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The influence of insemination dose on pregnancy per fixed-time artificial insemination in beef cows is affected by semen extender

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Abstract

The aim of this study was to compare the efficacy of two extenders (Tris-egg yolk - TRIS and Botu-Bov® - BB, Botupharma, Brazil) for bovine semen cryopreservation with a different number of sperm per straw (6, 12, 25 or 50 x 10^6 sperm per straw) on post-thaw seminal viability (experiment 1) and pregnancy per artificial insemination (P/AI; experiment 2). In experiment 1, higher values of linearity and straightness associated to low amplitude of lateral head displacement (ALH) were observed in samples cryopreserved with BB extender, when compared to samples cryopreserved with TRIS extender ($P < 0.05$) regardless of the sperm concentration per straw. The pregnancy rates were 57.63, 60.32, 59.26 and 62.50% respectively for 6, 12, 25 or 50 x 10^6 sperm/straw in BB samples and 45.61, 48.84, 60.34 and 70.59% respectively for the TRIS extender. Increasing the number of sperm had a significant effect on P/AI ($P < 0.05$) when TRIS extender was used. In conclusion, Botu-Bov® extender promotes better post-thaw sperm movement. The increase in the number of sperm cells per insemination dose improved P/AI rates in *Bos indicus* lactating beef cows inseminated when using Tris-egg yolk extender.

Keywords: bovine, cryopreservation, fixed time artificial insemination, semen extender, sperm number.

Introduction

The minimum sperm number necessary to obtain acceptable fertility rates is still a great challenge for the bovine artificial insemination (AI) industry (Foote and Kaproth, 2002). This minimal effective insemination dose is essential to optimize the use of genetically superior sires (Bucher *et al*., 2009) and thus guarantee higher numbers of inseminated cows (Den Daas *et al*., 1998) and better economic return.

It has previously been recognized that the number of spermatozoa inseminated could be a limiting factor in fertility (Bratton *et al*., 1954), especially due to the great variability among bulls (Den Daas *et al*., 1998). However different reports indicate that the increase in the number of sperm per insemination dose promotes higher numbers of accessory sperm per oocyte, resulting in better embryo quality, increasing fertilization (Dejarnette *et al*., 1992; Nadir *et al*., 1993) and conception rates (Gérard and Humblot, 1991; Shannon and Vishwanath, 1995; Andersson *et al*., 2004). One of the alternatives to increase the efficiency of AI technology for single and multiple ovulations in cows is the increase in the number of sperm per insemination dose (Dalton *et al*., 1999). This strategy could be especially interesting when associated with protocols for estrus synchronization or fixed-time AI (FTAI), resulting in higher pregnancy per insemination and, consequently, higher economic return.

Although extension of semen to low-sperm numbers per AI dose has been related to a decrease in bull sperm viability *in vitro* (Garner *et al*., 2001; Ballester *et al*., 2007), the effect of increasing the sperm concentration per straw (starting with a regular sperm AI dose) on post-thaw quality semen parameters has not been reported for bull semen. Additionally, the interaction between sperm cells and the extender is an essential factor in the preservation of sperm integrity and fertilizing ability (Manjunath *et al*., 2002). Furthermore, due to the reduced technological innovations on semen cryopreservation during recent years (Celeghini *et al*., 2008), the Tris-egg yolk-fructose extender is still the most commonly employed worldwide (Crespilho *et al*., 2012).

Thus, the objectives of the present study were to evaluate the effect of the cryopreservation extender and sperm concentration per 0.5 ml straw on *in vitro* bovine frozen semen viability (experiment 1) and pregnancy per AI (P/AI) observed in cows submitted to FTAI (experiment 2).

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Materials and Methods

Semen collection

Semen samples were obtained by electroejaculation from 14 Nellore (*Bos taurus indicus*) bulls aging 24 to 30 months old. For experiment 1, two ejaculates from each bull were obtained (the first used for initial screening of seminal quality and the second used for experimental procedures). The intervals between semen collections from the same bull varied between 2 and 7 days. The criteria adopted for animal inclusion in the study were total motility of fresh semen >70%, number of cells presenting major defects <20% and number of cells presenting minor defects <25%. For experiment *2*, ejaculates were obtained by electroejaculation from 7 bulls used in experiment 1.

Semen processing

Immediately after collection, the ejaculates were evaluated for total motility (0 to 100% scale) and sperm vigor (0 to 5 scale) under light microscope and total sperm concentration was determined by Neubauer counting chamber. Two freezing extenders were used: Tris-egg yolk fructose (TRIS; 30 g Tris-[hydroxymethyl aminomethane], 17 g citric acid, 12.5 g fructose, 0.20 g amikacin sulfate, 2 ml Orvum Est Pastum [OEP, Procter and Gamble, Cincinnati, Ohio, USA], 200 ml egg yolk and 64 ml 87% glycerol for 1000 ml total solution, according to Crespilho *et al*., 2012) and Botu-Bov® extender (BB; Botupharma Ltda, Botucatu, SP, Brazil). All the chemical reagents used (except the OEP) to prepare the Tris extender were from Merck Pharmaceutical (Darmstadt, Germany).

After collection, each semen sample was fractionated into 8 equal aliquots, diluted in TRIS or BB extender to either 6, 12, 25 or 50 x 10^6 total sperm concentrations, totaling 8 experimental groups (TRIS6 and BB6; TRIS12 and BB12, TRIS25 and BB25; TRIS50 and BB50, respectively).

After dilution, samples were packaged in 0.5 ml straws (IMV® Technologies, L'Aigle Cedex, France) and transferred to a digital programmable refrigerator (Minitübe®, Tiefenbach, Germany) to stabilize at 5ºC for 4 h.

Freezing was performed in nitrogen vapor (N_2) by placing the samples 5 cm above liquid nitrogen level in a 40 l isothermic box for 20 min. After this period, samples were immersed directly into N_2 and stored in a cryobiological container. Laboratorial analysis was performed after at least 3 days of storage.

Experiment 1

In the first experiment the effects of the freezing extender and sperm concentration were verified by computer-assisted sperm analysis (CASA - Hamilton Thorn Research IVOS-12, Beverly, USA) and evaluation of plasma membrane integrity.

CASA

Frozen samples were thawed in a water bath at 37°C for 30 sec, homogenized and evaluated in pre-warmed Makler chamber at 37ºC; five aleatory fields with at least 150 spermatozoa were observed.

Sperm parameters given by CASA and analyzed in the present study were total motility (MoT), 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 rapid cells (RAP). The parameters for motility analysis were: 30 frames acquired at 60 frames per second; minimum contrast 50, minimum cell size 6 pixels; lower VAP cut-off 30 µm/sec; lower VSL cut-off 20 µm/sec; VAP cut-off for progressive cells 40 µm/sec and straightness 60%, according to Celeghini *et al*. (2008).

Assessment of plasma membrane integrity

Plasma membrane integrity (PMI, %) was determined by a combination of fluorescent probes carboxyfluorescein diacetate (CFDA) and propidium iodide (PI), adapted from Harrison and Vickers (1990). One aliquot of 50 µl from each semen sample was diluted in 50 µl sodium citrate 2.94% solution at 37ºC, then another 50 µl of fluorescent working solution (1.0 ml 2.94% sodium citrate, 20 µl buffered formaldehyde saline, and 60 µl PI and 20 µl CFDA stock solutions) was added. Diluted samples were evaluated under epifluorescence microscope at 400X (Leika®, Solms, Germany) and two distinct groups of cells were identified: cells with intact plasma membrane (green) or cells with damaged plasma membrane (red). A total of 200 cells from each sample were evaluated.

Experiment 2

From the 14 bulls used in experiment 1, 7 were selected (based on phenotypic semen traits) for experiment 2. A second collection was performed 3 days after the first collection and semen was evaluated as described previously.

Semen processing

Ejaculates from the 7 selected bulls were collected during the summer in the Southern hemisphere, pooled and cryopreserved according to the method described in experiment 1. This pool of semen was divided into the same 8 experimental groups, according to freezing extender and sperm concentration

(TRIS or BB and 6, 12, 25 or 50 x 10^6 total sperm/straw). Semen samples were evaluated (4 replicates) according to the methodology described in experiment 1 to confirm sufficient maintenance of total motility (≥60%) and plasmatic membrane integrity (≥30%) post-thaw, prior to use in AI.

Fixed-time artificial insemination (FTAI)

Frozen samples of the semen pool were used for FTAI of 475 suckled Nellore (n = 410) or crossbreed cows (n = 65) maintained exclusively in *Brachiaria decumbens* pasture with mineral supplementation *ad libitum*. Only multiparous cows were included in this experiment. On the first day of the FTAI protocol all cows had their body condition evaluated using a 1-5 scale (1= emaciated, 5 = obese, according to Ayres *et al*., 2009). All FTAI programs were performed during the summer breeding season in a commercial farm in Mato Grosso do Sul State, Brazil (latitude -18° 55' 55"; longitude 54° 50' 39").

After calving, suckled cows were allocated into breeding groups according to calving date. Time of ovulation was synchronized in all cows by an estradiol/progestin based FTAI protocol. The protocol was initiated between 30 and 60 days postpartum. Regardless of the stage of the estrous cycle, on the first day of the synchronization protocol cows received an auricular implant (Crestar®, MSD Animal Health, Cruzeiro, Brazil) and i.m. injections of 3.0 mg Norgestomet and 5.0 mg estradiol valerate (both of MSD Animal Health, Cruzeiro, Brazil). Ear implants were removed after nine days and i.m. injection of 400 UI of eCG (Folligon®, MSD Animal Health, Cruzeiro, Brazil) was administered. Artificial inseminations were performed 50-54 h after implant removal by a single inseminator.

Ultrasound examinations

Ovaries were examined by transrectal ultrasonography (5 MHz, Aloka-SSD 500, Tokyo, Japan) at implant withdrawal, at FTAI and 48 h later. Ovulation was defined as the disappearance of a follicle ≥8.0 mm (Gimenes *et al*., 2008) between two consecutive ultrasound scannings. Ovulations were considered to have occurred in cows before FTAI (i.e. between implant withdrawal and FTAI exams), after FTAI (i.e. within 48 h after FTAI) or did not occur (i.e. the same large follicle was present at all ultrasound examinations).

All cows were examined for pregnancy by transrectal ultrasonography on day 30 after FTAI. The detection of an embryonic vesicle with a viable embryo (presence of heartbeat) was used as an indicator of pregnancy. Pregnancy per AI was calculated as the number of cows pregnant on day 30 after FTAI divided by the number of cows inseminated.

Statistical analysis

Sperm parameters given by CASA and fluorescent analysis were analyzed by univariate, repeated-measures analysis of variance using the GLM procedure SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). For experiment 1, freezing extenders and sperm concentration were considered to be fixed factors, whereas bull was considered to be random.

Conception results of experiment 2 were analyzed by multiple logistic regression using Proc Logistic 9.1.3 computer program (SAS; SAS Institute Inc., Cary, NC, 1999). The effects of body condition score of inseminated cows, semen extender, sperm concentration and their interactions were included in the model. Significant differences were considered when $P < 0.05$ and a tendency occurred when $0.1 < P \leq 0.05$.

Results

Experiment 1

Differences in mean values of ALH, BCF, STR and LIN were observed between extenders, with no effect of sperm concentration (Table1). Specifically for VCL, superior results ($P < 0.05$) were obtained with samples frozen in the TRIS extender when compared to those frozen in the BB extender (P = 0.040), except for samples frozen with 50 x 10^6 sperm/straw (BB50 = $122.5^a \pm 8.2$ *vs*. TRIS50 = 136.8 ± 8.4^a, P = 0.2312). The proportion of intact cells after thawing did not differ between the extenders used.

Samples frozen in the TRIS extender did not differ in any of the examined post-thaw parameters, regardless of the sperm concentration. When the Botu-Bov® extender was used, however, there were differences in STR (P = 0.0028) and LIN (P = 0.0168) as a function of the number of sperm per straw.

Experiment 2

Statistically significant differences were not observed among the 8 experimental pool semen groups in the percent total motility (BB6 = 70.00 ± 3.89 *vs*. TRIS6 = 63.25 ± 5.99; BB12 = 66.25 ± 1.38 *vs*. TRIS12 = 66.25 ± 1.55; BB25 = 72.00 ± 1.58 *vs*. TRIS25 = 65.25 ± 3.47; BB50 = 60.75 ± 2.69 *vs*. TRIS50 = 63.50 ± 0.65; P = 0.078) and percentage of intact membranes post-thaw (BB6 = 37.50 ± 2.36 *vs*. TRIS6 = 33.25 ± 4.03; BB12 = 35.50 ± 3.30 *vs*. TRIS12 = 37.25 ± 6.49; BB25 = 39.50 ± 1.55 *vs*. TRIS25 = 36.50 ± 2.50; BB50 = 32.50 ± 1.03 *vs*. TRIS50 = 34.25 ± 3.84; P = 0.811).

Averages of ovulation and conception rates in this experiment were 85.89% (408/475) and 49.47% (235/475), respectively. Considering only those cows presenting ovulation as a response to the synchronization protocol, the P/AI rate was 57.60% (235/408).

Linear or quadratic effects for body condition score (BCS) of inseminated cows were not observed (mean BCS = 2.4 ± 0.2). Nevertheless, a linear and compensatory effect of the increase in sperm concentration on conception rates for FTAI (P = 0.0132) was observed (Fig. 1).

A tendency for an effect of semen extender on conception rates was observed (P = 0.0830) and a significant interaction between number of sperm per insemination dose and TRIS extender was observed (P = 0.0126); the increase in the number of inseminated sperm did not increase conception rates when the BB extender was used (P = 0.6781; Table 1 and Fig. 2).

Table 1. Influence of bull semen extender and sperm concentration (6, 12, 25 or 50 x 10^6) on total motility (MoT, %), 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, %) rapid sperm (Rap, %), plasmatic membrane integrity (PMI, %) and average pregnancy per AI in fixed-time inseminated beef cows (P/AI, %).

	Treatments							
	TRIS				Botu-Bov®			
Parameters	6	12	25	50	6	12	25	50
MoT (%)	62.1 ± 4.2^{a}	65.5 ± 3.4^{a}	70.3 ± 3.9^{a}	58.9 ± 3.1^{a}	58.3 ± 2.8^{a}	66.3 ± 3.6^{a}	66.1 ± 3.1^{a}	63.9 ± 3.8^{a}
PM (%)	44.9 ± 4.0^{a}	48.1 ± 4.3^{a}	48.3 ± 4.4^{a}	40.2 ± 3.7^{a}	49.6 ± 3.6^{a}	57.3 ± 4.0^{a}	53.9 ± 3.6^{a}	48.6 ± 4.0^{a}
VAP (µm/s)	100.6 ± 8.0^{a}	95.6 ± 5.6^{a}	87.8 ± 5.7^{a}	83.7 ± 5.4^{a}	88.6 ± 5.1^{a}	8 7.3 ± 4.3^{a}	80.6 ± 3.9^{a}	82.2 ± 5.3^{a}
VSL (µm/s)	81.2 ± 5.9^{a}	78.2 ± 4.4^{a}	70.5 ± 4.2^{a}	68.7 ± 3.5^{a}	81.6 ± 5.3^{a}	79.3 ± 4.2^{a}	71.0 ± 4.0^{a}	72.3 ± 4.7^{a}
VCL (µm/s)	163.5 ± 13.5^{aA}	150.9 ± 10.1^{aA}	140.4 ± 9.9^{aA}	136.8 ± 8.4^{a}	120.8 ± 7.1^{aB}	121.1 ± 6.1^{aB}	115.9 ± 5.6^{aB}	122.5 ± 8.2^{a}
ALH (µm)	6.7 ± 0.4^{aA}	6.3 ± 0.4^{aA}	6.3 ± 0.3^{aA}	6.2 ± 0.2^{aA}	4.6 ± 0.4^{aB}	4.6 ± 0.3^{aB}	4.9 ± 0.3^{aB}	5.3 ± 0.3^{aB}
BCF (Hz)	24.4 ± 1.2^{aA}	25.3 ± 1.5^{aA}	26.7 ± 1.2^{aA}	25.4 ± 1.1^{aA}	31.1 ± 1.0^{aB}	31.7 ± 1.3^{aB}	30.6 ± 0.9^{aB}	28.6 ± 1.1^{aB}
STR (%)	82.4 ± 0.9^{aA}	83.3 ± 1.2^{aA}	81.2 ± 0.7^{aA}	81.3 ± 1.0^{aA}	91.4 ± 1.3^{aB}	90.7 ± 1.1^{abB}	87.6 ± 1.5^{abB}	84.9 ± 1.4^{bB}
LIN (%)	54.4 ± 1.5^{aA}	55.9 ± 1.8^{bA}	54.9 ± 1.7^{aA}	52.8 ± 1.2^{aA}	69.1 ± 2.4^{aB}	68.2 ± 2.3^{abB}	63.9 ± 2.5^{abB}	59.4 ± 2.1^{bB}
Rap (%)	54.8 ± 4.9^{a}	55.4 ± 4.7^{a}	58.6 ± 5.5^{a}	47.4 ± 4.5^{a}	51.0 ± 3.8^{a}	60.1 ± 4.2^{a}	57.5 ± 3.8^{a}	55.4 ± 5.0^{a}
PMI (%)	33.9 ± 2.3^{a}	31.8 ± 2.9^{a}	31.2 ± 2.5^{a}	26.9 ± 2.5^{a}	31.7 ± 2.5^{a}	31.1 ± 2.3^{a}	30.4 ± 2.2^{a}	30.2 ± 2.4^{a}
*P/AI (%)	45.61	48.84	60.34	70.59	57.63	60.31	59.26	62.50

^{a,b,c}Different letters in the same row indicate statistical differences between sperm concentrations within each semen extender (P < 0.05). ^{A,B}Capital letters in the same row indicate the differences between samples cryopreserved with BB or TRIS at the same sperm concentration. *Estimated extender effect, P = 0.0836; Interaction between concentration and TRIS dilluent, P = 0.0126; Interaction between concentration and BB, P = 0.6781; Effect of sperm concentration (for both extenders) per insemination dose, P = 0.0125.

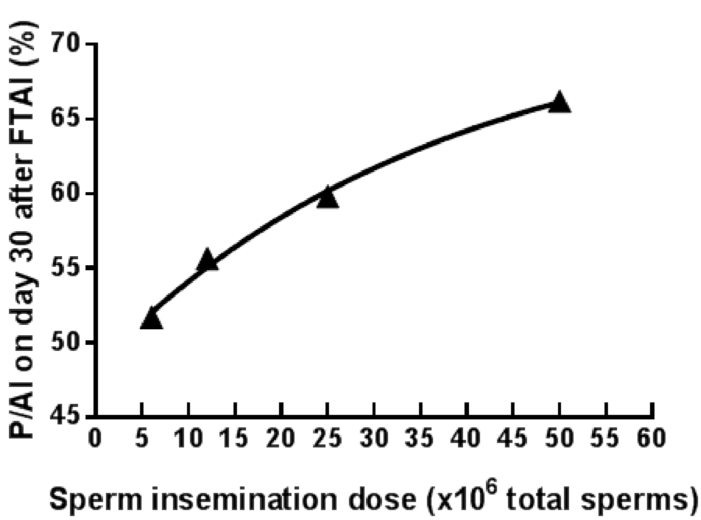

Figure 1. Effect of insemination dose on average pregnancy per artificial insemination (P/AI) obtained for cows inseminated after estrus synchronization and had confirmed ovulation with ultrasonography. There was a significant effect (P = 0.0132) for the number of sperm cells per insemination dose on the P/AI after fixed-time artificial insemination protocol in suckled *Bos indicus* cows.

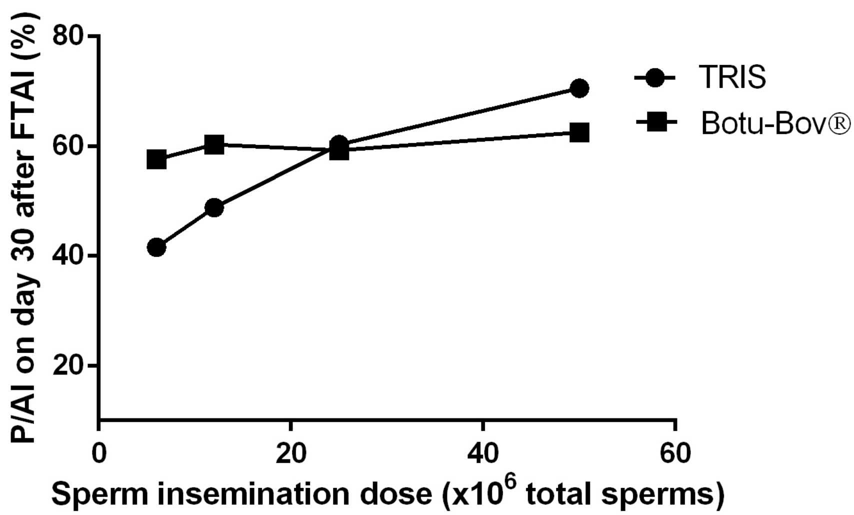

Figure 2. Relation between increasing number of sperm per insemination doses and average pregnancy per artificial insemination (P/AI) in fixed-time inseminated *Bos indicus* cows according to the semen extender. There was an improvement of pregnancy per AI due to the increase in the number of sperm per insemination dose using the TRIS extender (P = 0.0126), however no effect was observed (P = 0.6781) with an increase in the number of sperm per insemination dose when the BB extender was used.

Discussion

In the present study, there were no differences in progressive motility among all the 8 experimental groups, indicating that this parameter was not influenced by sperm concentration. Other authors, in contrast, reported a significant influence of the extender on the index of sperm presenting progressive motility (PM), as observed by a gradual decrease in PM when the viscosity of the medium was increased (Hirai *et al*., 1997). The lipid particles found on egg yolk based extenders could also play a deleterious role on progressive motility, acting as a physical barrier for spermatozoa, influencing natural sperm trajectory (Crespilho *et al*., 2012). In the present experiment, however, both extenders were centrifuged during the preparation, which may have diminished eventual differences in sperm progressivity due to the reduced viscosity of the extenders.

In relation to those variables that express sperm velocity during the pathway, significant differences were observed only in VCL values as a function of the extender in samples containing 6, 12 and 25 x 10^6 total sperm, pointing to higher post-thaw VCL results in samples cryopreserved with the TRIS-egg yolk fructose extender (Table 1). The relation between sperm velocity parameters and fertility rates is still not clearly defined in literature. However, Verstegen *et al*. (2002) reported that the values for VAP, VSL and VCL were significantly greater in samples that produced >50% of the fertilized oocytes than in those that fertilized <50% of the oocytes, demonstrating a positive correlation among sperm velocity and *in vitro* fertilization rates.

The results of the present study demonstrate that sperm cryopreserved in the TRIS extender present a smaller proportion of post-thaw straightness and linearity when compared to sperm processed in the BB extender, regardless of the concentration. It is possible to presume that for less linear - and thus more circular - sperm samples, a minor interference in straight movement patterns should be observed, which could justify the conflicting data presented for both extenders in this experiment.

Significant differences were also observed in ALH, BCF, STR and LIN parameters given by CASA, evidencing a markable effect of the extender on the higher values of BCF, STR and LIN presented by sperm cryopreserved with the BB extender indicating that those cells had more linear movement when compared to those cells preserved with the TRIS extender. Similar results were reported by Verberckmoes *et al*. (2005) who compared the efficiency of 3 extenders for the preservation of bovine fresh semen and suggested that higher values of VSL, BCF, STR and LIN promoted by the CEP-2 extender should be related to a better vigor and straightness of bovine sperm.

Among several parameters given by CASA, sperm linearity seems to be the parameter that is best correlated to the fertilizing potential of bovine frozen semen (Januskauskas *et al*., 2001; Martínez-Rodriguez, 2005). Hallap *et al*. (2004) reported a negative correlation (P < 0.05) between the proportion of motile non-linear sperm and non-return rates after artificial insemination.

Januskauskas *et al*. (2001) observed a significant positive correlation (r = 0.82) between sperm linearity and total post-thaw motility. In the present study, there was a significantly higher pattern of linearity presented by sperm cryopreserved with the BB extender when compared to those preserved by the TRIS extender, which may explain the numeric, though not significant (P = 0.078), superiority of total motillity promoted by the BB extender (Table 1).

Higher values of VCL and ALH observed in sperm cryopreserved with the TRIS extender may be related to an increased proportion of sperm hyperactivation with this extender. Hyperactivated bovine sperm present extremely lateralized movement, characterizing a pattern classified as "star-shaped movement" or "8-shaped movement" (Tardiff *et al*., 1997; Marquez and Suarez, 2004), which are a kinetic presentation that may be objectively evaluated by computer-assisted sperm analysis (Kavac *et al*., 2003). According to Verstegen *et al*. (2002), high values of VCL and ALH given by CASA may correspond to motion characteristics frequently used to describe a hyperactivated state.

Despite the physiologic importance of the hyperactivation process regarding gamete transport and oocyte penetration (Ho and Suarez, 2001), this motion pattern indicates a high degree of sperm cryoinjury (Centola *et al*., 1998; Hallap *et al*., 2004; Muiño *et al*., 2009), which may explain the poorer laboratorial post-thaw results obtained with TRIS extender.

Bilodeau and Panich (2002) indicated that the Tris-egg yolk extender presents a low capacity to neutralize reactive oxygen species (ROS) during the cryopreservation process, thus allowing the formation of substances primarily responsible for the control of cell permeability and capacitation. It is possible to presume that cells cryopreserved with the TRIS extender are more predisposed to undergo alterations compatible with the hyperactivated state. Reports similar to those obtained in the present study were reported by Tardiff *et al*. (1997), who observed an increased proportion of sperm hyperactivation with the use of the Tris extender when compared to the Cornell University extender.

Different studies report the marked variability among individual fertility rates ("bull effect"), with significant differences among animals on conception rates related to the number of inseminated sperm (Jondet, 1972; Den Daas *et al*., 1998; Nehring and Rothe, 2003; Andersson *et al*., 2004), the fertility rate and the number of recovered embryos after embryo transfer (Misra *et al*., 1999; Saacke *et al*., 2000), the cleavage rate and early embryonic development in IVF systems (Ward *et al*., 2001) and the maintenance of pregnancy after artificial insemination (Lima *et al*., 2004). In the present study, these interactions were minimized with the use of heterospermic insemination (pooled semen) and by the similarity in motion parameters and membrane integrity for the 8 experimental semen groups.

The average P/AI was 49.47% (for all cows inseminated) and 57.60% (only for cows with a synchronized ovulation, determined by three consecutive ultrasound ovarian scans), regardless of the extender and the insemination dose. These data agree with those reported by Sá Filho *et al*. (2009) and Crespilho *et al*. (2012) using the same insemination procedure.

In the present study, a significant and linear effect (P = 0.0132) of the increased sperm concentration on P/AI rate was observed in ovulated cows submitted to estrus and ovulation synchronization for FTAI (Fig. 1). Sperm failure to establish contact with the oocyte after AI represents the primary origin of failure in the process of fertilization (Hawk, 1986). Although there are a small number of sperm cells at the fertilization site when compared to the number of inseminated sperm (Januskauskas and Zilinskas, 2002), positive correlations are reported among increased insemination dose and the number of sperm reaching the oviduct and the number of accessory sperm cells per oocyte (Hawk, 1986; Larsson and Larsson, 1986; Saacke *et al.,* 2000). It is thus acceptable to suggest that the increase in insemination dose probably promotes higher numbers of viable sperm present in the cow's oviduct, which explains the significant increase in conception rates.

In view of the different experiments demonstrating the influence of semen extenders and their particular components on motion patterns of post-thaw bovine semen (Hirai *et al*., 1997; Moussa *et al*., 2002) and *in vitro* or *in vivo* fertility (Kommisrud *et al*., 1996; Thun *et al*., 2002; Amirat *et al*., 2005; Crespilho *et al*., 2012), it may be concluded that semen samples cryopreserved with Botu-Bov® extender present linear fertility with 6×10^6 total sperm or more. However, a significant positive effect of increased sperm concentrations on FTAI conception rates was observed when semen samples were cryopreserved with the Tris-egg yolk fructose extender (Fig. 2), suggesting that there is a compensatory effect of the insemination dose for decreased sperm parameters observed in laboratory analysis.

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