of Last Straw Group, the semen from the two-straw pair was pooled in a microcentrifuge tube and the same sperm variables analyzed.

The thermostatically controlled thawing bath was designed to maintain water temperature at 36 °C. However, because 10 frozen straws were simultaneously thawed, the water temperature was an important variable to consider. Thus, a digital thermometer (HI147-00, Hanna Instruments, Ann Arbor, MI, USA) was used to measure the temperature variation of water while the straws were in the thawing bath.

2.2.2. Computer-assisted semen analysis

Sperm motility and sperm thermal resistance were assessed by computer-assisted semen analysis (CASA) (Ivos-Ultimate; Hamilton Thorne Biosciences Inc. Beverly, MA, USA). However, semen samples used in this experiment were all cryopreserved in the same milk extender. Evaluation of bovine semen in lactose-based diluents has always been difficult, because of the presence of numerous fat globules [11]. Therefore, to make sperm more visible, sperm were stained with Hoechst 33342 (H33342) dye (H-1399; Molecular Probes, Inc., Eugene, OR, USA), which cause sperm to fluoresce in ultraviolet light.

For CASA analyses, the H33342 staining was prepared at a concentration of 5 mg/mL. The frozen semen straws had a sperm concentration between 25 and 35 $\times$ 10^6 sperm/mL. The CASA setup was preadjusted for bovine sperm analysis in IDENT option (Ident fluorescence system; Hamilton Thorne Biosciences Inc.) with the following parameters: number of frames: 30; frames per sec: 60 Hz; minimum contrast: 50; minimum cell size: 6 pixels; contrast with static cells: 30; straightness: 60%; average path velocity cutoff: 30 μm/sec; minimum average path velocity: 40 μm/sec; straight-line velocity cutoff: 20 μm/sec; cell size: 6 pixels; cell intensity: 80; static head size: 0.23 to 1.91; static head intensity: 0.56 to 1.20; static elongation: 8 to 92; magnification: X 1.89; video frequency: 60; illumination intensity: 2203; and temperature: 37 °C.

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 H33342 was added. It was incubated for 20 min at 37 °C. After incubation, postthaw sperm motility was evaluated, which involved placing 6 μL of H33342 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; μm/sec), straight-line velocity (VSL; μm/sec), curvilinear velocity (VCL; μm/sec), amplitude of lateral head displacement (ALH; μm), beat cross frequency (BCF; Hz), straightness (STR; %), linearity (LIN; %), and percentage of rapidly moving cells (RAPID; %).

2.2.3. Assessment of sperm thermal resistance after 2-h incubation

Sperm thermal resistance was assessed in order to verify possible differences in postthaw sperm longevity of semen samples. Hence, immediately after the thawing bath removal, an aliquot of 250 μL of frozen-thawed semen was put into a warmed microcentrifuge tube which remained incubated during 2 h at 37 °C. After 120 min of incubation, motility parameters were assessed by CASA. Thus, an aliquot of 100 μL of frozen-thawed semen was put into another warmed microcentrifuge tube and 2 μL of H33342 was added; it was incubated for 20 min at 37 °C and the same procedure described above (Section 2.2.2.) for CASA assessment was done.

2.2.4. Flow cytometry analyses

Flow cytometry analyses were carried out using FACSaria (Becton-Dickinson, San Jose, CA, USA) flow cytometer equipped with a 405 nm Near ultraviolet laser and 488 nm argon laser and filters (photomultiplier) 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 $\geq$ 80%). One aliquot was flash frozen in liquid nitrogen (to damage sperm membranes, resulting in a great proportion of damaged sperm) as described [12], whereas the other aliquot was used as a control (great proportion of membrane-intact sperm) [13].

Samples for flow cytometry analysis were diluted in a modified Tyrode's albumin lactate pyruvate 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'2f100 mL sodium penicillin. The pH of the medium was adjusted with NaOH (1 N) until it achieved pH 7.4. After addition of the dyes for each analysis, semen samples diluted in TALPm were analyzed in a flow cytometer, which was controlled by the BD FACSDiva 6.0 software (Becton-Dickinson). Samples were processed through the instrument at an acquisition rate of approximately 600 to 1000 events/s, acquiring 10 $\times$ 10^3 sperm per analysis [14].

2.2.4.1. Simultaneous assessment of plasma and acrosomal membranes To evaluate cells with intact plasma and acrosomal membranes, an aliquot was taken from the samples and added to TALPm. The resulting sam-

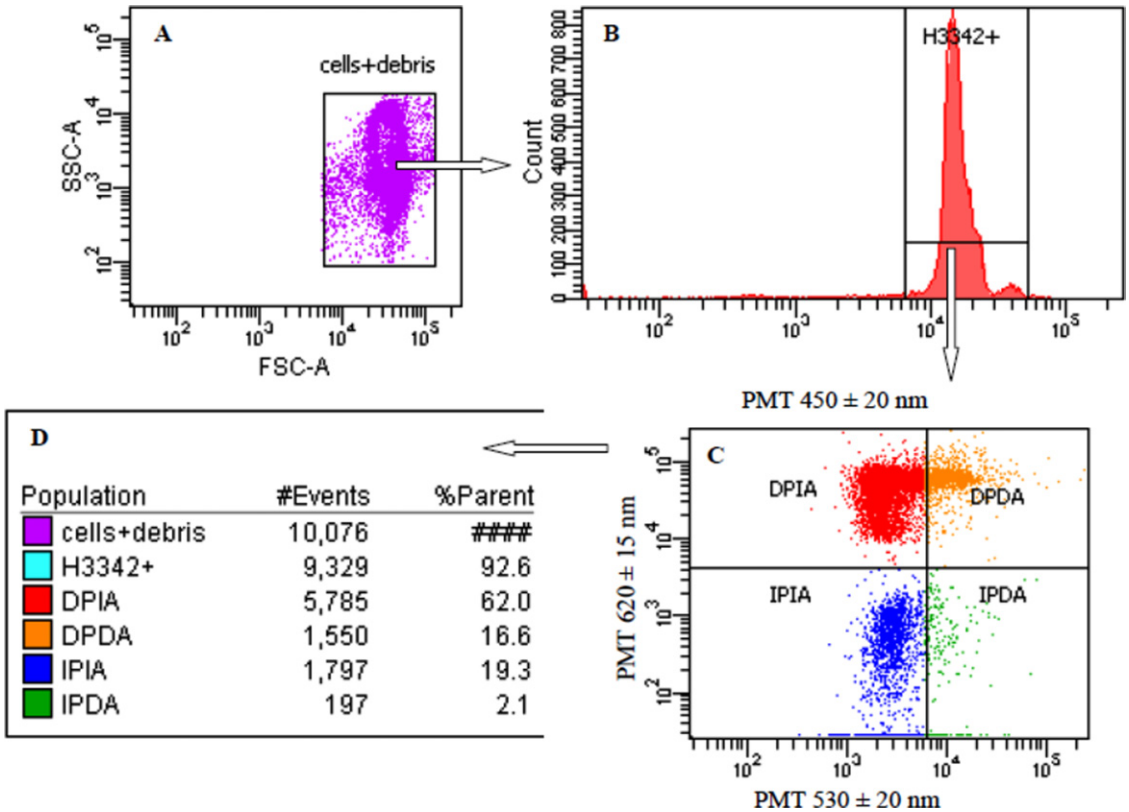

Fig. 2. Representative dot plots and histograms of frozen-thawed bovine sperm analyzed by flow cytometry. (A) Side Scatter × Forward Scatter dot plot. Quadrant "cell+debris" with population of cells and non-DNA containing particles. (B) Histogram of DNA containing particles (sperm) labeled with Hoechst 33342 (H33342) arises from quadrant "cell+debris". (C) sperm with intact plasma and acrosomal membranes (IPIA): propidium iodide (PI) (−), Pisum sativum agglutinin conjugated to fluorescein isothiocyanate (FITC-PSA) (−); sperm with an intact plasma membrane and a damaged acrosomal membrane (IPDA): PI (−), FITC-PSA (+); sperm with a damaged plasma membrane and an intact acrosomal membrane (DPIA): PI (+), FITC-PSA (−); and sperm with damaged plasma and acrosomal membranes (DPDA): PI (+) and FITC-PSA (+) [13]. (D) The mean percentage values for DPIA, DPDA, IPIA, and IPDA arising from dot plot (C), are shown in (D).

ples had a concentration of 5×10^6 sperm/mL in a volume of 148 μL. Then, 2 μL of H33342 (40 μg/mL) was added to stain the DNA of sperm, so particles with the same scatter properties as sperm were not included in the count. 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, MO, 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. The purpose of these probes was to stain the cells with damaged plasma membranes (PI positive) and those that had undergone an acrosome reaction (FITC-PSA positive) [12]. After 10 min of incubation at 37 °C, samples were diluted with 150 μL of TALPm to a final concentration of 2.5×10^6 sperm/mL and then analyzed by flow cytometry [13].

Two-dimensional dot-plots of FITC-PSA (Filter E) versus PI fluorescence (Filter C) from a total of 10 000 events were generated. Each quadrant represented one of the following sperm subpopulations: (1) sperm with intact plasma and acrosomal membranes (IPIA): PI (−), FITC-PSA (−); (2) sperm with intact plasma membrane and damaged acrosomal membrane (IPDA): PI (−), FITC-PSA (+); (3) sperm with damaged plasma membrane and intact acrosomal membrane (DPIA): PI (+), FITC-PSA (−); and (4) sperm with damaged plasma and acrosomal membranes (DPDA): PI (+) and FITC-PSA (+) [14]. Representative dot plots and histograms of frozen-thawed bovine sperm analyzed by flow cytometry, for assessment of plasma and acrosomal membranes integrity, is shown (Fig. 2).

2.2.4.2. Evaluation of lipid peroxidation A fluorescent fatty acid conjugate, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid

(C11-BODIPY$^{581/591}$), is a membrane probe whose fluorescence changes irreversibly from red to green upon exposure to reactive oxygen species (ROS) and has been used to assess lipid peroxidation in living bovine sperm [15].

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 sperm/mL and a final volume of 499.5 μL. Then, 0.5 μL of C11-BODIPY$^{581/591}$ (1 mg/mL; D-3861, Molecular Probes, Inc.) was added and samples were incubated for 30 min at 37 °C. Thereafter, 145 μL of this solution was transferred to another microtube and 2 μL of H33342 (40 μg/mL) was added. Samples were incubated for an additional 10 min at 37 °C. An H33342 probe was used to avoid particles with the same size and/or granularity of the sperm included in the count. After incubation with the H33342 probe, 3 μL of PI (0.5 mg/mL) was added to serve as a marker of cells with a damaged plasma membrane. Then, samples were incubated with PI for 5 min at 37 °C. Subsequently, semen was diluted with 150 μL of TALPm to a final concentration of 2.5×10^6 sperm/mL and then, analyzed by flow cytometry.

Two-dimensional dot plots of C11-BODIPY$^{581/591}$ (Filter E) versus PI fluorescence (Filter C) from a total of 10 000 events were generated. The following sperm subpopulations were considered in the analysis: (1) sperm with intact plasma membrane suffering lipid peroxidation (IPP): PI (−), C11-BODIPY$^{581/591}$ (+); and (2) sperm with intact plasma membrane with no lipid peroxidation detected (IPNP): PI (−), C11-BODIPY$^{581/591}$ (−) [16–18]. Representative dot plots and histograms of frozen-thawed bovine sperm analyzed by flow cytometry, for assessment of lipid peroxidation, is shown (Fig. 3).

2.2.5. Assessment of sperm chromatin structure and morphometry

A small aliquot was collected from microcentrifuge tubes containing the frozen-thawed semen samples and two smears were prepared for each sample, for later assessment of sperm chromatin structure and morphometry.

Sperm smears were fixed with ethanol acetic acid (3:1, vol/vol) for 1 min and, then in 70% ethanol for 3 min. 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 coverslipped.

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 X objective lens (immersion) coupled to a Leica ICC50 camera (Leica Microsystems, Inc.) connected to a PC. Displayed images of this sort 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 [19], at least 100 sperm heads were isolated for each smear. Sperm head segmentation was done using algorithms developed in numerical computation software (Scilab software; Scilab version 5.3.3, Scilab Enterprises, Versailles, France) through the Scilab Image Processing toolbox (SIP toolbox). The sperm heads were analyzed to obtain the average pixel value from every head. Six heads with the smallest pixel values were selected automatically and defined as standard heads. In theory, these heads had the most condensed chromatin. Subsequently, for each image, the difference between the standard value of the smear and the average value of each head analyzed was determined. This difference was transformed into a percentage of the average pixel value for the standard heads (Dif), which indicates sperm chromatin decondensation. The coefficient of variation (CV) of gray level intensity for each head, which indicates chromatin heterogeneity, was also calculated [20–23]. Area (A), perimeter (P), width (W), length (L) and width:length ratio (W/L) of all sperm heads was determined using other algorithms developed in the Scilab environment [19–23]. All of these parameters were calculated using procedures described by Beletti and Costa [19] and Beletti, et al. [20,21].

2.3. Statistical analyses

2.3.1. Statistical analysis of field experiment

Pregnancy/AI (CR) 40 days after AI was analyzed as a binary response variable using logistic regression by the LOGISTIC procedure of SAS (version 9.3, SAS Institute, Inc., Cary, NC, USA) fitted with a binary distribution and a logit link function. Variables considered in the initial models as fixed effects were breeding group (1 to 8, class), BCS ($\leq$ 2.5 and $>$ 2.5, class), progesterone intravaginal device (new or second use, class), AI technician (1 and 2, class) and sire (1, 2, and 3, class). The fixed factors straw sequence (1 to 10, class) and straw group (1, 2, or 3, class) were used separately with the other variables in different models that also included two-way interactions with other effects. Time of AI was used as a covariate.

A backward stepwise logistic regression model,

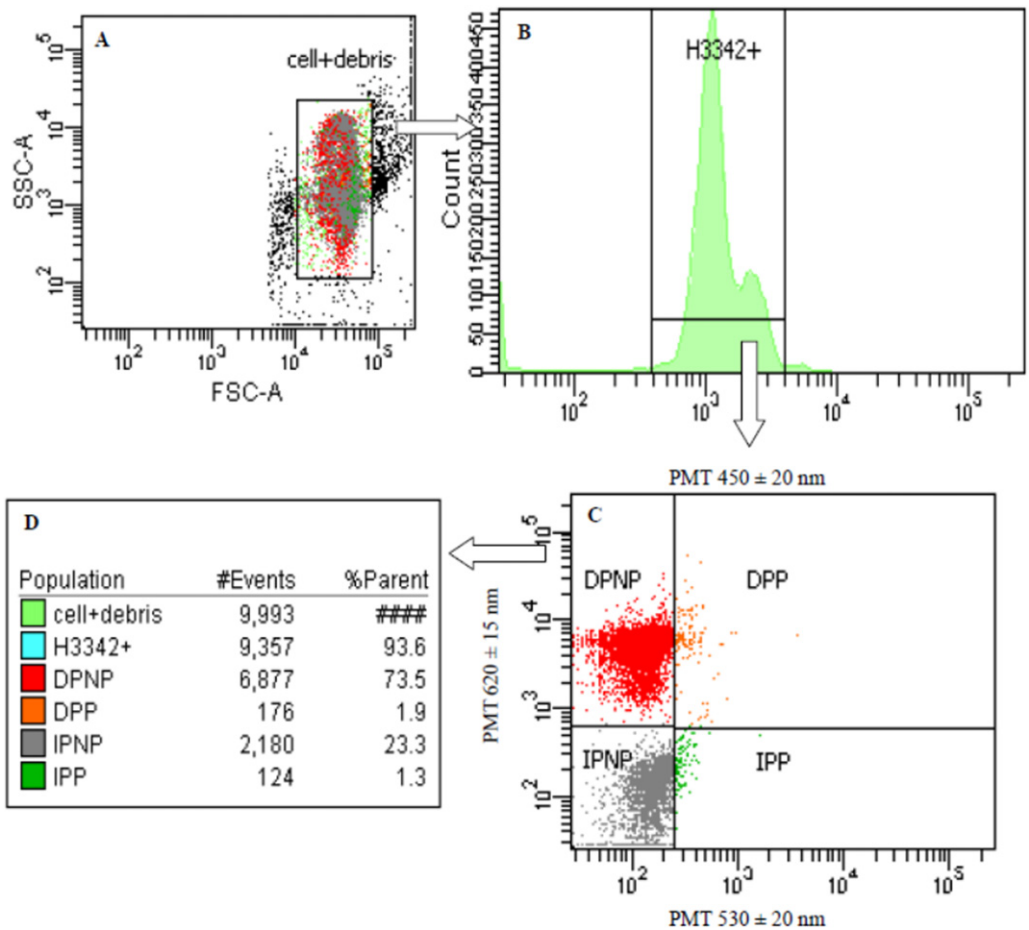

Fig. 3. Representative dot plots and histograms of frozen-thawed bovine sperm analyzed by flow cytometry. (A) Side Scatter × Forward Scatter dot plot. Quadrant "cell+debris" contains population of cells and non-DNA containing particles. (B) Histogram showing DNA containing particles (sperm) labeled with Hoechst 33342 (H33342) arises from quadrant "cell+debris". (C) Sperm with intact plasma membrane suffering lipid peroxidation (IPP): PI (−), 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY$^{581/591}$) (+); sperm with intact plasma membrane with no lipid peroxidation detected (IPNP): PI (−), C11-BODIPY$^{581/591}$ (−); sperm with damaged plasma membrane with no lipid peroxidation detected (DPNP): PI (+), C11-BODIPY$^{581/591}$ (−); sperm with damaged plasma membrane suffering lipid peroxidation (DPP): PI (+), C11-BODIPY$^{581/591}$ (+). (D) The mean intensity of fluorescence emission (a.u.), captured in the photomultiplier with a long pass of 502 and a band pass of 530 ± 15 nm, was analyzed in the viable sperm (MI) labeled with C11-BODIPY$^{581/591}$ and are represented in histogram. The mean percentage values for IPP, IPNP, DPNP, and DPP arising from dot plot (C), are shown in (D).

specified by the option Selection = Backward in SAS, was used and variables were continuously removed from the initial models by Wald statistic criterion when $P > 0.10$. The Hosmer and Lemeshow Goodness-of-Fit Test, specified by the LACKFIT option, were used to compare the final selected models against the nonreduced models. All final models were adequate with $P > 0.98$. The final models only considered straw sequence (1 to 10, class), sire (1, 2, and 3, class), and their interaction; or straw group (1, 2, and 3, class), sire (1, 2, and 3, class), and their interaction. Statistical differences in CR for the different straw sequence or straw groups, and sires were analyzed by logistic regression using least squares means (LS Means) statement of the GLIMMIX procedure. A significant difference between the levels of a classification variable was considered when $P < 0.05$.

2.3.2. *Statistical analysis of laboratory experiment*

In the present experiment, three semen batches of every bull were investigated. Hence, for each bull, three laboratory trials were considered.

Results obtained from all sperm variables of laboratory analyses were tested for normality of residues and homogeneity of variance. Dependent variables that did not meet these statistical premises were

Table 1
Conception rate (%; N/N) 40 days after timed AI in suckled multiparous Nelore cows.

Bull	Straw Group 1	Straw Group 2	Straw Group 3	Total
1	58.1 (50/86)^{ax}	60.2 (50/83)^{ax}	35.3 (42/119)^{bx}	49.3 (142/288)^{x}
2	40.2 (37/92)^{ay}	50.5 (49/97)^{ax}	51.7 (62/120)^{ay}	47.9 (148/309)^{x}
3	59.8 (64/107)^{ax}	51.0 (52/102)^{ax}	48.6 (67/138)^{ay}	52.7 (183/347)^{x}
Total	53.0 (151/285)^{a}	53.6 (151/282)^{a}	45.4 (171/377)^{b}	50.1 (473/944)

Within a column, values without a common superscript letter (x, y) differ ($P < 0.05$). Within a row, values without a common superscript letter (a, b) differ ($P < 0.05$).

submitted to arcsine transformation. To compare First and Last Straw Groups, data were submitted to ANOVA (PROC GLM) and treatment differences were separated using Tukey's test. Effects of sire, straw group (first vs. last), and the interaction of sire and straw were evaluated.

Additionally, the results from laboratory analysis were tested using a paired sample Student *t* test. However, for paired *t* test analyses, First and Last Straw Groups were compared separately for each bull. The results are presented as mean ± SD, and $P < 0.05$ was considered significant.

3. Results and discussion

In the present experiment, one of the three sires had reduced fertility for the group of straws associated with the longest interval from thawing to AI (Straw Group 3). However, semen from the other two bulls was not significantly different with respect to field fertility for any straw group (Straw Group 1, Straw Group 2, and Straw Group 3).

In beef cows, suckling and negative energy balance are two important factors affecting ovulation and pregnancy rates [5]. Furthermore, BCS and parity of cows, sire/semen quality and AI technician are critical factors that affect the success of timed AI programs. Also, differences between breeding groups within a farm may occur because of interactions of several factors affecting timed AI results [8]. However, in the present experiment, there was no effect of BCS ($P = 0.452$), breeding group ($P = 0.771$), AI technician ($P = 0.432$), progesterone device (new vs. second use; $P = 0.212$), or sire ($P = 0.346$). This was attributed to breeding groups that were well distributed across the timed AI variables and the use of only two skilled AI technicians, as well as only sires with high fertility. In addition, all animals of the present study were cows in the same category (suckled multiparous Nelore cows) with a small variation in BCS (2.22 ± 0.43) and range (1.75 to 3.25).

Nevertheless, there was an interaction between sire and straw group ($P = 0.002$) on CR. An effect of straw group ($P = 0.046$) on CR was also detected. The overall CR of Straw Group 3 was significantly lower than CR of Straw Groups 1 and 2. However, because an interaction of sire and straw group was detected, it is important to emphasize that the effect of straw group on CR was largely due to a single sire (Bull 1; Table 1).

Total CR obtained from cows inseminated with the first to the tenth straws in the sequence of insemination were Straw 1: 58.3% (N = 96); Straw 2: 47.4% (N = 95); Straw 3: 53.2% (N = 94); Straw 4: 52.1% (N = 94); Straw 5: 51.1% (N = 94); Straw 6: 57.4% (N = 94); Straw 7: 47.4% (N = 95); Straw 8: 50.0% (N = 94); Straw 9: 40.4% (N = 94); and Straw 10: 43.6% (N = 94); ($P = 0.287$). There was an interaction ($P = 0.0007$) between sire and straw sequence when only cows inseminated with first and tenth straws of the sequence were analyzed (CR of Sire 1: Straw 1 = 66.7% and Straw 10 = 31.3%; CR of Sire 2: Straw 1 = 37.9% and Straw 10 = 66.7%; CR of Sire 3: Straw 1 = 67.6% and Straw 10 = 37.1%). Overall CR of cows inseminated with first straw in the sequence (Straw 1) was 58% and of cows inseminated with tenth straw in the sequence (Straw 10) was 44% ($P = 0.092$).

For bovine AI, The Brazilian Association of Artificial Insemination recommends thawing a single frozen 0.5-mL semen straw for 30 sec in a water bath at 35 °C to 37 °C. This is the minimum interval routinely used on Brazilian farms. However, in the current study, a group of 10 straws was chosen, as we estimated this to represent the maximal number of straws simultaneously thawed in Brazil when timed AI is done in large herds. It is noteworthy that the sequence of AI was exactly the same as the sequence of straw removal from thawing bath. Moreover, for all cows, the interval between straw removal from thawing bath, AI gun preparation and semen deposition were closely related (with limited variation).

On average, the interval between straw removal from thawing bath to insemination was approximately 40 sec. Including the additional 30 sec of minimum thawing period, the interval from inserting the group of

10 straws into thawing bath to AI of the third cow in the sequence (Straw Group 1) was approximately 120 sec (Sire 1: 120 ± 42 sec; Sire 2: 119 ± 47 sec; Sire 3: 123 ± 48 sec), between inserting 10 straws into the thawing bath to AI of the sixth cow (Straw Group 2) was approximately 260 sec (Sire 1: 270 ± 69 sec; Sire 2: 253 ± 64 sec; Sire 3: 247 ± 57 sec), and between inserting 10 straws into the thawing bath to AI of the tenth cow (Straw Group 3) was approximately 420 sec (Sire 1: 412 ± 91 sec; Sire 2: 430 ± 114 sec; Sire 3: 412 ± 87 sec). Hence, with such a small time interval, it is intriguing how a difference of only few minutes of incubation could had interfered in CR from cows inseminated with Straw Group 3 compared with Straw Groups 1 and 2, even considering that this effect was observed for only one specific sire.

In general, the standard recommendations for cryopreserved bovine semen are to thaw no more straws than can be deposited in the female within 15 min and to maintain thermal homeostasis during this interval [24,25]. Goodell [26], in a study with 180 reproductive outcomes, reported a decreased CR for the third and fourth inseminations in the sequence, when more than two straws were thawed at once. However, DeJarnette et al. [24] reviewed the effects of sequence of insemination after simultaneous thawing on CR with data collected from several studies (N = 19 000 inseminations). The authors suggested that as many as four straws can be thawed at once with no considerable fertility concern, provided that inseminators strictly adhere to recommended semen handling procedures and respect the maximum interval of 15 to 20 min between thawing and insemination [24]. Likewise, Kaproth et al. [27] and Dalton et al. [28] demonstrated that experienced AI technicians can simultaneously thaw multiple semen straws and inseminate up to four cows within a 20-min interval, without adverse effects on field fertility. In a study similar to the present experiment, Sprenger et al. [29] observed that an interaction of herd by sequential insemination tended to influence field fertility outcomes. In one herd, straw number 5 had reduced CR compared with other straws in the sequence (1 until ≥ 7); in another herd, CR of straws number 6 and ≥ 7 were lower than straws 1 to 5. However, in the further 11 herds evaluated, sequence of insemination had no effect on CR. The authors concluded that, when recommended semen handling procedures are followed, more than two straws can be thawed at once without compromising semen fertility [29].

Hence, even thawing simultaneously 10 straws, the average of maximum interval period (389 ± 97 sec), as well as the highest absolute interval interval (630 sec), between thawing until AI was evidently short in the present experiment. Therefore, the hypothesis that additional factors other than incubation time could have affected the fertility for Straw Group 3 cannot be excluded.

An important consideration was the possibility of an influence of ambient temperature. According to Shepard (unpublished; cited by DeJarnette et al. [24]), an interaction of ambient temperature and interval to semen deposition might occur because of extended thaw duration. Shepard reported reduced CR for semen which thaw duration was > 10 min when ambient temperatures were higher than 17 °C (compared with ambient temperatures < 17 °C). Therefore, higher environmental temperatures may be problematic to post-thaw fertility maintenance. Similarly, Lee et al. [30] also suggested that greater sequences of insemination might compromise semen fertility when associated with the effects of high ambient temperatures and/or solar exposure. Because the current study was accomplished during summer season of a tropical region, the high ambient temperature during the experimental period (average maximum temperature approximately 30 °C) may have interacted with the effects of incubation time. In this case, semen fertility of a specific sire (Bull 1) was more susceptible to the effects of sequence of insemination under these conditions.

Thawing bath temperature during incubation was measured in a laboratory experiment. Although there was no substantial variation in water temperature during laboratory simulation (Fig. 4), the regular ambient temperature during laboratory simulation (20 °C) may not represent the real environmental temperature of a field experiment. Thus, temperature variation of the water bath during the field experiment was perhaps different from the laboratory experiment.

The results of the laboratory experiment were not able to explain the effect of simultaneous thawing on semen fertility in the field trial. Based on ANOVA, there were no effects of sire, straw group, and their interaction on the variables: TM, TM after 2 h, PM, PM after 2 h, VAP, VAP after 2 h, VSL, VSL after 2 h, VCL, VCL after 2 h, ALH, ALH after 2 h, BCF, BCF after 2 h, STR, STR after 2 h, LIN, LIN after 2 h, RAPID, RAPID after 2 h, DPIA, DPDA, IPIA, IPDA, IPNP, IPP, Dif, CV, area, perimeter, width, and length ($P > 0.05$). There was an interaction ($P = 0.024$) between sire and straw group on W/L (width: length ratio of morphometric analysis). This was the only sperm characteristic with a significant interaction between sire and straw group. For this characteristic, there

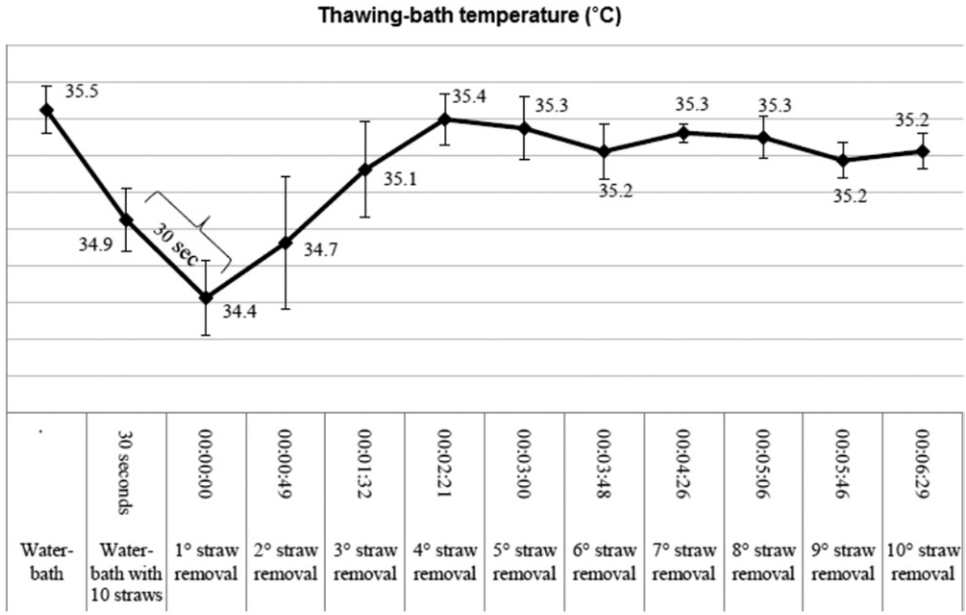

Fig. 4. Water temperature variation during the time that ten 0.5-mL frozen semen straws remained in the 400-mL thermostatically controlled thawing bath after simultaneous thawing, measured during the laboratory experiment.

was an effect of sire ($P < 0.0001$), but no effect of straw group ($P = 0.223$).

Because the main effect of field experiment was the interaction between bull and straw group, we also analyzed the laboratory data using a paired Student *t* test (First vs. Last Straw Group) separated by bull. According to this statistical analysis, there was no significant effect of straw group for any bull in the following variables: TM, TM after 2 h, PM after 2 h, VAP, VAP after 2 h, VSL, VSL after 2 h, VCL, VCL after 2 h, ALH after 2 h, BCF, BCF after 2 h, STR, STR after 2 h, LIN, LIN after 2 h, RAPID after 2 h, DPIA, IPIA, IPDA, IPP, Dif, CV, area, perimeter, width, and length ($P > 0.05$). Intriguingly, there was a straw effect for Bull 2 in the following variables: PM ($P = 0.044$), ALH ($P = 0.014$), RAPID ($P = 0.047$), DPDA ($P = 0.042$), and IPNP ($P = 0.043$). Conversely, for Bull 1 (whose field fertility was affected by sequence of insemination) there was an effect of straw group only for W/L, but as a trend ($P = 0.059$). The relevant laboratory results (analyzed by paired Student *t* test) are shown (Table 2).

Brown et al. [6] demonstrated in a laboratory experiment that as many as 10 straws (0.5 mL) can be simultaneously thawed in a 36 °C thermostatically controlled thawing bath with no effect on sperm motility [6]. However, in the present experiment, there were significant effects in the CASA analysis at 0-h evaluation, but only for Bull 2, which had reduced ($P < 0.05$) PM, ALH, and RAPID in the Last Straw Group compared with First Straw Group (Table 2). Nevertheless, in the present study, an incubation period of 20 min with H33342 was necessary to prepare semen samples for CASA evaluation. Thus, the values observed for motility parameters are not the real values of frozen-thawed semen used in the experiment. Regardless, given that all semen samples were submitted to the same staining challenge, differences between First and Last Straw Groups were the main outcome, and not the absolute values obtained.

The CASA analysis after 2 h of thermal incubation was intended to detect differences in thermal resistance of First and Last Straw Groups, which could had explained important differences in sperm survival capacity of Straw Group 1 versus Straw Group 3. However, because no straw effect was observed for any trial bull, the supposition that thermal environment of thawing bath could have interfered in sperm longevity of more susceptible semen was not confirmed. Additionally, considering that thawing bath incubation could have affected sperm viability, we expected significant differences in plasma and acrosomal membrane integrity between First and Last Straw Groups, mainly for Bull 1. Nevertheless, there was a significantly higher DPDA in Last Straw Group only for Bull 2 (Table 2).

Another concern of the present study was the occurrence of oxidative stress after incubation in the thawing bath. Sperm are susceptible to oxidation of their plasma

Table 2
Mean ± SD of in vitro sperm characteristics of bovine frozen-thawed semen from First Straw Group (straws removed from thawing bath after 30 sec) and Last Straw Group (straws removed from thawing bath 6 min and 29 sec after first straw removal), separated by bull.

Sperm variables	Sire 1		Sire 2		Sire 3	
	First Straw Group	Last Straw Group	First Straw Group	Last Straw Group	First Straw Group	Last Straw Group
TM (%)	51.04 ± 4.19	38.03 ± 7.10	33.59 ± 8.91	25.21 ± 8.15	46.12 ± 15.94	41.06 ± 17.71
PM (%)	44.28 ± 4.28	29.46 ± 9.78	27.97 ± 7.19[a]	21.48 ± 6.37[b]	32.02 ± 9.68	28.55 ± 11.43
ALH (μm)	3.88 ± 0.06	4.00 ± 0.33	4.24 ± 0.24[a]	4.02 ± 0.26[b]	4.45 ± 0.86	4.44 ± 0.52
RAPID (%)	45.99 ± 4.39	30.57 ± 10.04	29.26 ± 7.19[a]	21.99 ± 6.62[b]	35.39 ± 12.19	31.09 ± 13.02
TM after 2 h (%)	19.81 ± 10.74	12.70 ± 7.70	6.77 ± 3.14	5.44 ± 4.66	25.73 ± 15.24	22.96 ± 21.33
PM after 2 h (%)	15.96 ± 9.86	8.88 ± 7.47	4.60 ± 2.26	3.51 ± 2.94	15.76 ± 6.92	13.34 ± 11.89
ALH after 2 h (μm)	4.25 ± 0.17	3.67 ± 0.54	4.25 ± 0.45	3.75 ± 0.88	4.91 ± 0.40	4.33 ± 0.83
RAPID after 2 h (%)	16.86 ± 9.97	9.40 ± 8.07	4.96 ± 2.70	3.70 ± 3.15	17.46 ± 8.40	15.20 ± 13.54
IPIA (%)	39.10 ± 6.56	38.10 ± 7.62	36.47 ± 5.61	33.73 ± 6.57	47.17 ± 14.82	42.10 ± 11.23
DPDA (%)	30.77 ± 10.40	34.17 ± 14.20	30.30 ± 8.68[a]	37.77 ± 11.36[b]	20.50 ± 16.29	22.33 ± 15.41
IPP (%)	0.53 ± 0.21	0.33 ± 0.06	2.10 ± 1.77	0.57 ± 0.15	0.47 ± 0.25	0.40 ± 0.26
IPNP (%)	30.93 ± 3.45	29.43 ± 7.18	30.33 ± 5.12[a]	24.90 ± 4.68[b]	40.80 ± 16.26	37.50 ± 18.30
W/L	0.518 ± 0.002[A]	0.525 ± 0.005[B]	0.532 ± 0.004	0.544 ± 0.004	0.522 ± 0.010	0.514 ± 0.005
Dif (%)	4.61 ± 0.91	7.56 ± 3.67	7.14 ± 1.56	8.72 ± 4.33	8.70 ± 6.71	5.80 ± 1.67
CV (%)	8.14 ± 3.74	8.61 ± 3.67	10.64 ± 0.39	11.09 ± 1.68	8.47 ± 2.50	8.33 ± 1.59

Within a row, values without a common superscript lowercase letter differ ($P < 0.05$). Values without a common superscript capital letter differ ($P < 0.06$).

ALH, amplitude of lateral head displacement; CV, chromatin heterogeneity measured by coefficient of variation of the gray-level; Dif, chromatin decondensation measured by percentage of gray-level differences; DPDA, sperm with damaged plasma and acrosomal membranes; IPIA, sperm with intact plasma and acrosomal membranes; IPNP, sperm with intact plasma membrane with no lipid peroxidation detected; IPP, sperm with intact plasma membrane suffering lipid peroxidation; PM, progressive motility; RAPID, percentage of rapidly moving cells; TM, total motility; W/L, width:length ratio of sperm head.

membranes because of polyunsaturated fatty acids [31]. Elevated ROS concentrations may become cytotoxic if they overcome the natural defense mechanisms of cell and extending medium. It can inhibit sperm motility and damage proteins, nucleic acids, and membrane lipids [32]. In the present study, there was a significant decrease in IPNP cells in the Last Straw Group for Bull 2 (Table 2). It is interesting to note that the impairment of in vivo sperm parameters for Bull 2 seemed to be associated. However, the speculation that reduced fertility of Straw Group 3 observed for Bull 1 could have been because of oxidative stress caused by thermal environment of thawing bath, was not confirmed in the laboratory. Regardless, it is noteworthy that the negative effect of some ROS-generating systems does not require lipid peroxidation to induce cytotoxic changes [33]. In that regard, Guthrie and Welch [33] reported that menadione and H_2O_2 decreased the percentage of motile sperm, but had no effect on BODIPY oxidation.

Kasimanickam et al. [34] reported that bulls with higher sperm lipid peroxidation were more likely to have high DNA fragmentation index and low plasma membrane integrity. They also observed that bulls with higher sperm lipid peroxidation and DNA fragmentation had lower sperm fertilization capacity [34]. However, in the present study, there were no significant differences in chromatin parameters for First versus Last Straw Group for any sire evaluated. Nevertheless, interestingly, the parameter W/L of sperm morphometry seemed to indicate some relationship with field results. In ANOVA, there was an interaction ($P < 0.05$) of sire and straw group for this sperm characteristic. Likewise, the trend ($P < 0.06$) observed in the paired *t* test (Table 2) seemed to indicate that W/L was different between First and Last Straw Group, but only for Bull 1.

The use of computational image analysis for morphologic characterization allows identification of minor morphometric alterations of sperm head [20]. However, little is known about the influence of such abnormalities on bull fertility [21]. Because mammalian sperm heads consist almost entirely of chromatin, even minor changes in chromatin organization might affect sperm head shape [35]. Nonetheless, morphologic alterations in sperm head are not always caused by alterations in chromatin condensation. Likewise, chromatin abnormalities are not always followed by evident morphologic irregularities [20,21,35]. According to Beletti et al. [20], the parameter W/L has inverse correlation with sperm ellipticity. When W/L increases, the sperm head tends to be more spherical [20]. Hence, sperm heads of

Table 3
Mean ± SD of in vitro sperm characteristics of bovine frozen-thawed semen from First Straw Group (straws removed from thawing bath after 30 sec) and Last Straw Group (straws removed from thawing bath 6 min and 29 sec after first straw removal), separated according to field fertility outcomes (affected fertility sire or unaffected fertility sires).

Sperm variables	Affected fertility sire (Bull 1)		Unaffected fertility sires (Bulls 2 and 3)	
	First Straw Group	Last Straw Group	First Straw Group	Last Straw Group
TM (%)	51.04 ± 4.19	38.03 ± 7.10	39.85 ± 13.43[a]	33.13 ± 15.08[b]
PM (%)	44.28 ± 4.28	29.46 ± 9.78	29.99 ± 7.94[a]	25.02 ± 9.14[b]
ALH (μm)	3.88 ± 0.06	4.00 ± 0.33	4.34 ± 0.58	4.23 ± 0.43
RAPID (%)	45.99 ± 4.39	30.57 ± 10.04	32.33 ± 9.56[a]	26.54 ± 10.50[b]
TM after 2 h (%)	19.81 ± 10.74	12.70 ± 7.70	16.25 ± 14.31	14.20 ± 16.81
PM after 2 h (%)	15.96 ± 9.86	8.88 ± 7.47	10.18 ± 7.65[a]	8.43 ± 9.44[b]
ALH after 2 h (μm)	4.25 ± 0.17	3.67 ± 0.54	4.58 ± 0.52	4.04 ± 0.83
RAPID after 2 h (%)	16.86 ± 9.97	9.40 ± 8.07	11.21 ± 8.82	9.45 ± 10.82
IPIA (%)	39.10 ± 6.56	38.10 ± 7.62	41.82 ± 11.61[a]	37.92 ± 9.42[b]
DPDA (%)	30.77 ± 10.40	34.17 ± 14.20	25.40 ± 12.85[a]	30.05 ± 14.77[b]
IPP (%)	0.53 ± 0.21	0.33 ± 0.06	1.28 ± 1.44	0.48 ± 0.21
IPNP (%)	30.93 ± 3.45	29.43 ± 7.18	35.57 ± 12.21[a]	31.20 ± 13.79[b]
W/L	0.518 ± 0.002[A]	0.525 ± 0.005[B]	0.527 ± 0.009	0.529 ± 0017
Dif (%)	4.61 ± 0.91	7.56 ± 3.67	7.92 ± 4.44	7.26 ± 3.34
CV (%)	8.14 ± 3.74	8.61 ± 3.67	10.64 ± 0.39	11.09 ± 1.68

Within a row, values without a common superscript lowercase letter differ ($P < 0.05$). Values without a common superscript capital letter differ ($P < 0.06$).

Affected fertility sire, bull that presented reduced fertility in Straw Group 3 of field experiment; ALH, amplitude of lateral head displacement; CV, chromatin heterogeneity measured by coefficient of variation of the gray-level; Dif, chromatin decondensation measured by percentage of gray-level differences; DPDA, sperm with damaged plasma and acrosomal membranes; IPIA, sperm with intact plasma and acrosomal membranes; IPNP, sperm with intact plasma membrane with no lipid peroxidation detected; IPP, sperm with intact plasma membrane suffering lipid peroxidation; PM, progressive motility; RAPID, percentage of rapidly moving cells; TM, total motility; Unaffected fertility sires, bulls which field fertility were not affected in any straw group of field experiment; W/L, width:length ratio of sperm head.

Bull 1 were less elliptical in Last Straw Group than sperm heads of First Straw Group (Table 2). Ostermeier et al. [35] reported that the average shape of sperm from high-fertility bulls was more tapered and elongated than average shape of sperm from low-fertility bulls [35]. Regardless, small variations in sperm head shape can exist without interfering with hydrodynamic properties or sperm fertilizing capacity [20]. Because no other alteration in sperm shape or chromatin characteristic, nor significant sperm damage, were observed in Last Straw Group of Bull 1, it is difficult to identify possible causes for such ellipticity changes. Regardless, the biological relevance of this finding and its association with semen fertility encourages additional future investigations.

Regarding incubation-associated diminished sperm quality, laboratory results for all three bulls were also separated into two categories: unaffected fertility bulls (Sires 2 and 3) and the bull with diminished field fertility (Sire 1), as shown (Table 3). Again, the results were puzzling and, in general, in vitro sperm characteristics of First versus Last Straw Group were not significantly different. Perhaps the main reason for the lack of statistical differences in laboratory analyses was the small number of bulls, and/or batches, tested. Moreover, an important factor probably interfering with these results was the intriguing fact that in vitro semen quality of Last Straw Group was inferior to First Straw Group mainly for Bull 2, which was an unaffected fertility bull.

Taking all together, a notable consideration confirmed in the present study was the importance of field trials when definitive conclusions are made regarding semen fertility. Because the high environmental temperature during the field experiment may have potentiated the effects of incubation time on semen quality, the possibility that the thermal environment of thawing bath could have interfered on sperm fertility of Bull 1, should not be excluded.

In summary, semen fertility of some sires appeared to be more negatively affected by sequence of insemination than others. The number of straws that can be simultaneously thawed without compromising semen fertility seems to vary for each bull. Unfortunately, the laboratory analyses did not account for the effect of interaction between sire and straw group observed in the field experiment. Either the number of sires used was small, or laboratory trial was not able to correctly simulate field trial, or the aspects being tested were not pertinent to assess semen fertility over the range of

factors of this study. Nevertheless, the reason why semen from some bulls seems to be more susceptible to specific thawing environments and/or procedures remains to be clarified.

In conclusion, the sequence of insemination after simultaneous thawing of multiple semen straws might affect fertility outcomes, depending on the sire used. Under these environmental conditions, 10 semen straws (0.5 mL) should not be simultaneously thawed, because it could affect conception rates, depending on the semen used. Therefore, in similar routine procedures of timed AI programs consisting of large herds, it seems more cautious to not exceed six semen straws (0.5 mL) for simultaneous thawing. Nevertheless, the effect of this thawing bath environment on in vitro sperm characteristics, which could explain the impairment of in vivo semen fertility, was not elucidated.

Acknowledgments

The authors thank Dr. José Ricardo de Garla Maio (Ouro Fino Agronegócio, Cravinhos, SP, Brazil) and the staff of Ouro Fino Saúde Animal for their support with the timed AI protocol and hormones; to statistical consultant Pablo Daniel Reeb, Ph.D. (MSU Department of Fisheries & Wildlife) for his help and suggestions with statistical analyses; and also the staff, employees, and proprietors of Agropecuária Fazenda Brasil (Nova Xavantina, MT, Brazil) for their help handling the cattle, and for allowing the use of their animals and facilities for this study. In addition, 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 the form of grants and research assistance.

References

[1] Sá Filho MF, Crespilho AM, Santos JE, Perry GA, Baruselli PS. Ovarian follicle diameter at timed insemination and estrous response influence likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled Bos indicus cows. Anim Reprod Sci 2010; 120:23–30.

[2] Pursley JR, Mee MO, Wiltbank MC. Synchronization of ovulation in dairy cows using PGF2α and GnRH. Theriogenology 1995;44:915–23.

[3] Bó GA, Baruselli PS, Martínez MF. Pattern and manipulation of follicular development in Bos indicus cattle. Anim Reprod Sci 2003;78:307–26.

[4] Baruselli PS, Reis EL, Marques MO, Nasser LF, Bó GA. The use of hormonal treatments to improve reproductive performance of anestrous beef cattle in tropical climates. Anim Reprod Sci 2004;8283:479–86.

[5] Meneghetti M, Sá Filho OG, Peres RF, Lamb GC, Vasconcelos JL. Fixed-time artificial insemination with estradiol and progesterone for Bos indicus cows I: basis for development of protocols. Theriogenology 2009;72:179–89.

[6] Brown DW Jr, Senger PL, Becker WC. Effect of group thawing on post-thaw viability of bovine spermatozoa packaged in .5-milliliter French straws. J Anim Sci 1991;69:2303–9.

[7] Perry GA, Smith MF, Roberts AJ, MacNeil MD, Geary TW. Relationship between size of the ovulatory follicle and pregnancy success in beef heifers. J Anim Sci 2007;85:684–9.

[8] Sá Filho OG, Meneghetti M, Peres RF, Lamb GC, Vasconcelos JL. Fixed-time artificial insemination with estradiol and progesterone for Bos indicus cows II: strategies and factors affecting fertility. Theriogenology 2009;72:210–8.

[9] Galvão KN, Santos JE. Factors affecting synchronization and conception rate after the Ovsynch protocol in lactating Holstein cows. Reprod Domest Anim 2010;45:439–46.

[10] Houghton PL, Lemenager RP, Moss GE, Hendrix KS. Prediction of postpartum beef cow body composition using weight to height ratio and visual body condition score. J Anim Sci 1990; 68:1428–37.

[11] Van Demark NL, Estergreen VL Jr, Schorr R, Kuhlman DE. Use of fluorescent dyes for observing bovine spermatozoa in opaque media. J Dairy Sci 1959;42:1314–9.

[12] Celeghini EC, de Arruda RP, de Andrade AF, Nascimento J, Raphael CF. Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. Reprod Domest Anim 2007;42:479–88.

[13] De Andrade AF, Zaffalon FG, Celeghini EC, Nascimento J, Tarragó OF, Martins SM, et al. Addition of seminal plasma to post-thawing equine semen: what is the effect on sperm cell viability? Reprod Domest Anim 2011;46:682–6.

[14] Leite TG, do Vale Filho VR, de Arruda RP, De Andrade AF, Emerick LL, Zaffalon FG, et al. 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 2010;120:31–8.

[15] Brouwers JF, Gadella BM. In situ detection and localization of lipid peroxidation in individual bovine sperm cells. Free Radic Biol Med 2003;35:1382–91.

[16] De Andrade AFC. Effects that seminal plasma addition has on capacitation-like changes (cryocapacitation) in cryopreserved equine spermatozoa. Ph.D. Dissertation. Pirassununga, SP: University of São Paulo, 2009.

[17] Zaffalon FG. Capacitation-like changes in bovine semen after cryopreservation using extenders within egg yolk or soy lecithin. Masters Degree Thesis. Pirassununga, SP: University of São Paulo, 2009.

[18] De Andrade AF, Zaffalon FG, Celeghini EC, Nascimento J, Bressan FF, Martins SM, et al. Post-thaw addition of seminal plasma reduces tyrosine phosphorylation on the surface of cryopreserved equine sperm, but does not reduce lipid peroxidation. Theriogenology 2012;77:1866–72.

[19] Beletti ME, Costa LdaF. A systematic approach to multispecies sperm morphometric characterization. Anal Quant Cytol Histol 2003;25:97–107.

[20] Beletti ME, Costa LF, Guardieiro MM. Morphometric features and chromatin condensation abnormalities evaluated by toluidine blue staining in bull spermatozoa. Braz J Morphol Sci 2005;22:85–90.

[21] Beletti ME, Costa LdaF, Viana MP. A comparison of morphometric characteristics of sperm from fertile Bos taurus and Bos indicus bulls in Brazil. Anim Reprod Sci 2005;85:105–16.
[22] Kanayama CY, Beletti ME. Computational evaluation of chromatin condensation and morphometric characteristics of rabbit (Oryctolagus cuniculus) sperm head. Arq Bras Med Vet Zootec 2011;63:94–9.
[23] Oliveira LZ, Santos RM, Hossepian de Lima VFM, Arruda RP, De Andrade AFC, Guimarães Junior V, et al. Chromatin and morphometry alterations of bovine sperm head after Percoll density gradient centrifugation. Livest Sci 2011;141:267–71.
[24] DeJarnette JM, Shepard RW, Kaproth MT, Michael NA, Dalton JC, Goodell GM, et al. Effects of sequential insemination number after batch-thawing on conception rates of cryopreserved bovine semen: a review, in Proceedings of the 19th Technical Conference on Artificial Insemination and Reproduction, NAAB, 2002; 102–8.
[25] DeJarnette JM, Marshall CE, Lenz RW, Monke DR. Sustaining the fertility of artificially inseminated dairy cattle: the role of the artificial insemination industry. J Dairy Sci; 2004; 87(E Suppl): E93–104.
[26] Goodell G. Comparison of AI pregnancy rates in dairy cattle by order of preparation of insemination straws. J Anim Sci 2000; 78(Suppl 1):229.
[27] Kaproth MT, Parks JE, Grambo GC, Rycroft HE, Hertl JA, Gröhn YT. Effect of preparing and loading multiple insemination guns on conception rate in two large commercial dairy herds. Theriogenology 2002;57:909–21.
[28] Dalton JC, Ahmadzadeh A, Shafii B, Price WJ, DeJarnette JM. Effect of simultaneous thawing of multiple 0.5-mL straws of semen and sequence of insemination on conception rate in dairy cattle. J Dairy Sci 2004;87:972–5.
[29] Sprenger MJ, DeJarnette JM, Marshall CE. Conception rates of sequential inseminations after batch-thawing multiple straws of semen: a professional technician case study. J Anim Sci 2001; 79(Suppl 1):253.
[30] Lee CN, Huang TZ, Sagayaga AB. Conception rates in dairy cattle are affected by the number of semen straws thawed for breeding. J Dairy Sci 1997;80(Suppl 1):151.
[31] Aitken J, Fisher H. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. Bioessays 1994;16:259–66.
[32] Storey BT. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Mol Hum Reprod 1997;3:203–13.
[33] Guthrie HD, Welch GR. Use of fluorescence-activated flow cytometry to determine membrane lipid peroxidation during hypothermic liquid storage and freeze-thawing of viable boar sperm loaded with 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid. J Anim Sci 2007;85:1402–11.
[34] Kasimanickam R, Kasimanickam V, Thatcher CD, Nebel RL, Cassell BG. Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters, and competitive index in dairy bulls. Theriogenology 2007;67: 1004–12.
[35] Ostermeier GC, Sargeant GA, Yandell BS, Evenson DP, Parrish JJ. Relationship of bull fertility to sperm nuclear shape. J Androl 2001;22:595–603.