v. 3, n. 10, 2023

Journal of Agricultural Sciences Research

VIABILITY OF SPERMATOZOA REFRIGERATED AT 5 °C FOR 120 HOURS: EFFICIENCY OF DIFFERENT EXTENDERS IN THE MAINTENANCE OF BUFFER SEMEN FOR USE IN FTAI

Jaci de Almeida
http://lattes.cnpq.br/0496544397311361

Osvaldo Almeida Resende
http://lattes.cnpq.br/8880214526674810

Abstract: The objective of the present study was to verify the effectiveness of different extenders used for cooling buffalo semen in relation to maintenance of sperm longevity throughout processing. Seminal samples of 11 Murrah bulls aged 5 to 6 years were preserved at 5 °C for 120 hours in a passive refrigeration system (refrigerated counter) using TRIS extenders with 10% LDL (replacing egg yolk), Tolera D® (soy lecithin replacing egg yolk) and (Botu-Bov®). These samples were subjectively evaluated at T0 (post-collection), T1, T24, T48, T72, T96 and T120 hours post-dilution and refrigeration through computerized sperm movement analysis (CASA), plasmatic membrane integrity (Hyposmotic Test). Each male had his ejaculate analyzed for the physical and morphological characteristics of the semen, which were divided into three aliquots for each of the extenders. The values obtained were submitted to the Friedman Test ($P<0.05$). From the results achieved, it can be concluded that there was a better preservation of the samples kept under refrigeration with the Botu-Bov® extender at 5 °C/120 hours, providing greater protection against injuries and integrity of the spermatic membrane, when compared to the others. Thus, the Botu-Bov® extender can be an alternative for the use of refrigerated buffalo semen in AI and TAI for up to 48 hours.
Keywords: sperm longevity, computerized semen analysis, hyposmotic test.

INTRODUCTION

The economic viability of a livestock production system is directly linked to productivity rates and these are directly affected by reproductive efficiency and genetic selection of herds. Increased productivity in national livestock can be obtained with the use of biotechniques that optimize the production system, seeking greater production per hectare and thus reducing production costs. Celeghini (2005) reported that the use of biotechniques applied to reproduction makes it possible to maximize the use of superior genetics, enabling an increase in productivity.

Several reproductive biotechniques are used in Brazil, however, the most widespread is artificial insemination (AI). AI with cryopreserved semen is a more affordable reproductive biotechnique that serves as a tool for the genetic improvement of herds, including the Bubalus bubalis species. However, AI for buffalo is based on the use of frozen semen (Ribeiro et al., 1994). Garner et al. (2001) reported that despite the advances made in recent years, cell freezing still damages sperm organelles and membranes, also inducing changes in sperm capacitation and acrossomal reaction. According to Rasul et al. (2001), when analyzing buffalo spermatozoa using the CASA method (computerized semen analysis), concluded that the process causes damage to the axoneme, reducing the ability of sperm to move.

The decline in sperm viability and fertility related to cryopreservation motivated the development of new studies to address the preservation of semen in refrigerated form for the buffalo species (Dhami and Sahni, 1994; Adeel et al., 2009; Akhter et al., 2011b ; Singh et al., 2012; Almeida et al., 2015; Almeida et al., 2016a; Almeida et al., 2017 and Almeida et al., 2020a, 2020b; 2021), also low-density lipoproteins (LDLs) have been the subject of research for chilled (Almeida et al., 2016b) and frozen buffalo semen (Akhter et al., 2011a; Brito, 2014; El-Badry et al., 2015 and Almeida et al., 2020a, 2020b; 2021). However, there is a paucity of information on the use of LDLs for chilled buffalo semen. The main advantage of this type of processing is the prevention of injuries related to the freezing process, ensuring greater sperm viability which in turn allows for a reduction in the insemination dose, optimization of high genetic merit breeders in

artificial insemination programs, lower cost of storage, in addition to its ease of use in AI studies (Verberckmoes et al., 2005 and Bucker et al., 2009). All these associated advantages act positively in reducing animal production costs, enabling the implementation and dissemination of techniques such as AI and TAI in the most diverse national livestock production systems.

Considering that the storage time influences the viability of refrigerated semen (Batellier et al., 2001), one of the main indications for the use of buffalo semen preserved under refrigeration are FTAI programs (Almeida et al., 2015; Almeida et al, 2016a; Almeida et al., 2017; Almeida et al., 2021, 2022). The protocols allow the insemination of a large number of animals, in a short period of time (Bucker et al., 2009), allowing the incorporation of semen cooling technology even to the detriment of the low longevity of these samples. In this sense, refrigerated buffalo semen represents a promising alternative to cryopreserved semen, and can act to increase conception rates in AI and FTAI programs, reducing their final costs (Almeida et al., 2020c; 2021; 2022).

This study was carried out with the aim of comparing the pre- and post-refrigeration quality of buffalo semen diluted in three different diluting media (TRIS 10% LDL, Tolera D® and Botu-Bov®) in relation to sperm kinetics patterns by analysis (CASA) and sperm membrane integrity by the Hypoosmotic Test (HOST) for use in AI and TAI programs for buffaloes.

MATERIAL AND METHODS

The study was carried out after approval by the Committee on Ethics in the Use of Animals (CEUA) of the Veterinary School of ``Universidade Federal de Minas Gerais`` (EV/UFMG), with the approval of number 368/2015, according to the ethical principles of animal experimentation.

The experiment was conducted at the Bubalinoculture Biotechnology Center, located at the UFMG's Experimental Sustainable Production Farm, in Pedro Leopoldo, Minas Gerais, with an average altitude of 710 meters, latitude 19o 37' 05" S and longitude 44o 02' 35" W.

Eleven Murrah buffaloes, aged 4 and 5 years, were kept on pasture throughout the experiment, with salt and water ad libitum. These animals were already conditioned to semen collection with an artificial vagina and were maintained on a weekly collection basis throughout the entire period of the experiment.

Semen collections were performed using an artificial vagina at a temperature of 42-45 °C, with a false mount (in order to increase sperm volume and concentration) prior to semen collection (second mount). Immediately after collection, each ejaculate was submitted to analyzes of the physical and morphological characteristics of the semen according to the CBRA Manual (2013). Only ejaculates with ≥ 70% motility, vigor ≥ 3 and sperm abnormalities ≤ 30% and with at least 600x106 SPTZ/mL were used. Each ejaculate was divided into 3 aliquots, each sample being diluted in one of the extenders to obtain 50x106 SPTZ/mL. The bottling was carried out in straws of 0.5 mL, packed in a plastic bag submerged in a container with water (1.4 L) at 27 ºC.

The flask was placed in an environment at 5 ºC (refrigerated counter) obtaining a cooling curve of 0.25 ºC/minute (from 27 to 5 ºC/ average of the evaluated period). The straws were kept refrigerated at 5°C post-dilution for up to 72 hours.

To obtain LDLs (replacing egg yolk), fresh eggs produced on a commercial farm were used. The eggs were properly cleaned and submitted to the technique described by Neves et al. (2008). Composition of TRIS: TRIS

(2.42g), citric acid (1.36 g), Fructose (1.0 g), Amikacin (83.4 µg/mL), Equex 0.4%, LDL (10 mL) and Autoclaved Milli-Q® H2O (complete 100 mL), adapted from Brito (2014), with all reagents used from Sigma-Aldrich®. The other two extenders used are commercial products, the first with soy lecithin replacing the egg yolk (Tolera D® - ``Inprenha Bioctecnologia e Desenvolvimento Avançado`` S.A., São Paulo, Brazil) and the second (Botu-Bov® - BotuPharma Biotecnologia Animal, São Paulo Brazil).

The evaluation of sperm motility immediately after the final dilution was performed subjectively (Nikon 200 phase contrast microscope - T0 hours). Before the subsequent evaluations (T1, T24, T48, T72, T96 and T120 hours post-dilution with refrigeration at 5 oC), one straw being reheated at 37 ºC/30 seconds. An aliquot of each sample was deposited in a 1.5 mL Eppendorf® tube and incubated in a "dry bath" at 37 °C/5 minutes, until they were submitted to motility analysis by the CASA computerized system (model Sperm Class Analyzer - SCA ® v.4.0). The setup used for buffalo semen analysis was: particle area (20 to 70 microns2), VCL (10 < Slow < 25; > 25 and Medium < 50), Progressivity (> 70% STR), Circular (< 50% LIN), Points for the VAP (5) and Connectivity (12). For CASA analysis, 5 µL drops of each sample were placed between slide and cover slip, previously maintained at 37 °C, using 5 captured fields, homogeneous, with a minimum of 200 spermatic cells in each one for the analysis of each sample from them.

Following the methodology proposed by Farell et al. (1998) used, for the weightings on the effectiveness of the extenders used, regarding the kinetic parameters of the CASA, that set of parameters that, in the aforementioned study, presented the highest correlation with fertility. These parameters were: Total Motility (MT), Progressive Motility (MP), Curvilinear Velocity (VCL), Path Velocity (VAP), Progressive Velocity (VSL), Linearity (LIN), Straightness (STR), Oscillation Index (WOB), Lateral Head Displacement Amplitude (ALH) and Tail Beat Frequency (BCF).

To perform sperm concentration, cell count was used in the Neubauer hematocytometric camera at a dilution of 1:200, in formalin-saline-buffered solution (Hancock, 1957), also under conventional optical microscopy at 400x magnification (CBRA, 2013).

For the evaluation of sperm morphology, an aliquot of 50 µL of semen was added to 1000 µL of formalin-saline-buffered solution (Hancock, 1957), depositing these evaluation contents in conical Eppendorff® tubes of 1.5 mL, and the analysis was performed in a humid chamber, with a phase contrast microscope at 1000x magnification. The pathologies were classified into major defects (DM) and normal, as recommended by (CBRA, 2013).

For the hyposmotic test (HOST), an aliquot of 50 µL of semen was added to 500 µL of a hyposmotic solution containing fructose-sodium citrate at 121 mOsm/L (2.702 g of fructose + 1.324 g of sodium citrate). This solution was incubated for 30 minutes in a dry bath at 37 ºC and fixed in 200 µL of buffered saline formol, for later analysis in a phase contrast microscope (x1000), 200 cells being counted according to the presence or absence of a tail coiled (Melo et al., 2005).

In order to carry out the statistical analysis, a sample design was developed using 3 extenders, 6 evaluations carried out at different times and 11 breeders, configuring a blocking scheme, characterizing three sources of variation (extensor, refrigeration time and bull). Because it is a randomized block design with repeated measurements for each treatment and animal, the Friedman test was the most appropriate, which was carried out by comparing pair by pair (pairwise

comparison). The statistical package used was STATA 12.0 Statistical Analysis Software (Statacorp, 2012).

RESULTS AND DISCUSSION

The physical characteristics of the in natura ejaculates used for the experiment are presented in table 1.

Semen analysis is a simple assessment, but with a high degree of importance. In Brazil, according to the Manual of andrology and semen evaluation (CBRA, 2013), to be considered a fertile buffalo, the animal must have the following characteristics in fresh semen 300-1200x106SPTZ/mL, vortex ≥ 3, above 70% motile spermatozoa, vigor ≥ 3 and more than 70% of spermatozoa with normal morphology, and in frozen semen: ≥ 10x106SPTZ motile, motility ≥ 30%, vigor ≥ 2, normal SPTZ ≥ 70% and major defects ≤ 10.

For in natura semen, all 11 bulls analyzed (Table 1) had values for sperm parameters above the minimum values recommended by CBRA (2013). The association of the good values obtained in the studied seminal parameters, allows to make a good estimate of the fertilizing capacity of a given sample (Severo, 2009), however the andrological selection can provide the choice of superior sires, but by itself it is not able to predict effectively the cooling and freezing potential of semen. This is because the extender often does not equally protect the spermatozoa from the ejaculate of different bulls, highlighting the differences between the animals and their genetic material.

The good parameters found for the in natura semen of the animals studied are partly due to the fact that they have been in a weekly seminal collection regime for at least 3 years. And also associated with performing a false mount (diversion of the penis without performing the semen collection, which was only performed on the second copulation attempt, this to obtain an ejaculate with greater volume and better concentration, since the animal will be prestimulated). However, sperm volume and concentration are variable, due to seasonality, climate, time of day, period of sexual rest of the animal and collection method (artificial vagina, electroejaculator and/or massage of the ampoules of the vas deferens).

Salvador et al. (2008) reported that high initial sperm motility in in natura semen does not, by itself, guarantee good performance in freezing, but allows animals with high rates of motility drop to still show approval in post-freezing semen. According to Cox et al. (2006) sperm motility is commonly identified as one of the most important characteristics associated with the fertilizing ability of sperm. Verstegen et al. (2002) reported that CASA-assisted sperm analysis provides an objective classification of a given sperm population and is considered to be one of the most reliable methods for assessing semen quality.

According to Chacur (1996) and Chacur et al. (1997), the analysis of multiple attributes of sperm movement using the CASA technique and the determination of sperm viability using cytoplasmic membrane analysis techniques can add greater sensitivity to the in vitro evaluation of buffalo semen.

Computerized semen analysis was standardized using 5 μL of each sample, waiting 5 minutes for sedimentation and then choosing five fields from a total of nine, using a concentration of 50x106 SPTZ/mL, trying to maintain the maximum possible homogeneity between the analyzed samples.

For the post-dilution semen, there was no significant difference (P>0.05) for the sperm motility parameters (Table 2), with these being above 96% for Total Motility (MT) and 79% for Motility Progressive (MP).

There is a scarcity of literature on the

Animals	Vol. (mL)	Turb. (0-5)	MS (%)	Force (0-5)	Concentration (x10^6/mL)	DM (%)	Normal (%)
1	3	3	85	3	840	7	76
2	1,8	3	80	3	625	8	72
3	5	3	80	3	3195	5	81
4	1	3	85	4	1190	6	78
5	1	3	85	3	1135	10	70
6	1,1	4	90	4	1005	4	81
7	1	3	85	3	1190	7	71
8	2,1	3	80	3	930	9	75
9	2,1	4	90	4	985	6	79
10	3,5	4	90	3	1645	9	72
11	2,6	5	95	4	1425	3	86
Average ±DP	**2,2±1,3**	**3,5±0,7**	**85,9±4,9**	**3,4±0,5**	**1287,7±658,2**	**6,7±2,1**	**76,5±4,8**

Caption: Vol. = volume; Turbo. = Whirlwind; MS = Subjective motility; DM = Major defects; SD = Standard Deviation.

Table 1 - Characteristics of the ejaculates of 11 buffaloes evaluated immediately after collection.

Sperm parameters	Time (hours)	Extenders		
		TRIS 10% LDL	Tolera D®	Botu-Bov®
MT (%)	1	96,3^a	96,1^a	96,5^a
	24	90,9^b	89,2^b	93,8^a
	48	88,0^a	87,5^a	91,3^a
	72	78,4^{ab}	75,7^b	85,1^a
	96	62,0^a	55,9^b	73,7^a
	120	44,2^b	45,1^{ab}	57,0^a
MP (%)	1	81,4^a	79,5^a	82,2^a
	24	72,8^a	64,8^b	75,8^a
	48	64,2^a	62,4^a	67,2^a
	72	50,4^{ab}	48,5^b	61,2^a
	96	35,0^b	29,6^b	48,5^a
	120	21,6^b	23,1^b	34,6^a
VCL (µm/s)	1	89,0^{ab}	98,0^a	80,6^b
	24	82,2^a	94,6^a	71,4^b
	48	89,9^a	81,8^{ab}	71,2^b
	72	85,0^a	69,8^b	63,8^b
	96	77,9^a	67,5^b	72,5^{ab}
	120	57,4^{ab}	64,1^a	50,8^b
VAP (µm/s)	1	57,2^b	76,2^a	63,0^b
	24	56,2^b	70,9^a	63,6^b
	48	55,3^a	62,5^a	56,2^a
	72	56,3^a	44,5^a	47,4^a
	96	48,4^a	43,1^a	46,2^a
	120	32,6^{ab}	37,1^a	29,0^b

VSL (µm/s)	1	32,0[b]	48,4[a]	49,2[a]
	24	32,6[b]	53,2[a]	52,1[a]
	48	27,9[b]	44,2[a]	47,6[a]
	72	21,7[b]	39,8[a]	43,9[a]
	96	25,4[b]	29,0[ab]	41,4[a]
	120	18,1[a]	20,1[a]	22,2[a]
LIN (%)	1	36,5[c]	51,1[b]	59,4[a]
	24	36,9[c]	56,2[b]	68,9[a]
	48	33,2[c]	57,4[b]	65,7[a]
	72	31,3[c]	48,8[b]	69,9[a]
	96	31,5[c]	41,7[b]	65,9[a]
	120	29,1[c]	37,4[b]	44,8[a]
STR (%)	1	59,5[c]	68,8[b]	80,4[a]
	24	58,3[c]	74,4[b]	85,0[a]
	48	45,1[c]	75,1[b]	85,1[a]
	72	49,4[c]	65,7[b]	89,0[a]
	96	57,9[c]	63,8[b]	90,3[a]
	120	48,7[c]	63,7[b]	75,5[a]
WOB (%)	1	62,3[b]	73,7[a]	74,9[a]
	24	65,4[c]	73,8[b]	79,4[a]
	48	67,4[b]	77,8[a]	78,1[a]
	72	62,4[b]	74,0[a]	78,0[a]
	96	62,2[b]	62,6[b]	73,3[a]
	120	54,6[a]	60,0[a]	60,5[a]
ALH (µm)	1	3,4[c]	3,0[b]	2,7[a]
	24	3,3[b]	3,2[b]	2,2[a]
	48	2,9[b]	2,6[ab]	2,2[a]
	72	3,0[b]	2,7[b]	2,0[a]
	96	3,0[a]	3,0[a]	2,8[a]
	120	2,7[a]	2,5[a]	2,4[a]
BCF (Hz)	1	10,0[b]	8,9[b]	11,1[a]
	24	10,3[a]	10,9[a]	11,5[a]
	48	10,2[b]	11,1[ab]	12,4[a]
	72	10,3[b]	10,7[b]	12,5[a]
	96	10,3[b]	10,2[b]	11,9[a]
	120	9,7[a]	10,4[a]	10,1[a]

Different letters in each sperm characteristic (line) differ statistically from each other (P<0.05). VCL: Curvilinear Velocity; VAP: Travel Speed; VSL: Progressive Speed; LIN: Linearity; STR: Straightness; WOB: Oscillation Index; ALH: Lateral Head Offset Amplitude; BCF and Tail Beat Rate. Values discussed by time for each extender and kinetic parameter (line).

Table 2 - Effects of different extenders used on semen from 11 buffaloes (1 ejaculate/donor), post-dilution (T1 hours - room temperature), and refrigerated (T24, T48, T72, 96 and 120 hours) for kinetic parameters sperm.

distribution of sperm velocity in fresh and chilled buffalo semen analyzed by the computerized CASA system. For fresh semen, the values of MT, MP and sperm concentration found in this study are higher than those reported by (Mahmoud et al., 2013). Similar values for MT were also reported by Becerra (2017) when evaluating fresh buffalo semen with TRIS extender medium with 10% LDL. However, for MP the author obtained a lower motility (< 40%).

For the other kinetic parameters, there was a statistically significant difference (P<0.05), observed in the seminal post-dilution, verifying that the Botu-Bov® extender presented the highest values for LIN, STR, WOB, ALH and BCF. This probably occurred due to the difference in composition and concentration of the components present for each of the extenders used, causing the osmolarity and viscosity of the medium to contribute to a variation in sperm kinetics.

Results similar to those found by Celegnini (2005) and that had been clarified by Mortiner (1997) and Hiraí et al. (1997), stating that the density of the medium can directly influence sperm velocity. Another point that contributed to the difference in viscosity is the presence of glycerol in the composition of the commercial media, which is not present in the TRIS 10% LDL medium, because the commercial media are indicated for cryopreservation. However, Almeida et al. (2015 - unpublished data) when testing the TRIS extender with 10 and 5% LDL with and without glycerol for cooling buffalo semen at 5 ºC did not find differences between treatments.

For the time of 24 hours after refrigeration, no statistically significant difference was observed between the TM extensors (P>0.05), but the MP showed a significant difference (P<0.05), with the highest percentages in favor of the TRIS medium with 10% LDL and Botu-Bov®, which remained above 70% (Table 2). However, it can be observed that the motility for the extensors did not have a sharp drop between in natura and refrigerated semen for up to 24 hours, showing that the refrigeration process was efficient in preserving the spermatozoa.

Becerra (2017) in a study with refrigerated semen for 24 hours, reported a MT close to 90% and MP below 50%, using the TRIS extender with 10% LDL. Tarig et al. (2017) when studying the effects of different concentrations of soy lecithin (LS) and virgin coconut oil in a TRIS extender for the quality parameters of bovine semen, found a motility of 81.0% for the extender with 1.25 % of LS.

Almeida et al. (2020a) reported MT and MP values greater than 89% and 64%, respectively, for 24 hours of refrigeration at 5 oC, when testing four different extenders. Previously Almeida et al. (2016b), when studying the cooled semen of buffaloes in different extenders for 24 hours, reported having found mean values for TM and PM of 81.8 and 53.5%, respectively. A lower result (60.0%) was obtained by Sing et al. (2012), when evaluating buffalo semen refrigerated at 5 ºC in TRIS extender medium for 24 hours.

As for the kinetic parameters VSL, LIN, STR, WOB, ALH and BCF, the Botu-Bov® extender was statistically superior to the others (P<0.05). However, it was verified that all extenders were efficient in preserving the semen during the first 24 hours of refrigeration, corroborating Almeida et al. (2020a). The Botu-Bov® extender presented the lowest values for ALH for the 24-hour evaluation period, indicating that the lateral displacement of the sperm head towards this medium tended to interfere less with cell progression.

Arruda (2003) reported that the highest numerical value of ALH translates into worse sperm quality. However, this value was mathematically determined and maximum

and minimum limits have not yet been established to say how much displacement of the head could interfere with the progressive motility or even the fertilization process of the oocyte (Celeghini, 2005 and Celeghini et al., 2008).

BCF was the same for extenders in refrigerated semen for 24 hours. This suggests that the extensor means were efficient in preserving the flagellar structures in the first 24 hours of refrigeration. However, given the superiority of the Botu-Bov® stent found for most of the evaluated parameters, this would be the stent chosen for use with 24 hours of refrigeration at 5 oC.

After 48 hours of refrigeration, no statistical difference was observed between the extenders for TM and MP (P>0.05), maintaining an MP above 60% for both extender media (Table 2). A similar result was obtained by Almeida et al. (2020a), when they evaluated buffalo semen refrigerated for up to 72 hours in four different extenders.

For the 48-hour period, the Botu-Bov® stent presented the highest values for the evaluated kinetic parameters, except for the VCL, where TRIS stents with 10% LDL and Tolera D® were superior. A conclusive explanation for this finding was not found, since the TM and MP did not differ between the extensors. However, Blesbois et al. (1999) reported the effects of refrigeration on the lipid components of the plasmatic membrane of bird spermatozoa, noting a decrease in total lipids from 820 to 620 µg/109 spermatozoa after 48 hours of storage at 5 oC and also a decrease in phospholipids of 75 to 60%. The authors concluded that there was a lysis of lipids, peroxidation and/or an endogenous metabolism of lipids in the sperm membrane capable of altering its composition, metabolism and fusion capacity.

Almeida et al. (2020a) reported values greater than 87.5 and 62.4% for MT and MP in buffalo semen refrigerated at 5 oC for 48 hours, respectively. Becerra (2017) found an MT close to 70% and an MP lower than 40% for semen refrigerated for 48 hours. Almeida et al. (2016b) when researching the refrigerated semen of buffaloes at 5 oC in different extenders for 48 hours, found for TM and PM (70.5 and 42.4%, respectively). A similar result for motility (41.7%) was obtained by Sing et al. (2012) when evaluating refrigerated semen of buffaloes at 5 ºC for 48 hours. A possible explanation for this lower motility value found by the authors is probably due to the different periods of semen collection and the different animals used. It is possible to observe that ALH was lower for the Botu-Bov® extender, with less interference in sperm progressivity (Table 2), which is desirable for an efficient extender, and the extender is recommended for use after 48 hours of refrigeration.

TRIS extenders with 10% LDL and Botu-Bov® were similarly efficient MT and MP for 72 hours of refrigeration (Table 2), with values greater than 78 and 50% being found for MT and PM, respectively. It becomes evident that the refrigeration for buffalo semen is highly efficient in preserving the spermatozoa, corroborating with Dhami and Sahni (1994). Similar results were obtained by Almeida et al. (2020a) when evaluating four different thinners for these same refrigeration times at 5 oC. However, lower results for TM (< 50%) and MP (< 20%) were found by Becerra (2017) when analyzing buffalo semen refrigerated for 72 hours.

Tarig et al. (2017) reported a motility of 63.5% for bovine semen refrigerated for 72 hours in medium containing 1.3% LS, a result higher than the 31.7% found by Sing et al. (2012), using the TRIS extender for 72 hours in the cooling of buffalo semen at 5 ºC. Working with refrigerated buffalo semen at 5 oC for 72 hours Almeida et al. (2016b) reported obtaining 55.9 and 30.9% for TM and PM.

The Botu-Bov® and Tolera D® stents showed similar values (P>0.05) for the VCL, VSL and WOB kinetic parameters. But for ALH and BCF, the Botu-Bov® medium was superior to the others (P<0.05), given the results obtained, the Botu-Bov® extender was superior to the others for most of the characteristics analyzed after refrigeration for 72 hours.

After 96 hours of refrigeration, the semen continues with good motility, with a MT greater than 70% being found for the Botu-Bov® extender. For PM Botu-Bov® showed a statistically significant difference (P<0.05) being superior to other extenders with more than 48% PM.

In a study carried out by Becerra (2017), the values found for TM and MP remained below 30%, indicating under the conditions in which the experiment was carried out, that refrigeration after 72 hours was not effective in preserving spermatozoa, thus preventing its use in reproductive insemination programs. A similar result was found by Almeida et al. (2016b) when studying the refrigerated semen of buffaloes for 96 hours in the CASA system, obtained 28.4% for TM and 18.1% for PM.

The kinetic parameters (LIN, STR, WOB and BCF) also remained superior for the Botu-Bov® extender. This statistical difference in favor of Botu-Bov® is probably due to a lower density and viscosity of the extender, since the motility for this medium was superior to Tolera D® and equal to TRIS with 10% LDL, indicating that with the As the days go by, the extender with soy lecithin tends to agglutinate and make it difficult for the sperm to move. As for VAP and ALH, the values found for both extenders were similar, showing that both extenders were efficiently able to preserve the refrigerated semen.

In the last evaluation with 120 hours, it is possible to verify a greater MT and MP for the Botu-Bov® extender, with values above 57 and 34%, respectively.

Making a comparison with frozen semen for the buffalo species, it appears that international standards postulate that at least 50% of sperm must be motile after thawing (Zhang et al., 1999). In the CBRA manual (2013), there is a recommendation to use post-thawed buffalo semen that has a minimum of 30% motility. As it can be seen in Table 2, if these assumptions are met, semen refrigerated for up to 120 hours can be used without a problem. However, given the ease of collection of the bull (conditioned to the artificial vagina) once present on the farm where the insemination work will be carried out and being able to use fresh semen after dilution and even refrigerated for 24 and 48 hours, it is not justified to use a semen with lower motility than that obtained with 48 hours. Not to mention that the risk of a reduced pregnancy rate after 48 hours of refrigeration is much greater.

Furthermore, maintaining a semen collection interval of 24 to 48 hours, it is possible to perform 2 to 3 collections per week from the same animals in order to use them in AI and FTAI programs, thus optimizing the animals and achieving inseminate a large number of females at the same time. The other kinetic parameters remained constant between the evaluated times, with the highest values always found for the Botu-Bov® extensor.

Becerra (2017) after 120 hours of refrigeration for buffalo semen, reported values for MT and PM of less than 20 and 10%, respectively.

The hyposmotic test (HOST) was chosen to assess the integrity of the plasma membrane, since a proven correlation was found between the results of the HOST test and the in vivo fertility rate (Brito et al., 2003). This test can therefore be used to predict fertility. In the post-dilution period, HOST showed a statistically significant difference between extenders (P<0.05), even so, all means were

efficient in protecting spermatozoa, as shown in Table 3.

The percentages of HOST-reactive spermatozoa found in this study for in natura semen were higher than the 84.5% reported by Mahmoud et al. (2013). Besides, it is higher than the 83.3% found by Becerra (2017) for in natura buffalo semen, using the TRIS extender with 10% LDL. Almeida et al. (2020a) obtained values ranging from 77.1 to 83.8% for in natura buffalo semen when comparing four different extenders for refrigeration.

Sperm parameters	Time (hours)	Extenders		
		TRIS 10% LDL	Tolera D®	Botu-Bov®
HOST (%)	1	91,5[b]	92,3[ab]	92,9[a]
	24	83,1[b]	83,6[b]	86,8[a]
	48	75,7[b]	76,6[b]	80,1[a]
	72	68,4[b]	69,6[b]	73,4[a]

Different letters in each sperm characteristic (line) differ statistically from each other (P<0.05). HOST: Hyposmotic Test. Values discussed by time for each extender and kinetic parameter (line).

Table 3 - Effect of different extenders used for semen from 11 buffaloes (1 ejaculate/donor), post-dilution (T0 hours - room temperature), and refrigerated at 5 oC (T1, T24, T48 and T72 hours) for sperm membrane integrity (HOST).

Another study, carried out with refrigerated semen of buffaloes at 5 ºC in a TRIS extender, using HOST to verify the integrity of the sperm membrane Sing et al. (2012) reported 62.0% of reactive sperm for post-dilution semen. In the present study, the HOST, even with high values for all the stents, was significantly higher (P<0.05) for the Botu-Bov® medium.

For spermatic membrane integrity after 24 hours of cooling, there were significant differences between the extenders (P<0.05), being favorable to the Botu-Bov® extender. Values lower than those found in this study for HOST were reported by Tarig et al. (2017) for bovine semen refrigerated for 24 hours (72.1%), in TRIS extender with coconut oil with 1.25% LS. Becerra (2017) reported a percentage of 70.8% of HOST-reactive spermatozoa for the 24-hour period of evaluation of buffalo semen refrigerated at 5 ºC. Sing et al. (2012) reported 48.7% of reactive sperm for semen 24 hours after refrigeration using the TRIS extender for buffalo semen.

After 48 hours of refrigeration at 5 ºC, there were significant differences between the stents (P<0.05), once again in favor of the Botu-Bov® stent. With the values obtained, it is possible to verify a high percentage of spermatozoa reactive to HOST, demonstrating that the extenders were efficient in protecting the spermatozoa.

Becerra (2017), for buffalo semen refrigerated for 48 hours in a TRIS extender with 10% LDL, obtained 74.7% of spermatozoa with intact spermatic membrane. In another study using the TRIS extender, Sing et al. (2012) reported 37.0% of reactive sperm for refrigerated buffalo semen.

As it can be seen in table 3, the extenders were effective in protecting the integrity of the spermatic membrane, for up to 72 hours, and for up to this observation time, the Botu-Bov® extender remained superior to the others. Lower value was reported by Tarig et al. (2017) for bovine semen refrigerated for 72 hours in a TRIS extender with coconut oil with 1.25% LS (58.7%). In another study related to sperm membrane integrity, Becerra (2017) reported having found 61.8% of HOST-reactive spermatozoa for buffalo semen refrigerated for 72 hours in TRIS extender with 10% LDL.

Ahmed et al. (2016) reported that the main parameters to predict the fertility of frozen and field-thawed buffalo semen are MP, rapid velocity, VAP, VSL and supravital plasma membrane integrity. If the same kinetic parameters are taken for the refrigerated semen of buffaloes at 5 ºC, it is observed that

the Botu-Bov® extender presented similar or superior values to the other means for most of the parameters in all studied times.

During the evaluations of the three extenders for refrigeration, for a period of up to 120 hours, there were several alternations between the extenders for some kinetic parameters analyzed, thus it is believed that the components present in the commercial extenders, mainly in Botu-Bov®, could be acting by stimulating the frequency of sperm cell beats through the production of ATP. However, the exact composition of these extenders is not disclosed due to industrial secrecy, making it impossible to comment further.

Even though there is no indication in the package insert for the use of commercial means (Botu-Bov® and Tolera D®) for the buffalo species, these as well as TRIS with 10% LDL were efficient in preserving the motility and integrity of the spermatic membrane, during refrigeration times under the conditions in which the experiment was carried out, thus allowing to recommend its use for refrigerated semen for up to 48 hours to be used in AI and TAI programs.

So far, cryopreservation methods and simple insemination techniques are not able to provide pregnancy percentages similar to those found with natural mating. Therefore, it is extremely important to develop research that seeks more effective methods, aiming at the production of excellent quality semen samples, which would allow obtaining good fertility rates. In view of the results obtained in this study and data from other studies mentioned here, refrigerated semen appears as an alternative to improve pregnancy rates in AI and TAI programs in buffalo farms.

CONCLUSIONS

It is concluded that the Botu-Bov® diluent was equal to or superior to most of the parameters evaluated by CASA, constituting a new viable option for an extender medium for cooling buffalo semen.

Sperm membrane integrity was better preserved by the Botu-Bov® extender for buffalo semen refrigerated at 5°C for up to 72 hours.

Based on the results obtained, it is possible to conclude that the Botu-Bov® extender shows potential to maintain semen quality, with individual motility, viability and integrity of the spermatic membrane during the liquid preservation of buffalo semen at 5 ºC. Therefore, it can be used as an alternative in AI and TAI programs for buffaloes for up to 48 hours, aiming to improve pregnancy rates.

THANKS

To the National Institute of Science and Technology (``INCT da Pecuária``) and the Coordination for the Improvement of Higher Education Personnel (CAPES), for providing part of the resources for the purchase of material for the experiment and for granting the scholarship, an indispensable factor for the progress and completion of the course.

REFERENCES

ADEEL, M.; IJAZ, A.; ALEEM, M.; REHMAN, H.; YOUSAF, M.S.; JABBAR, M.A. **Improvement of liquid and frozen-thawed semen quality of Nili-Ravi buffalo bulls (Bubalus bubalis) through supplementation of fat.** Theriogenology, v.71, p.1220-1225, 2009.

AHMED, H.; ANDRABI, S.M.H.; ANWAR, M.; JAHA, S. **Use of post-thaw semen quality parameters to predict fertility of water buffalo (Bubalus bubalis) bull during peak breeding season.** Andrologia, p.1-6, 2016.

AKHTER, S.; ANSARI, M.S.; RAKHA, B.A.; ANDRABI, S.M.H.; KHALID, M.; ULLAH, N. **Effect of low density lipoproteins in extender on freezability and fertility of buffalo (Bubalus bubalis) bull semen.** Theriogenology, v.76, p.759-764, 2011a.

AKHTER, S.; ANSARI, M.S.; RAKHA, B.A.; ULLAH, N.; ANDRABI, S.M.H.; KHALID, M. **In vitro evaluation of liquid-stored buffalo semen at 5 ºC diluted in soya-lecithin based extender (Bioxcell®), Tris-citric egg yolk, skim milk and egg yolk-citrate extenders.** Reproduction in Domestic Animals, v.46, p.45-49, 2011b.

ALMEIDA, J.; BRITO, M.F.; BECERRA, V.A.B.; NEVES, B.P.; AULER, P.A.; HADDAD, J.P.; HENRY, M. **Effectiveness of TES-TRIS or TRIS association with low density lipoprotein on in vitro longevity of refrigerated buffalo semen.** Arquivo Brasileiro de Medicina Veterinária e Zootecnia (online), v.72, p.729-736, 2020a.

ALMEIDA, J.; BRITO, M.F.; NEVES, B.P.; BECERRA, V.A.B.; AULER, P.A.; BARUSELLI, P.S.; HENRY, M. **Evaluation of pregnancy rates in milk buffaloes submitted to FTAI with Ovsynch or P4/E2 and eCG based protocols with refrigerated or frozen semen during favorable or unfavorable breeding season.** Abstracts: 34th Annual Meeting of the Brazilian Embryo Technology Society (SBTE). Animal Reproduction, v.17, n.3, 2020c.

ALMEIDA, J.; BRITO, M.; NEVES, B.P.; BECERRA, V.A.B.; AULER, P.A.; HADDAD, J.P.; BARUSELLI, P.S.; HENRY, M. **Use of cooled buffalo semen as a strategy to increase conception rates in fixed-time artificial insemination programs during unfavorable reproductive periods.** Arquivo Brasileiro de Medicina Veterinária e Zootecnia (online), v.73, n.3, p.560-570, 2021.

ALMEIDA, J.; BRITO, M.; NEVES, B.P.; AULER, P.A.; DUTRA, P.A.; SILVA, D.F.; BERGMAN, L.; PEÑUELA, P.A.O.; BARUSELLI, P.S.; HENRY, M. **Evaluation of the pregnancy rates in primiparous buffaloes submitted to FTAI with refrigerated semen vs. frozen during the unfavorable breeding station.** Proceedings of the 31st Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Cabo de Santo Agostinho, PE, Brazil, August 17th to 19th, 2017. Abstracts. 666, Animal Reproduction, Jul./Sept., v.14, n.3, p.666, 2017.

ALMEIDA, J.; HENRY, M.; RESENDE, A.O. **Viabilidade dos espermatozoides refrigerados a 5 °C por 120 horas: eficiência de diferentes por 120 horas: eficiência de diferentes diluidores na manutenção do sêmen bubalino para utilização na IATF.** Anais da VI Reunião Anual da ABRAA, Campinas, SP, 10 a 11 de junho, 2022, Animal Reproduction, p.220-221, 2022.

ALMEIDA, J.; NEVES, B.P.; BRITO, M.F.; BECERRA, V.A.B.; FREITAS, R.F.; LACERDA, L.G.; GRAPIUNA, L.S.; HADDAD, J.P.; AULER, P.A.; HENRY, M. **Impact of *in vitro* fertilization by refrigerated versus frozen buffalo semen on developmental competence of buffalo embryos.** Animal Reproduction, v.17, n.4, p.1-11, 2020b.

ALMEIDA, J.; NEVES, B.P.; BRITO, M.F.; AULER, P.A.; BARUSELLI, P.S.; HENRY, M. **Pregnancy rates of buffaloes (*Bubalus bubalis*) using cooled or frozen semen at fixed time artificial insemination (FTAI) - Preliminary results.** XI Congresso Mundial de Búfalos - Cartagena - Colômbia, Medicina Veterinária y Zootecnia, 183, Septiembre/Diciembre, 2016a.

ALMEIDA, J.; BECERRA, V.A.B.; NEVES, B.P.; AULER, P.A.; ANDRADE, G.O.; BRITO, M.F.; HENRY, M. **Refrigeração de sêmen bubalino (*Bubalus bubalis*) frente a diferentes extensores e avaliação da motilidade espermática no CASA.** Proceedings of the 30th Annual of the Brazilian Embryo Technology Society (SBTE), Foz do Iguaçú, PR, Brazil. August 25th to 27th, 2016, and 32th Meeting of the European Embryo Transfer Association (AETE), Barcelona, Spain, Septembre 9th and 10th, 2016. Abstracts. Animal Reproduction, Jul./Sept., v.13, n.3, p.557, 2016b.

ALMEIDA, J.; BRITO, M.F.; AULER, P.A.; MORAIS, C.M.L.; ANDRADE, G.O.; NEVES, B.P.; BARUSELLI, P.S.; HENRY, M. **Avaliação das taxas de prenhez em búfalas com o uso de sêmen refrigerado vs. congelado em programas de IATF durante a estação reprodutiva desfavorável.** Congresso Brasileiro de Reprodução Animal, Belo Horizonte, MG. Anais...Belo Horizonte: CBRA, v.21, n.99, 2015.

ARRUDA, R.P.; BALL, B.A.; GRAVANCE, C.G.; LIU, I.K.M. **Avaliação dos efeitos de diluidores e crioprotetores para espermatozoides de garanhões utilizando análises computadorizadas da motilidade (CASA) e citometria de fluxo.** Acta Scientiae Veterinariae, 31 (Suplemento), Porto Alegre: UFRGS, p.228-229, 2003.

BATELLIER, F.; VIDAMENT, M.; FAUQUANT, J.; DUCHAMP, G.; ARNAUD, G.; YVON, J.M.; MAGISTRINI, M. A**dvances in cooled semen technology.** Animal Reproduction Science, v.68, p.181-190, 2001.

BECERRA, V.A.B. **Efeito da adição de *Aniba canelilla,* blueberry e polifenol de chá verde sobre a viabilidade *in vitro* de espermatozoides de búfalo (*Bubalus bubalis*) resfriados a 5 ºC.** 2017. Dissertação (Mestrado) - Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, 69p., 2017.

BLESBOIS, E.; GRASSEAU, I.; HERMIER, D. **Changes in lipid content of fowl spermatozoa after liquid storage at 2 a 5 ºC.** Theriogenology, v.52, p.325-334, 1999.

BUCHER, A.; KASIMANICKAM, R.; HALL, J.B.; DEJARNETTE, J.M.; WHITTIER, W.D.; KÄHN, W.; XU, Z. **Fixed-time AI pregnancy rate following insemination with frozen-thawed or fresh-extended semen in progesterone supplemented CO-Synch protocol in beef cows.** Theriogenology, v.71, p.1180-1185, 2009.

BRITO, M.F. **Uso de lipoproteínas de baixa densidade, extraídas da gema de ovo, para a criopreservação do sêmen de búfalos.** 2014. Dissertação (Mestrado) - Universidade Federal de Minas Gerais, Belo Horizonte, 96p., 2014.

BRITO, L.F.; BARTH, A.D.; BILODEAU-GOESEEIS, S.; PANICH, P.L.; KASTELIC, J.P. **Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with *in vitro* fertilization rate.** Theriogenology, v.60, p.1539-1551, 2003.

Colégio Brasileiro de Reprodução Animal - **Manual para exame andrológico e avaliação de sêmen animal.** 3ª ed. Colégio Brasileiro de Reprodução Animal, Belo Horizonte. 104p., 2013.

CELEGHINE, E.C.C. **Efeitos da criopreservação do sêmen bovino sobre a membranas plasmática, acrossomal e mitocondrial e estrutura da cromatina dos espermatozoides utilizando sondas fluorescentes.** 2005. Tese (Doutorado) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 186p, 2005.

CELEGHINI, E.C.C.; ARRUDA, R.P.; ANDRADE, A.F.C.; NASCIMENTO, J.; RAPHAEL, C.F.; RODRIGUES, P.H. **Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin.** Animal Reproduction Science, v.104, n.2-4, p.119-131, 2008.

CHACUR, M.G.M. **Avaliação da congelação de sêmen bubalino com diluidores Glicina-Gema, Tryladil® e TES em diferentes tempos de equilíbrio**. 1996. Dissertação (Mestrado) - Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, 105p., 1996.

CHACUR, M.G.M.; OBA, E.; GONZALES, C.I.M. **Equilibrium time influence on motility, vigor and membrana integrity of thawed buffalo semen using Tryladil®, glycine-egg-york and TES extenders.** In: Word Buffalo Congress, 5, Caserta. Anais… Caserta, Italy, p.846-849, 1997.

COX, J.F.; ALFARO, V.; MONTENEGRO, V.; RODRIGUEZ-MARTINEZ, H. **Computer-assisted analysis of sperm motion in goats and its relationship with sperm migration in cervical mucus.** Theriogenology, v.66, p.860-867, 2006.

DHAMI, A.J.; SAHNI, K.L. **Effects of various cooling from 30 ºC to 5 ºC, equilibration and diluent treatments on freezability, post-thaw thermoresistance, enzyme leakage and fertility of bubaline spermatozoa.** Buffalo of Journal, v.2, p.147-159, 1994.

EL-BADRY, D.A.; RAWASH, Z.M.; MANAL, B.; ELAAL, A.B.D.; ALI, A.H. **The effect of LDL from different bird's eggs on the freezability of buffalo spermatozoa.** Global Veterinaria, v.14, n.2, p.158-165, 2015.

FARRELL, P.B.; PRESICCE, G.A.; BROCKETT, C.C.; FOOTE, R.H. **Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility.** Theriogenology, v.49, p.871-879, 1998.

GARNER, D.L.; THOMAS, C.A.; GRAVANCE, C.G.; MARSHALLB, C.E.; DEJARNETTE, J.M.; ALLENC, C.H. **Seminal plasma addition attenuates the dilution effect in bovine sperm.** Theriogenology, v.56, p.31-40, 2001.

HANCOCK, J.L. **The morphology of boar espermatozoa.** Journal of Reproductive Microscopy Society, v.76, p.84-97, 1957.

HIRAI, M.; CERBITO, W.A.; WIJAYAGUNAWARDANE, M.P.B.; BRAUN, J.; LEIDL, W.; OHOSAKI, K.; MATSUZAWA, T.; MIYAZAWA, K.; SATO, K. **The effect of viscosity of semen diluents on motility of bull spermatozoa**. Theriogenology, v.47, p.1463-1478, 1997.

MAHMOUD, K.G.M.; EL-SOKARY, A.A.E.; ABOU, EL ROOS, M.E.A.; ABDEL GHAFFAR, A.D.; NAWIT, M. **Sperm Characteristics in cryopreserved buffalo bull semen and field fertility.** Iranian Journal of Applied Animal Science, v.3, n.4, p.777-783, 2013.

MELO, M.I.V.; HENRY, M.; BEKER, A.R.C.L. **Teste hiposmótico para avaliação da viabilidade do sêmen equino resfriado com diferentes diluidores.** Arquivo Brasileiro de Medicina Veterinária e Zootecnia, v.57, n.6, p.757-763, 2005.

MORTIMER, S.T. **A critical review of the physiological importance and analysis of sperm movement in mammals.** Human Reproduction Update, v.3, n.5, p.403-439, 1997.

NEVES, M.M. **Extração das lipoproteínas de baixa densidade da gema do ovo de Gallus domesticus e sua aplicação na criopreservação do sêmen canino.** 2008. Tese (Doutorado) - Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, 116p., 2008.

RASUL, Z.; AHMAD, N.; ANZAR, M. **Changes in motion characteristics, plasma membrane integrity, and acrossome, morphology during cryopreservation of buffalo spermatozoa.** Journal of Andrology, v.22, n.2, p.278-283, 2001.

RIBEIRO, H.F.L.; LÁU, H.D.; SILVA, A.O.A.; SOUSA, J.S.; VALE, W.G. **Preliminary report on artificial insemination in buffaloes of the Amazon region, through imported semen.** In: Congresso mundial dos criadores de búfalos, 4, 3, 1994, São Paulo. Anais... São Paulo: Associação Brasileira dos Criadores de Búfalos, p.591-593, 1994.

SALVADOR, D.F.; ANDRADE, V.J.; VALE FILHO, V.R.; DIAS, J.C.; NOGUEIRA, L.A.G. **Associação entre o perfil andrológico e a congelação de sêmen de touros da raça Nelore aos dois anos de idade, pré-selecionados pela classificação andrológica por pontos (CAP).** Arquivo Brasileiro de Medicina Veterinária e Zootecnia, v.60, n.3, p.587-593, 2008.

SEVERO, N.C. **Influência da qualidade do sêmen bovino congelado sobre a fertilidade.** Hora Veterinária, ano 28, Jan-Fev, v.167, p.36-39, 2009.

SINGH, A.K.; SINGH, V.K.; NARWADE, B.M.; MOHANTY, T.K.; ATREJA, S.K. **Comparative quality assessment of buffalo (Bubalus bubalis) semen chilled (5 ºC) in egg yolk- and soya milk-based extenders**. Reproduction of Domestical Animal, v.47, p.596-600, 2012.

STATACORP. **Stata: Release 12. Statistical Software.** College Station, TX: StataCorp LP, 2012.

TARIG, A.A.; WAHID, H.; ROSNINA, Y.; YIMER, N.; GOH, Y.M.; BAIEE, F.H.; KHUMRAN, A.M.; SALMAN, H.; ASSI, M.A.; EBRAHIM, M. **Effect of different concentrations of soybean lecithin and virgin coconut oil in Tris-based extender on the quality of chilled and frozen-thawed bull semen.** Veterinary World, 10, June, p.772-778, 2017.

VERBERCKMOES, S.; SOOM, A.V.; DEWULF, J.; KRUIF, A. **Comparison of three diluents for the storage of fresh bovine semen.** Theriogenology, v.63, p.912-922, 2005.

VERSTEGEN, J.; IGUER-OUADA, M.; OCLIN, K. **Computer assisted semen analyzers in andrology research and veterinary pratice**. Theriogenology, v.57, n.1, p.149-179, 2002.

ZHANG, B.R.; LARSSON, B.; LUNDEHEIM, N.; HAARD, M.G.; RODRIGUEZ-MARTINEZ, H. **Prediction of bull fertility by combined in vitro assessments of frozen-thawed semen from young dairy bulls entering an AI-Programme.** International Journal of Andrology, v.22, p.253-260, 1999.