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Effect of the addition of sodium caseinate on the viability of cryopreserved buffalo semen

Efeito da adição de caseinato de sódio sobre a viabilidade do sêmen bubalino criopreservado

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Highlights:
An egg-yolk bovine media was evaluated for cryopreservation of buffalo semen.
The effect of adding sodium caseinate to this diluent was also studied.
The diluent was suitable for cryopreserving buffalo semen.
Freezing caused more damage to sperm cells than refrigeration.
Sodium caseinate did not lessen the harmful effects of cryopreservation.

Abstract

The use of cooled semen in artificial insemination operations results in higher pregnancy rates than the use of frozen semen. This result seems to be related to the more severe damage triggered by the freezing process than that observed during refrigeration. Due to its ability to bind to sperm-binding proteins and calcium ions, sodium caseinate has been studied as a substance capable of preventing early sperm capacitation, a significant cause of the decreased pregnancy rate resulting from the use of frozen semen. The first objective of this study was to evaluate whether a commercial egg yolk diluent developed for frozen bovine semen could be used for buffalo semen cryopreservation; the second objective was to investigate the effect of this diluent in combination with sodium caseinate during the procedures of buffalo sperm cryopreservation using flow cytometry and computer-assisted sperm analysis. In the first part of the study, comparing the results of spermatic kinetics and plasma and acrosomal membrane integrity, it was observed that the freezing process resulted in more cell damage than the cooling process. In the second part of the study, no effects of the addition of sodium caseinate to the egg yolk diluent were observed. From the results of the present study, it was possible to conclude that the egg yolk-based diluent was suitable for buffalo semen cryopreservation and that the addition of sodium caseinate did not decrease the harmful effects related to seminal cryopreservation.
Key words: BSP. Buffalo. Casein. Cryopreservation. Sperm.

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Resumo

O uso de sêmen resfriado em operações de inseminação artificial resulta em taxas de prenhez mais altas do que o uso de sêmen congelado. Esse resultado parece estar relacionado aos danos mais severos desencadeados pelo processo de congelação, quando comparado com o de refrigeração. Devido à sua capacidade de se ligar **às** proteínas ligadoras de espermatozoides e íons cálcio, o caseinato de sódio foi estudado quanto a sua capacidade de prevenir a capacitação espermática precoce, uma causa significativa de diminuição da taxa de prenhez com o uso de sêmen congelado. O primeiro objetivo deste estudo foi avaliar se um diluidor comercial a base de gema de ovo, destinado à congelação de sêmen bovino, poderia ser usado para a criopreservação de sêmen bubalino; o segundo objetivo foi investigar o efeito da adição do caseinato de sódio ao diluidor estudado durante os procedimentos de criopreservação de espermatozoides bubalinos. Foram empregadas a citometria de fluxo e a análise computadorizada do movimento espermático como métodos de avaliação seminal. Na primeira fase do estudo, comparando-se os resultados da cinética espermática e da integridade das membranas plasmática e acrossomal, observou-se que o processo de congelação promoveu mais danos celulares que o processo de resfriamento. Na segunda fase do estudo, não foram observados efeitos benéficos da adição de caseinato de sódio ao diluente empregado. A partir dos resultados do presente estudo, foi possível concluir que o diluente à base de gema de ovo foi adequado para a criopreservação do sêmen de bubalino e que a adição do caseinato de sódio não diminuiu os efeitos deletérios desencadeados pelo processo de criopreservação seminal.

Palavras-chave: BSP. Búfalo. Caseína. Criopreservação. Espermatozoide.

Introduction

Artificial insemination is performed predominantly using frozen semen; however, freezing procedures involve a series of potentially deleterious processes to spermatozoa, such as the formation of free radicals and intracellular ice crystals, which decrease sperm viability after thawing (Watson, 2000; Souza & Ferreira, 2007; Ugur et al., 2019).

A new alternative in fixed-time artificial insemination (FTAI) programs in cattle is the use of refrigerated semen with or without glycerol. In a study involving Nellore bulls, the conception rate was significantly higher with the use of 24-hour cooled semen diluted with Botu-Bov® with 7% glycerol than with 24-hour cooled semen diluted with Botu-Bov® without glycerol or frozen with the same diluent with 7% glycerol (51%, 44%, 41%, respectively) (Papa et al., 2015).

The reason that frozen semen presents with lower fertility rates than cooled semen is related to the higher degree of damage and cellular alteration inflicted on sperm by the freezing process, particularly those related to plasma membrane lipid loss (Watson, 2000).

Casein is an essential component of milk and represents approximately 75-80% of its proteins (Fox & McSweeney, 1998); this protein is an important molecule responsible for the cryoprotective properties of milk (Battelier et al., 1998, 2001). Similar to egg yolk, casein prevents the loss of lipids from the sperm membrane and decreases the binding of sperm-binding proteins (BSPs) to spermatozoa (Batellier, Magistrini, Fauquant, & Palmer, 1997; Bergeron, Brindle, Blondin, & Manjunath, 2007; Lusignan, Bergeron, Lafleur, & Manjunath, 2011; Pagl, Aurich, Müller-Schlösser, Kankkofer, & CAurich, 2006), a fundamental process in the adequate cryopreservation of bovine semen (Bergeron et al., 2007).

In a recent study conducted in our laboratory studying the effect of the addition of sodium caseinate to the commercial media Botu-Bov®, higher post-thawed sperm motility and a higher pregnancy rate after FTAI were obtained when the media contained 2% sodium caseinate (Diniz, 2017).

Thus, based on the previous results obtained in bovines, the objective of this work was to evaluate whether egg yolk-based Botu-Bov supplemented with 7% glycerol, with or without the addition of 2% sodium caseinate, is suitable for the cryopreservation of buffalo semen.

Material and Methods

Ethical aspects

The project was carried out according to the ethical recommendation of the Brazilian College of Animal Experimentation (CONCEA) and approved by the FMVZ-Unesp's Ethics Committee on the Use of Animals (permit number 0166/2018).

Animals

Nine Murrah bulls *(Bubalus bubalis)* aged two to ten years old were used. After semen collection performed with an artificial vagina, total motility, vigor, and spermatic concentration were determined. Then, using wet sperm preparation techniques, morphological evaluation of spermatozoa was performed under phase-contrast microscopy (400 X - Leyca - DM 500, Wetzlaretzlar - Germany). Only ejaculates with at least 70% motility, vigor 3 (on a scale of 0-5), major defects below 10%, and minor defects below 20% (Colégio Brasileiro de Reprodução Animal [CBRA], 2013) were used.

Experiment 1 (n= 9 ejaculates)

Seminal samples diluted to $30x10^6$ spermatozoa/mL with commercial egg yolk media Botu-Bov® with 7% glycerol (Botupharma, Botucatu/Brazil) were packed in 0.25 mL straws (IMV Technologies, L'Aigle Cedex, France) and kept in a polystyrene thermal box at 12°C for three hours while being transported from the farm to the faculty laboratory, where they were processed and evaluated according to the following groups: Control: assessed upon arrival at the laboratory; Refrigerated 24 H: refrigerated for 24 hours at 5°C; Refrigerated 48 H: refrigerated for 48 hours at 5°C; and Frozen. Before each evaluation, the samples were heated in a water bath at 37°C for 5 min.

Experiment 2 (n=12 ejaculates)

The seminal samples were divided into two aliquots and diluted to $30x10^6$ spermatozoa/mL with the same media as experiment 1 (Botu-Bov® with 7% glycerol) with or without 2% sodium caseinate. Then, the samples were packed in 0.25 mL straws (IMV Technologies, L'Aigle Cedex, France) and kept in a polystyrene thermal box at 12°C for three hours while being transported from the farm to the laboratory, where they were processed and evaluated according to the following groups: Control and Caseinate Control: assessed upon arrival at the laboratory; Refrigerated 24 H and Refrigerated Caseinate 24 H: refrigerated for 24 hours at 5°C; Refrigerated 48 H and Refrigerated Caseinate 48 H: refrigerated for 48 hours at 5°C; and Frozen and Frozen Caseinate. Before each evaluation, the straws were heated in water baths at 37°C for 5 min.

Cryopreservation protocols

In both experiments, the seminal samples were cooled in a passive cooling box (Botu-Flex®: Botupharma Ltda., Botucatu, São Paulo, Brazil) at a rate of 0.5°C/min down to 5°C.

For freezing, the initial cooling of the samples was performed in an automatic cooler (MiniTub®: Minitub do Brasil Ltda., Porto Alegre, Rio Grande do Sul, Brazil) at a rate of 3°C/min down to 5°C, and the samples were maintained at this temperature for four hours. Subsequently, the straws were transferred to a 45-L polystyrene isothermic box where they remained 3 cm above a 3.5 cm layer of a liquid nitrogen (NL) slide for 20 min; after

this period, the straws were immersed in the NL and then stored in a cryogenic container at -196°C (Zorzetto, 2013).

Semen analyses

For the evaluation of sperm kinetics, total motility (MT, %), progressive motility (MP, %), and percentage of sperm with rapid movements (RAP, %) were determined using computer-assisted sperm analysis (CASA, HTM-IVOS 12, Hamilton Thorne Research, Beverly, MA, USA).

Additionally, the samples from the frozen groups were submitted to a Slow Thermal Resistance Test (STRT), maintained at 37°C for two hours in a water bath, and then again evaluated for sperm kinetics.

Flow cytometry

After the evaluation of sperm kinetics, all samples, except those from the Control and Refrigerated groups in experiment 2, were analyzed by flow cytometry with a BD LSR Fortessa device (Becton Dickinson, Mountain View, CA, USA) equipped with the following excitation lasers: blue, 488 nm, 100 mW, with emission filters 530/30 nm and 695/40 nm; red, 640 nm, 40 mW with emission filter 660/20 nm; and violet, 405 nm, 100 mW, with emission filter 450/50 nm. In each test, at least 10,000 cells per sample were analyzed, and the results were analyzed by BD FACSDiva software™ v 6.1.

Initially, the samples were diluted at a concentration of 5 x 10^6 spermatozoa/mL in modified TALP-PVA medium (100 mM NaCl; 3.1 mM KCl; 25.0 mM $NaHCO_3$; 0.3 mM NaH_2PO_4; 21.6 mM DL-sodium lactate 60%; 2.0 mM $CaCl_2$; 0.4 mM $MgCl_2$; 10.0 mM HEPES-free acid; 1.0 mM sodium pyruvate; 1.0 mg/mL polyvinyl-PVA alcohol; and 25 µg/mL gentamicin) (Parrish, Susko-Parrish, Winer, & First, 1988) plus 7 µM Hoechst 33342 for elimination of debris and correct acquisition of only cellular events.

The association of propidium iodide (PI) and FITC-PSA (Pisum sativum agglutinin conjugated to fluorescein isothiocyanate) was used to evaluate the integrity of plasma and acrosome membranes according to Freitas-Dell'Aqua et al. (2012). For this, in a sample of 200 µL of diluted semen, 1.5 µM PI and 2 ng FITC-PSA were added, and the resulting mixture was incubated for 5 min under light.

In the evaluation of mitochondrial potential (PM) and superoxide (O_2^-) production in the mitochondrial matrix, the association of Yopro (YP; cell marker of destabilized plasma membranes), MitoStatus Red (MST; mitochondrial potential) and MitoSOXTM Red (MSR; generation of O_2^-anions in the mitochondrial matrix) was assessed according to the protocol already described (Diniz, 2017). For this, in a sample of 500 µL of diluted semen, 25 nM YP, 20 µM MST, and 2 µM MSR were added, and this mixture was incubated at 37°C for 20 min.

The association of CM-H2DCFDA (C6827, Life Technologies) with propidium iodide was used to evaluate the intracellular production of hydrogen peroxide (H_2O_2. For this purpose, 1.5 µM propidium iodide and 1 µM CM-H2DCFDA were added to 500 µL of diluted semen and incubated for 20 min at 37°C.

The probe C11-BODYPY (D-3861; Molecular Probes) was used for the evaluation of lipid peroxidation (PERO). For this, in a sample of 500 µL of diluted semen, 0.5 µL of C11BODIPY581/591 (1 mg/mL), 5 µL of IP (50 µg/mL), and 5 µL of H342 (100 µg/mL) were added and incubated for 30 min at 37°C. After incubation, two consecutive washes per centrifugation at 300 g for 5 min with TALP-PVA were performed, and the pellet was resuspended in 300 µL of TALP-PVA (Guasti et al., 2012).

Statistical analysis

All analyses were performed using the SigmaPlot 11.0 program. When only two treatments were compared, data with normal distributions were

evaluated by the t-test. For nonnormally distributed data, the Mann-Whitney nonparametric U test was employed. For the comparison of three or more treatments, ANOVA was used for data with normal distributions, and the Kruskal-Wallis test was used for nonnormally distributed data.

The results are presented as the means ± standard errors of the mean (SEMs), with $P < 0.05$ considered significant.

Results

Experiment 1

The values of MT, MP, and RAP were not different between the Control and Refrigerated 24 H groups; however, the frozen group presented lower values of these variables than the Control and Refrigerated 24 H groups. The Refrigerated 48 H group showed values similar to those of the Refrigerated 24 H group; however, its MT and RAP values were similar to those of the frozen group (table 1).

Table 1
Mean values ± EPM of the kinetics parameters of Murrah Buffalo *(B. bubalis)* semen cryopreserved with egg yolk-based media* (n= 9)

	Control	Refrigerated 24 H	Refrigerated 48 H	Frozen-thawed
MT (%)	85.0 ± 1.7^{a}	83.0 ± 3.0^{a}	74.0 ± 5.0^{ab}	43.0 ± 7.0^{b}
MP (%)	71.0 ± 1.9^{a}	66.0 ± 3.3^{ab}	56.0 ± 4.1^{b}	35.0 ± 5.6^{c}
RAP (%)	84.0 ± 1.8^{a}	81.0 ± 2.6^{a}	72.0 ± 5.3^{ab}	40.0 ± 6.7^{b}

* Botu-Bov® with 7% glycerol (Botupharma, Botucatu/Brazil), MT: Total motility, MP: progressive motility, RAP: percentage of spermatozoa with rapid movement. Different letters in the same line indicate statistical difference ($P<0.05$).

The concentration of O_2^- in the Control and Refrigerated 24 H groups was lower than that in the frozen group. The PERO indexes were similar between the Control and Refrigerated 24 H and 48 H groups and between the Refrigerated 24 H and 48 H and Frozen groups; however, the Frozen group had higher PERO indexes than the Control group. The concentration of H_2O_2 was lower in the frozen group only when compared with that in the Refrigerated 48 H group (table 2).

Table 2
Mean values ± EPM of the oxidative stress parameters of Murrah buffalo *(B. bubalis)* semen cryopreserved with egg yolk-based media* (n = 9)

	Control	Refrigerated 24 H	Refrigerated 48 H	Frozen-thawed
O_2^- (%)	21.8 ± 2.1^{a}	28.3 ± 3.2^{ab}	39.1 ± 5.8^{bc}	70.1 ± 6.5^{c}
O_2^- (UA)	106.0 ± 22.0^{a}	181.0 ± 49.0^{a}	356.0 ± 123.0^{ab}	940.0 ± 209.5^{b}
PERO (%)	2.5 ± 0.7^{a}	7.4 ± 2.2^{ab}	5.9 ± 1.6^{ab}	19.6 ± 4.6^{b}
H_2O_2 (UA)	65.0 ± 17.0^{ab}	60.0 ± 8.9^{ab}	70.1 ± 9.3^{a}	39.1 ± 3.6^{b}

* Botu-Bov® with 7% glycerol (Botupharma, Botucatu/Brazil), O_2^- (%): Percentage of cells with a high concentration of superoxide anion, O_2^- (UA): Level of superoxide anion in the total sample, PERO (%): Percentage of cells with lipid peroxidation, H_2O_2 (UA): hydrogen peroxide concentration in whole cells. Different letters in the same line indicate statistical difference ($P<0.05$).

The percentage of cells with plasma and acrosome membrane integrity (MPAI) and the mitochondrial potential of cells without membrane destabilization (PM) were lower in the frozen group. The percentage of cells without membrane destabilization (CSDM) and with high mitochondrial potential (APM) was lower in the Refrigerated 48 H and frozen groups (table 3).

Table 3
Mean values ± EPM of the sperm membranes destabilization and mitochondrial potential parameters of Murrah buffalo *(B. bubalis)* semen cryopreserved with egg yolk-based media* (n= 9)

	Control	Refrigerated 24 H	Refrigerated 48 H	Frozen-thawed
APM (%)	78.2 ± 2.1[a]	75.7 ± 2.2[a]	69.2 ± 3.3[b]	29.5 ± 5.6[c]
MPAI (%)	80.4 ± 2.3[a]	78.9 ± 2.2[a]	72 ± 3.2[a]	34.3 ± 6.3[b]
CSDM (%)	78.9 ± 1.9[a]	76.7 ± 2.3[a]	70.7 ± 3.4[b]	30.8 ± 5.7[b]
PM (UA)	12128.0 ± 2025.0[a]	9775.0 ± 1905.0[a]	6784.0 ± 1701.0[a]	1689.0 ± 542.0[b]

* Botu-Bov® with 7% glycerol (Botupharma, Botucatu/Brazil), APM: Percentage of cells with high mitochondrial potential; MPAI: Percentage of cells with intact plasma membrane and acrosome membrane; CSDM; Percentage of cells without membrane destabilization; PM: Qualitative evaluation of the mitochondrial potential of cells without membrane destabilization. Different letters on the same line indicate statistical difference (P<0.05).

Experiment 2

There was no effect of treatment on MT, MP, or RAP (table 4). The differences appeared only when pre- and post- STRT values were compared within the frozen groups (frozen and frozen caseinate groups) (Table 5).

Table 4
Mean values ± EPM of the sperm kinetics parameters of Murrah Buffalo *(B. bubalis)* semen cryopreserved with egg yolk-based media* with (CA) or without (BB) 2% sodium caseinate (n = 12).

	Control		Refrigerated 24 H		Refrigerated 48 H	
	Ca	Bb	Ca	Bb	Ca	Bb
MT (%)	89.2 ± 8.1	89.3 ± 8.1	87.0 ± 4.1	86.0 ± 5.1	70.9 ± 9.9	84.0 ± 5.3
MP (%)	40.6 ± 4.1	45.2 ± 5.2	49.0 ± 4.6	52.0 ± 5.5	36.8 ± 7.2	45.0 ± 5.8
RAP (%)	63.0 ± 6.9	69.1 ± 7.2	60.0 ± 8.0	64.0 ± 8.2	46.0 ± 10.0	54.0 ± 8.0

* Botu-Bov® with 7% glycerol (Botupharma, Botucatu/Brazil), MT: Total motility, MP: progressive motility, RAP: percentage of spermatozoa with rapid movement. CONTROL: diluted semen with diluent for freezing, 24H: 24 hours of refrigeration, 48 H: 48 hours of refrigeration.

Table 5
Mean values ± EPM of sperm kinetics parameters of Murrah Buffalo *(B. bubalis)* semen frozen with egg yolk-based media* with (CA) or without (BB) 2% sodium caseinate (n = 12)

	MT (%)		MP (%)		RAP (%)	
	Frozen-thawed	STRT	Frozen-thawed	STRT	Frozen-thawed	STRT
Ca	45.7 ± 6.5[aA]	11.0 ± 5.2[aB]	37.5 ± 5.5 [aA]	7.0 ± 3.8[aB]	42.5 ± 6.3[aA]	9.0 ± 5[aB]
Bb	42.9± 7.0[aA]	8.0 ± 4.8[aB]	36.6 ± 5.8[aA]	6.0 ± 4 [aB]	41.4 ± 6.9[aA]	6.8 ± 4[aB]

* Botu-Bov® with 7% glycerol (Botupharma, Botucatu/Brazil), MT: total motility, MP progressive motility, RAP: percentage of sperm with rapid movement. STRT: Frozen-thawed evaluated after slow thermal resistance test (2 h at 37°C). Different lowercase letters in the same column indicate statistical difference; different uppercase letters in the same row indicate statistical difference (P<0.05).

When the frozen and frozen caseinate groups were compared in terms of the morphofunctional variables related to oxidative stress, there was no difference between them, except for the percentage of cells with a high concentration of O_2^- after the STRT test, which was higher in the frozen group (table 6).

Table 6
Mean values ± EPM of oxidative stress parameters of Murrah buffalo *(B. bubalis)* semen frozen with egg yolk based media* with (CA) or without (BB) 2% sodium caseinate (n = 12)

	Frozen-thawed		STRT	
	CA	BB	CA	BB
O_2^- (%)	77.7 ± 4.8[a]	70.1 ± 6.1[a]	79.8 ± 4.0[a]	75.7 ± 5.1[a]
O_2^- (UA)	651.5 ± 54.7[a]	801.7 ± 97.3[a]	649.5± 47.5[a]	920.1 ± 85.8[b]
H_2O_2 (UA)	333.3 ± 167.7[a]	342.0 ± 254.7[a]	89.4 ± 8.3[a]	86.7 ± 13.6[a]
PERO (%)	24.5 ± 5.6[a]	21.3 ± 5.4[a]	12.2 ± 3.3[a]	24.5 ± 8.4[a]

* Botu-Bov® with 7% glycerol (Botupharma, Botucatu/Brazil), STRT: Frozen-thawed evaluated after slow thermal resistance test (2 h at 37°C), O_2^- (%): Percentage of cells with a high concentration of superoxide anion, O_2^- (UA): concentration of superoxide anion in the total sample, PERO (%): Percentage of cells with lipid peroxidation, H_2O_2 (UA): hydrogen peroxide concentration in total cells. Different letters in the same line indicate statistical difference (P<0.05).

Similarly, no differences were observed between the frozen and frozen caseinate groups for the variables related to the evaluation of sperm membranes, except for the PM, which was lower in the frozen caseinate group after STRT (table 7).

Table 7
Mean values ± EPM of the sperm membranes destabilization and mitochondrial potential parameters of Murrah buffalo *(B. bubalis)* semen frozen with egg yolk-based media* with (CA) or without (BB) 2% sodium caseinate (n = 12)

	Frozen-thawed		STRT	
	CA	BB	CA	BB
APM (%)	19.9 ± 5.1[a]	28.5 ± 5.9[a]	17.3 ± 3.4[a]	23.1 ± 5.2[a]
MPAI (%)	24.0 ± 5.6[a]	33.9 ± 6.7[a]	20.1 ± 3.6[a]	26 ± 5.7[a]
CSDM (%)	91.4 ± 2.86[a]	87.8 ± 3.5[a]	91.0 ± 3.3[a]	88.1 ± 3.7[a]
PM (UA)	1257.7 ± 702.2[a]	1222.7 ± 542.4[a]	509.6 ± 26.8[a]	549.6 ± 27.5[b]

* Botu-Bov® with 7% glycerol (Botupharma, Botucatu/Brazil), STRT: Frozen-thawed evaluated after slow thermal resistance test (2 h at 37°C), APM (%): Percentage of cells with high mitochondrial potential, MPAI (%): Percentage of cells with intact plasma membrane and acrosome, CSDM (%): Percentage of cells without membrane destabilization. PM (AU): Qualitative evaluation of the mitochondrial potential of cells without membrane destabilization. Different letters in the same line indicate statistical difference (P<0.05).

Discussion

The results of experiment 1 indicate that cooling buffalo semen is a promising possibility for use in FTAI programs, mainly if considered the results obtained in the first 24 hours of refrigeration. In cattle, the use and superiority of refrigerated semen, compared to post-thawed semen, in FTAI programs was reported for the first time in a study conducted in our department (Papa et al., 2015). Since then, its use has been progressively increasing (Borges-Silva et al., 2016; Tarragó, 2017; J. C. B. Silva et al., 2019). Similarly, the commercial media tested, Botu-Bov®, supplemented with 7% glycerol, although developed for cattle, proved to be suitable for buffaloes.

The results obtained after 24 hours of refrigeration were systematically higher than those obtained after thawing, except for the percentage of PERO and concentration of H_2O_2, which were similar between these two groups. Although a drop in the sperm quality index after 48 hours of refrigeration has previously been verified, the results observed in the Control group were quite similar to those noted for the Refrigerated 24 H group. When these results are analyzed from a biological point of view, they indicate the possibility of using semen refrigerated for 48 hours in FTAI programs. It should be noted that for some variables (MT, H_2O_2, APM, MPAI, PM), the results observed for Refrigerated groups were better than those found for the frozen group.

As well characterized in cattle, the reduced post-thawed fertility is due to the severity of cell damage that occurs during the freezing process (Watson, 2000). The evaluations carried out in our study also demonstrated that the freezing process was more harmful to buffalo spermatozoa than refrigeration. These results partially explain why Almeida (2018), employing the same media as our study, obtained a higher pregnancy rate in a buffalo FTAI program with 24-hour refrigerated semen than with post-thawed samples regardless of whether the inseminations were performed during favorable or unfavorable reproductive seasons (57.8% versus 31.1% and 48.2% versus 34.6%, respectively).

Cryocapacitation consists of a series of changes that occur in the plasma membrane of spermatozoa during the freezing process, which are quite similar to those observed during physiological capacitation (Bailey, Blodeau, & Cormier, 2000). Cryocapacitation is an important phenomenon that explains why the freezing process decreases sperm motility and the fertility of post-thawed semen (Watson, 1995).

Several indicators can be used to infer the occurrence of cryocapacitation, including the generation of reactive oxygen species and plasma membrane changes (Bailey et al., 2000). In experiment 1, cryocapacitation-signaling alterations, such as a higher percentage of cells with a high concentration of $O2^-$ and PERO, a higher level of $O2^-$ and H_2O_2, and a lower rate of cells without membrane destabilization, were evident in the frozen group.

Buffalo ejaculates with higher levels of BSPs have worse post-thawed quality (Singh et al., 2014); as BSPs are involved in the lipid changes of the capacitation process, the higher amount of these proteins can result in excessive removal of lipids from the plasma spermatozoa membrane (Manjunath, 2012). As sodium caseinate can bind to BSPs, preventing excessive membrane lipid loss, and sequester Ca^{+2}, it has been indicated as a potential molecule for preventing early capacitation (Batellier et al., 1997; Bergeron et al., 2007; Silva, Seidel, Squires, Graham, & Carnevale, 2012).

Unlike the observations by Diniz (2017) in cattle, in buffaloes, it was not possible to identify additional advantageous effects of the addition of sodium caseinate to the semen media studied. As the results of the post-thawed sperm variables were quite similar among the frozen and frozen caseinate groups, it was not possible to infer the protective effect of this molecule in preventing cryocapacitation in buffaloes. Even after STRT, these groups did not differ from each other.

Finally, at the concentrations used in this study, it is also worth mentioning that no harmful effect was detected regarding the addition of sodium caseinate to the commercial media used in our research. As casein is the main milk protein (Fox & McSweeney, 1998) and is responsible for its cryoprotective properties (Battelier et al., 1998, 2001), our results suggest that it is a potential substitute for milk in semen media. This possibility is of great importance for the development of new, chemically defined, and biologically safe media, an important goal in the seminal cryopreservation area (Olson & Seidel, 2000; Emamverdi et al., 2015).

Conclusion

Botu-Bov media®, with 7% glycerol, was successfully used in the cryopreservation of buffalo semen. However, the better-quality indicators observed for samples refrigerated for 24 hours compared with those found for the frozen samples indicate that refrigeration is an excellent alternative for FTAI programs.

The addition of sodium caseinate to the Botu-Bov media® did not prevent the appearance of cryocapacitation in the post-thawed samples, and no additional cryoprotective effect of its use was detected in this study. However, the addition of sodium caseinate also did not result in harmful effects on sperm quality, suggesting that this molecule may be an excellent candidate for the composition of chemically defined seminal media.

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