Ciências Agrárias
SEMINA

DOI: 10.5433/1679-0359.2022v43n6p2743

Equine semen cooled with chemically defined extender containing soy lecithin

Sêmen equino refrigerado com diluidor quimicamente definido contendo lecitina de soja

William Morais Machado[1]*; Thalita Marques de Brito[2]; Larissa Rodrigues Santana[3]; Maíra Guimarães Kersul[3]; Paola Pereira das Neves Snoeck[4]

Highlights

Inclusion of 2 to 6% soy lecithin in BWW medium preserves cooled semen.
BWW with 2, 4 and 6% soy lecithin preserves MT, VCL and LIN like BotuSemen®.
BWW containing lecithin preserves the structural integrity of cooled sperm.
BWW containing 1% lecithin is not able to preserve functional integrity.

Abstract

Equine semen has historically been chilled using milk-based media. However, the use of animal-based components presents several potential concerns, such as variability in formulations, microbial contamination and regulatory issues. We aimed to evaluate the potential of including different concentrations of soy lecithin (LS) in chemically defined Biggers, Whitten and Whittingham (BWW) medium for cooling equine semen to 15°C. Ejaculates were diluted as six different experimental groups: 1) BotuSêmen® (control); 2) BWW; 3) BWW + 1% LS; 4) BWW + 2% LS; 5) BWW + 4% LS and 6) BWW + 6% LS. BWW medium, did not preserve motility, velocity, straightness (STR), linearity (LIN), amplitude of lateral sperm head displacement (ALH), cross flagellar beat frequency (BCF), functional and structural integrity of equine spermatozoa during 24 h of refrigeration when compared to BotuSêmen® ($P < 0.05$). The use of BWW for cooling equine semen was only possible with the addition of LS, being the concentrations equal or higher than 2% better, because they preserved total motility, curvilinear velocity (VCL) and LIN with the same potential of BotuSêmen® ($P > 0.05$). Nevertheless, BotuSêmen® showed superiority in preserving the percentage of sperm progressive motility, average path velocity (VAP), linear progressive velocity (VSL) and BCF during cooling compared to the other extenders ($P < 0.05$). The inclusion of soy lecithin, from 2 to 6% in the BWW medium, allowed maintaining the viability of equine semen cooled at 15ºC for up to 24 hours.

Key words: Cryopreservation. Sperm. Soy Phosphatidylcholine. Skimmed milk. Stallion.

[1] Prof. Dr., Faculdade de Irecê, FAI, Irecê, BA, Brazil. E-mail: machadowm.vet@gmail.com
[2] Master's Student of the Animal Science Postgraduate Program, Universidade Estadual de Santa Cruz, UESC, Ilhéus, BA, Brasil. E-mail: thalitamarquesvet@gmail.com
[3] Doctoral Students of the Animal Science Postgraduate Program, UESC, Ilhéus, BA, Brazil. E-mail: larirodriguesvet@gmail.com; marykersul@gmail.com
[4] Profª Drª, Laboratory of Animal Reproduction, UESC, Ilhéus, BA, Brazil. E-mail: paolasnoeck@uesc.br
* Author for correspondence

Received: Apr. 12, 2022 - Approved: Jan. 07, 2023

Resumo

O sêmen equino tem sido historicamente refrigerado usando meios à base de leite. No entanto, o uso de componentes de origem animal causa várias preocupações potenciais, como variabilidade nas formulações, contaminação microbiana e questões regulatórias. Objetivou-se avaliar o potencial de inclusão de diferentes concentrações de lecitina de soja (LS) no meio quimicamente definido BWW - Biggers, Whitten e Whittingham para refrigeração de sêmen equino e armazenamento na temperatura de 15°C. Os ejaculados foram diluídos em seis diferentes grupos experimentais: 1) BotuSêmen® (controle); 2) BWW; 3) BWW + 1% lecitina de soja (LS); 4) BWW + 2% LS; 5) BWW + 4% LS e 6) BWW + 6% LS. O meio BWW, não preservou a motilidade, a velocidade, a retilinearidade (STR), a linearidade (LIN), a amplitude do deslocamento lateral da cabeça (ALH), a frequência de batimento flagelar cruzado (BCF), a integridade funcional e estrutural dos espermatozoides equino durante 24 h de refrigeração quando comparado ao BotuSêmen® (P <0,05). O uso de BWW para refrigeração de sêmen equino só foi possível com adição de lecitina de soja, sendo as concentrações igual ou superior a 2% melhores, pois preservaram a motilidade total, a velocidade curvilinear (VCL) e LIN com mesmo potencial do BotuSêmen® (P >0,05). Ainda assim, o diluidor comercial BotuSêmen® apresentou superioridade em preservar o percentual de espermatozoides progressivamente móveis, a velocidade média da trajetória (VAP), a velocidade linear progressiva (VSL) e a frequência do batimento flagelar cruzado (BCF) durante a refrigeração comparado aos demais diluidores (P <0,05). A inclusão de lecitina de soja, de 2 a 6% no meio BWW, permitiu a manutenção da viabilidade do sêmen equino refrigerado a 15ºC por até 24 horas.

Palavras-chave: Criopreservação. Espermatozoides. Fosfatidilcolina de soja. Leite desnatado. Garanhão.

Introduction

Cooled semen is a biotechnique widely used in the equine industry to maximize the reproductive potential of stallions, mainly, because between 20% to 40% of them are considered "bad freezers" (Vidament et al., 1997), besides, frozen semen has a higher cost per pregnancy (Murphy et al., 2014). Although, cooled semen results in better pregnancy rates in artificial insemination (AI), compared to frozen semen, cooling curve can cause irreversible damage, reducing the fertilizing potential compared to fresh semen (Watson & Morris, 1987).

Seminal extenders used in semen preservation processes at low temperatures have as a main objective to protect and minimize sperm damage (Alamaary et al., 2019), mainly that resulting from cold shock (Sorrenti et al., 2014), being possible by adding macromolecule sources, such as egg yolk, milk, and its derivatives in specific formulations to act against the deleterious effects of temperature reduction (Oliveira et al., 2013). A disadvantage of these components of animal origin is their inherent health risks (Bousseau et al., 1998), in addition to resulting in an extenders without standardization (Aurich et al., 2007).

The search for standardization of sperm extenders resulted in chemically defined media formulations, considered as viable alternatives and which generate satisfactory sperm quality results (Ricker et al., 2006), since the proposed media maintain

sperm viability similar to that of conventional extenders. In this sense, the Biggers, Whitten and Whittingham (BWW) medium described by Gibb et al. (2015) and Swegen et al. (2016) for the transport and storage of spermatozoa at room temperature (22°C), maintaining viable sperm parameters for 114 hours in AI protocols in equine species and with superior protection to conventional extenders based on animal products (egg yolk and milk), with indications for its use in conservation of equine semen at 5°C or 15°C.

The use of plant-derived lipoproteins such as soy lecithin as a component os extenders minimizes the health risks of using animal products (Papa et al., 2011), as well as aiding in the standardization of the diluting formulation (Aurich et al., 2007). Soy lecithin is a phospholipid fraction isolated from soybean that is mainly composed of phosphatidylcholine (Nadri et al., 2019), with potential to replace egg yolk or milk in seminal extenders, presenting components with protective potential for spermatozoa during cryopreservation and for cooling processes (Papa et al., 2011), and maintaining sperm viability parameters and sperm fertilization potential (Ricker et al., 2006; Papa et al., 2011).

Soy lecithin is a suitable alternative for replacement of macromolecules of animal origin in seminal extenders for cooling and freezing of spermatozoa from different species; however, its efficacy has never been evaluated in association with BWW medium. Thus, the aim of this study was to test the inclusion of different concentrations of soy lecithin in BWW extender in the cooling of equine semen at 15 °C for 24 h.

Materials and Methods

The present study was approved by the Ethics Committee on Animal Experimentation CEUA/UESC of the Universidade Estadual de Santa Cruz, Ilhéus, Bahia, Brazil under protocol number 004/16.

Semen collections were performed in two stud farms located in the municipality of Cabaceiras do Paraguaçu, Bahia, Northeast, Brazil (latitude 12°32'9"S, longitude 39°11'27"W) and in the municipality of Itabuna, Bahia, Northeast, Brazil (Latitude: 14º47'08"S and Longitude: 39º16'49"W). Eight Mangalarga Marchador stallions (four stallions per stud farm), aged between six and ten years, were used. Before the beginning of the experiment, all stallions were submitted to the depletion of their extragonadal sperm reserves through serial semen collection for seven consecutive days, using an artificial vagina model Botucatu® (Botupharma, Botucatu, SP, Brazil) and the help of a female in estrus as a mannequin. After three days of the depletion process, an ejaculate from each stallion was collected only once, obtaining a total of eight ejaculates.

All ejaculates were macro- and microscopically evaluated according to Brazilian College of Animal Reproduction [CBRA[, (2013) and met the minimum criteria of minimum motility of 60%, spermatozoa vigor of 3, with 70% of morphologically normal spermatozoa. The commercial extender used as control was BotuSêmen® and the tested extender was the BWW medium (Biggers et al., 1971) modified by Gibb et al. (2015).

The tested extenders 1%, 2%, 4% and 6% of soy lecithin (LS; p/v) (P5638, Sigma, St. Louis, MO, USA) were prepared by adding in the formulation a defined volume

of water with maintenance in hydration for 1 h at room temperature (25ºC). Then, the suspensions were homogenized using a magnetic stirrer (Fisatom, 752ª) until the formation of a homogeneous solution. Subsequently, the solutions were kept in an ultrasonic bath (25ºC) for 30 min for fragmentation of micelles, following the methodology described by Mozafari (2010). After sonication, debris were removed and the solution clarified by centrifugation (2,200 × g for 30 min) and double filtration (first in a 3 µm and then 0.45µm filter) as described by De Paz et al. (2010). Due to the possibility of this soy lecithin inclusion methodology causing a loss of up to 50% of phospholipids, the initial values of this lipoprotein source were adjusted taking into account the maximum loss during processing.

For the cooling process, the dilution was adjusted to 50 × 106 sperm/mL, forming the experimental groups: D1) BotuSêmen® (control); D2) BWW; D3) BWW + 1% LS; D4) BWW + 2% LS; D5) BWW + 4% LS and D6) BWW + 6% LS. After dilution, the motility and vigor parameters of the spermatozoa were again evaluated and the semen was then packed in a storage and transportation system BotuFlex® (Botupharma, Botucatu, SP, Brazil) set up to reach a storage temperature of 15°C. The diluted semen remained in the transport box for a minimum period of 06h which is the time described to reach the desired temperature, when the samples were transferred to an automated refrigeration system MiniTube®, stabilized at a temperature of 15°C. After 24 h of storage at 15°C, the samples were removed from the MiniTube® and placed in a dry bath at 37°C for a period of three to five min, followed by evaluation of kinematic parameters, functional and structural integrity of the plasma membrane, chromatin fragmentation, and sperm morphology for all samples.

The kinematics of chilled semen were evaluated using a computerized system Sperm Class Analyser® - CASA (SCA evolution, Microptics S.L, Barcelona, Spain). The standards used to adjust the equipment were: 25 images/second with 25 Hz; particle size captured between 4 and 75 µm/m2; sperm considered immobile < 10 µm/s, slow < 45 µm/s, medium from 45 to 90 µm/s and fast > 90 µm/s. The following parameters were evaluated: Total Motility (TM), Progressive Motility (PM), Linearity (LIN), Straightness (STR), expressed in percentages (%); Curvilinear Velocity (VCL), Linear Progressive Velocity (VSL) and Average Path Velocity (VAP), expressed in micrometers per second (µm/s); Amplitude of Lateral Sperm Head Displacement (ALH), expressed in micrometers (µm) and Cross Flagellar Beat Frequency (BCF), expressed in Hertz (Hz).

Functional integrity of the plasma membrane was evaluated by determining the percentage of spermatozoa reactive to the hyposmotic test (HOST) after dilution of 50 µL of the sample in 500 µL of 100 mOsmol/L sucrose solution. The samples were first incubated in a dry bath at 37°C for 30 min and were subsequently fixed with 250 µL of 4% formolized sodium citrate solution, followed by evaluation of 200 cells under a phase-contrast microscope (1000 ×; Olympus® CX 31). The percentage of functionally intact cells was calculated as follows: HOST% = % tail region changes after HOST - % tail region changes before HOST, according to the method of Melo and Henry (1999). Tail changes before and after incubation in hypoosmotic solution were analyzed using the wet preparation technique. Morphological

changes were evaluated after fixation of the samples in a 4% formolized sodium citrate solution using a phase-contrast microscope at 1000 × magnification (Olympus® CX 31).

The structural integrity of the plasma and acrosomal membranes were evaluated under a fluorescence microscope (400 ×; Olympus® CX 31) using the fluorochromes carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) according to the method of Harrison and Vickers (1990). Staining with CFDA was evaluated using the standard fluorescein filter set, while staining with PI was evaluated using a standard rhodamine filter set. 200 spermatozoa were analyzed per sample. Spermatozoa were classified into three subpopulations: structurally intact, with intact plasma and acrosomal membranes (PI-, CFDA+); partially intact, with damaged plasma membrane and intact acrosomal membrane (PI+, CFDA+); total loss of integrity, with damaged plasma and acrosomal membranes (PI+, CFDA-). To evaluate the efficiency of extenders, only the percentage of spermatozoa with intact plasma and acrosomal membranes was considered.

Sperm chromatin integrity was assessed by toluidine blue-induced metachromasia (Naves et al., 2004). Smears were taken with a 10 µL aliquot of the sample, dried at room temperature and fixed for 1 min in Carnoy's solution (3:1, 75 mL 100% alcohol + 25 mL acetic acid) and then in 70% alcohol for 3 min. Hydrolysis was carried out with 4 N hydrochloric acid for 15 min, washed in distilled water and dried at room temperature. For staining of the smear, 20 µL of 0.025% toluidine blue solution (0.00125 g of toluidine blue in 5 mL of McIlveine solution, pH 4.0) was deposited between a slide and coverslip and 500 cells were evaluated under phase-contrast microscopy at 1000 × magnification (Olympus® CX 31). Spermatozoa were classified as having compact chromatin (head region stained light blue) and with decompacted chromatin (head region stained in dark blue or violet).

The experimental design was a randomized block design, considering the stallion as a block. Only one semen collection was made for each stallion, dividing the ejaculates among the six groups and totaling 48 evaluations. Analysis of variance was used to compare treatments, and the necessary assumptions were verified. Tukey's test was applied for multiple comparisons of means at a 5% significance level. All analyses were performed using SPSS Statistics software (IBM, v.23.0 for Windows).

Results and Discussion

Initial dilution of semen with the different extenders tested: BotuSêmen®, BWW, and BWW containing different concentrations of soy lecithin guaranteed minimum total motility of 70%, minimum progressive motility of 50% and minimum vigor of 3 (0-5). BWW extender should not be used for cooling semen from Mangalarga Marchador stallions at 15°C for a period of 24 h because it does not preserve important sperm viability characteristics such as motility, velocity, STR, LIN, ALH, BCF, functional, structural, and sperm chromatin integrity ($P < 0.05$; Tables 1 and 2). The inclusion of soy lecithin in BWW medium ensured that spermatozoa could be cooled similarly to BotuSêmen®, an extender widely used for this purpose, and the concentration of 2% to 6% was better, as it maintained MT, VCL and LIN equal to the commercial extender ($P > 0.05$).

Table 1
Sperm kinematics of Mangalarga Marchador stallions cooled at 15ºC, for 24 hours, in BotuSêmen® extender and BWW, with different concentrations of soy lecithin (LS)

Parameters	Experimental extenders						EPM	*P*-value
	Botu Sêmen®	BWW	BWW 1%LS	BWW 2%LS	BWW 4%LS	BWW 6%LS		
MT (%)	61,9^{a}	4,6^{c}	27,2^{cb}	46,6^{ab}	49,0^{ab}	50,3^{ab}	3,7	0,0001
MP (%)	26,2^{a}	0,17^{c}	2,5^{b}	9,7^{b}	8,5^{b}	10,5^{b}	1,7	0,0001
VCL (µm/s)	55,0^{a}	10,9^{c}	27,6^{bc}	39,9^{ab}	37,5^{ab}	36,9^{ab}	2,6	0,0001
VAP (µm/s)	32,8^{a}	4,6^{c}	11,9^{bc}	19,8^{b}	18,8^{b}	19,2^{b}	1,6	0,0001
VSL (µm/s)	23,9^{a}	2,3^{c}	6,5^{b}	12,1^{b}	11,3^{b}	12,4^{b}	1,2	0,0001
STR (%)	62,0^{a}	24,5^{c}	51,9^{ab}	54,1^{ab}	54,1^{ab}	57,7^{ab}	2,4	0,0001
LIN (%)	36,5^{a}	10,8^{c}	20,5^{bc}	25,2^{ab}	25,5^{ab}	29,1^{ab}	1,6	0,0001
ALH (µm)	1,4^{a}	0,4^{c}	0,9^{ab}	1,2^{ab}	1,2^{ab}	1,2^{ab}	0,1	0,0001
BCF (Hz)	10,4^{a}	0,9^{c}	2,5^{bc}	5,4^{b}	4,6^{bc}	5,0^{b}	0,6	0,0001

Parameters evaluated by the SCA® after cooling: total motility (MT); progressive motility (PM); curvilinear velocity (VCL); average path velocity (VAP); linear progressive velocity (VSL); straightness (STR); linearity (LIN); amplitude of lateral sperm head displacement (ALH) and cross flagellar beat frequency (BCF). ^{abc}Superscript letters indicate differences within the line (P <0.05).

Table 2
Parameters of sperm viability in the semen of Mangalarga Marchador stallions cooled at 15ºC for 24 hours in BotuSêmen® and BWW extender, with different concentrations of soy lecithin (LS)

Parameters	Experimental extenders						EPM	*P*-value
	Botu Sêmen®	BWW	BWW 1%LS	BWW 2%LS	BWW 4%LS	BWW 6%LS		
HOST (%)	37,4^{a}	22,1^{b}	34,8^{a}	36,8^{a}	43,8^{a}	40,0^{a}	1,3	0,0001
CFDA+ (%)	34,1^{ab}	1,2^{c}	13,3^{ab}	44,5^{a}	55,1^{a}	44,9^{a}	3,9	0,0001
DNA + (%)	95,4	96,1	95,2	95,5	96,2	95,6	0,3	0,9210
MDef (%)	7,0	7,9	10,0	7,5	10,9	7,3	0,4	0,5740
mDef (%)	13,3	14,0	11,1	13,5	12,3	12,0	0,7	0,6740
Normal (%)	80,3	77,9	79,5	79,0	76,8	80,6	0,6	0,7140

Sperm with functional integrity by the hyposmotic test (HOST). Spermatozoa with intact structural membrane (CFDA+ / PI-). Spermatozoa with compacted chromatin (DNA +). Spermatozoa with major defect (MDef). Sperm with minor defect (mDef). Morphologically normal spermatozoa (Normal). ^{abc}Superscript letters indicate differences within the line.

BWW medium was not developed for cooling semen, being produced for the maintenance and embryonic development of mice at room temperature (Biggers et al., 1971) and later in the conservation of equine spermatozoa for up to 144 h at 22ºC (Gibb et al., 2015; Swegen et al., 2016). The inefficiency of this medium for cooling may be associated with the absence of lipoproteins in the formulation (Florez-Rodriguez et al., 2014). There are no reports of its use to maintain chilled equine semen at temperatures of 5°C or 15°C or the addition of different sources of lipoproteins such as soy lecithin, rich in phosphatidylcholine and other phospholipids, as presented in this study.

Many studies using different extenders have confirmed the potential of soy lecithin in preventing damage that the cryopreservation process causes to spermatozoa, mainly by controlling the efflux of cholesterol and phospholipids from the sperm membrane, resulting in its stabilization (De Paz et al., 2010; Dalmazzo et al., 2019; Zhao et al., 2021). The inclusion of soy lecithin to BWW medium allowed the dilution and refrigeration of semen at 15ºC, because it did not cause toxicity and preserved several kinematic and integrity parameters similar to BotuSêmen®, a fact verified by Papa et al. (2011) with the inclusion of 45 g/L of soy lecithin in equine semen freezing extender.

On the other hand, we observed that the inclusion of 1% to 6% of soy lecithin in BWW medium resulted in many particles in suspension, visualized only microscopically, indicating phase separation, i.e., the emulsion was not fully incorporated and dissolved in the extender. The process of inclusion of soy lecithin followed the methodology described by Mozafari (2010) with filtrations according to De Paz et al. (2010), avoiding the use of substances with toxic effects on spermatozoa for its solubilization. However, this component is a fat, an emulsifier, composed mainly of three types of phospholipids: phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (John et al., 2015). Emulsifiers can undergo certain phenomena that affect their stability, such as coalescence, flocculation, cream formation, sedimentation, Ostwald ripening, and phase inversion (Deng, 2021). The destabilization of soy-derivative stabilized emulsions can be affected by the nature of molecules present in three dimensional structures, hydrophobicity, charge, solubility, and molecular flexibility, in addition to environmental conditions such as pH and ionic strength (Ashaolu, 2020). Such occurrence was also reported in ovine semen freezing extenders (Forouzanfar et al., 2010). The low solubility of soy lecithin may also result from the wide varieties of this molecule available in the market, some lyophilized and others not, the degree of purification, and form of processing for inclusion of this substance in seminal extenders (Nguyen et al., 2019).

BWW medium did not preserve spermatozoa during cooling and resulted in lower values of motility, VCL, VAP, VSL, STR, LIN, ALH, BCF and functional and structural integrity of the membranes when compared to BotuSêmen® ($P < 0.05$; Tables 1 and 2), evidencing the importance of lipoprotein-rich sources as a component of seminal extenders for cryopreservation. Corroborating these findings, Brasileiro et al. (2019) demonstrated that cooling semen in ACP-105® extender, chemically defined and without lipoprotein-rich components in its formulation, resulted in lower kinematic parameters when compared

to BotuSêmen® and ACP-105® with the inclusion of skim milk or containing different concentrations of egg yolk (2.5% and 5%). According to Akhter et al. (2011), the highest values of motility and plasma membrane integrity after 24 h of cooling buffalo semen were in samples processed in extenders containing soy lecithin or milk.

The inclusion of concentrations > 1% of soy lecithin in BWW medium resulted in maintenance of total motility, VCL, STR, LIN, ALH characteristics and functional and structural integrity of sperm membranes similar to BotuSêmen® (P >0.05; Tables 1 and 2), which highlights its potential in refrigerating equine semen at 15ºC. Caldevilla et al. (2020) reported that addition of soy lecithin to Andromed® extender was able to preserve LIN and STR parameters equally to extenders containing egg yolk during freezing of equine semen. Promising results were also reported by Zhao et al. (2021) who found that the addition of 0.5% soy lecithin in Tris medium allowed the refrigeration of sheep semen at 0°C for 216 h, with sperm motility and pregnancy rate similar to samples processed in Tris containing egg yolk.

It is believed that the mechanism of action of soy lecithin is due to increased spermatozoa plasma membrane fluidity, as well as the improvement of signal transduction pathways that consequently increase the fertilizing potential (Sun et al., 2021). The employment of soy lecithin as a lipid source in seminal diluents is described in several species, being used alone or in association with other macromolecules of animal origin (Zhao et al., 2021; Papa et al., 2011; Nouri et al., 2013). This is because, in its composition there is a large proportion of phosphatidylcholine and a fraction of low density lipoproteins (LDL), already described as components with the ability to protect the integrity of sperm membranes during cryopreservation (Nadri et al., 2019). That is, the mechanisms of action of this emulsifier are similar to the mechanisms of protection of LDL of egg yolk during conservation processes at low temperatures, which justifies its use in seminal extenders (Forouzanfar et al., 2010) for cooling and freezing.

Soy lecithin has antioxidant properties, since in its composition alpha, gamma and delta tocopherols are present (Wang & Wang, 2008). The main antioxidant mechanism of this macromolecule is based on the synergistic effect between amino alcohol phospholipids, as well as the gamma and delta tocopherols (Judde et al., 2003), which act directly in decreasing the amount of malonaldehyde and potentiating the endogenous enzymes superoxide dismutase and catalase (Sun et al., 2021) that control the formation of reactive oxygen species (ROS) and lipid peroxidation of membranes. Another important property protecting the membrane integrity of spermatozoa is the ability to form a biofilm or aggregate on plasma membranes during storage at refrigeration or freezing temperatures, which would reduce the loss of components of the membrane, leading to greater stability (Ricker et al., 2006).

The best parameters of progressive motility, VAP, VSL and BCF obtained in the samples cooled in BotuSêmen® when compared to the other diluents tested (P <0.01; Table 1) may be due to a more efficient mechanism of cell protection by the higher solubility and availability of proteins present in milk. It is known that casein micelles and aggregates of milk proteins interact directly with seminal plasma proteins, preventing

them from causing the efflux of cholesterol molecules from the plasma membrane, which stabilizes the membrane during the transition phase in the cooling process, leading to a higher resistance of spermatozoa to such processes (Plante et al., 2015).

Despite the promising results of including soy lecithin in BWW medium found in the present study, its efficacy is limited or controversial for semen cryopreservation, mainly in equine species (Papa et al., 2011, Caldevilla et al., 2020), as it has been shown to be inferior to other sources of macromolecules that function as externally acting cryoprotectants (Dalmazzo et al., 2019; Papa et al., 2011, Caldevilla et al., 2020), especially when compared to skim milk (Campos et al., 2020; Novello et al., 2020).

The non-maintenance of some sperm viability parameters in this study at a level similar to BotuSêmen®, in addition to other literature reports indicating that soy lecithin can protect spermatozoa less efficiently than other lipoprotein sources, may be due to lower (bio)availability of the fractions of this compound responsible for increasing cryotolerance. It is known that the particle size of soy lecithin when dissolved in diluent media is an important factor for the quality of cryopreserved semen (Mousavi et al., 2019). Another factor that may be linked to the reduction in viability parameters would be the inclusion of high concentrations of soy lecithin, because this leads to increased viscosity of the seminal extender and may cause the appearance of larger micelles, directly influencing the kinematic parameters of progressive motility, velocity, linearity, and straihtness and cause reduction of sperm fertility (De Paz et al., 2010; Forouzanfar et al., 2010).

Conclusions

The inclusion of soy lecithin in BWW medium allows the cooling of equine semen at 15ºC for up to 24 h, preserving parameters of sperm viability with concentrations between 2% and 6%. Further studies regarding how to incorporate this source of lipoproteins in order to maximize the cryoprotective action of equine semen cooling extenders are necessary, ensuring chemically defined and sanitary controlled media.

Acknowledgments

To the owners of Mangalarga Marchador stallions from Itabuna and Cabaceiras do Paraguaçu, Bahia, Brazil, for making the animals available for the experiment; to the student Diego Passos for his help in field and laboratory activities; to the research promotion agency of the state of Bahia, FAPESB, for the financial support (DTE 0009/2015) and to Capes for granting the doctoral scholarship.

References

Akhter, S., Ansari, M. S., Rakha, B. A., Ullah, N., Andrabi, S. M., & Khalid, M. (2011). In vitro evaluation of liquid stored buffalo semen at 5c diluted in soya lecithin-based extender (Bioxcell), tris-citric egg yolk, skim-milk and egg yolk-citrate extenders. *Reproduction in Domestic Animals, 46*(1), 45-49. doi: 10.1111/j.1439-0531.2009.01561.x

Alamaary, M., Haron, A. W., Ali, M., Hiew, M., Adamu, L., & Peter, I. (2019). Effects of four extenders on the quality of frozen semen in Arabian stallions. *Veterinary World,*

12(1), 34-40. doi: 10.14202/vetworld.2019.34-40

Ashaolu, T. J. (2020). Applications of soy protein hydrolysates in the emerging functional foods: a review. *International Journal of Food Science & Technology, 55*(2), 421-428. doi: 10.1111/ijfs.14380

Aurich, C., Seeber, P., & Müller-Schlösser, F. (2007). Comparison of different extenders with defined protein composition for storage of stallion spermatozoa at 5 degrees C. *Reproduction in Domestic Animals, 42*(4), 445-448. doi: 10.1111/j.1439-0531.2006.00828.x

Biggers, J. D., Whitten, W. K., & Whittingham, D. G. (1971). The culture of mouse embryos in vitro. In J. C. Daniels (Ed.), *Methods in mammalian embryology* (pp. 86-116). San Francisco, CA: Freeman.

Bousseau, S., Brillard, J. P., Marquan-Le Guienne, B., Guerin, B., Camus, A., & Lechat, M. (1998). Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin-based diluents. *Theriogenology, 50*(5), 699-706. doi: 10.1016/s0093-691x(98)00175-7

Brasileiro, L. S., Segabinazzi, L. G. T. M., Menezes, E., Salgueiro, C. C., Novello, G., Scheeren, V. F. D. C., Alvarenga, M. A., & Nunes, J. F. (2019). Coconut water as an extender component for cooled equine sperm. *Journal of Equine Veterinary Science, 78*(1), 69-73. doi: 10.1016/j.jevs.2019.03.213

Caldevilla, M., Ferrante, A., & Neild, D. (2020). Utilización de un medio con lecitina de soja para congelar semen equino. *InVet, 22*(1), 41-49.

Campos, G. A., Garcia, V. F. C., Freitas-Dell'Aqua, C. P., Segabinazzi, L. G. T. M., Maciel, L. F. S., Alvarenga, M. A., Papa, F. O., & Dell'Aqua, J. A. (2020). Sodium caseinate and cholesterol improve bad cooler stallion fertility. *Journal of Equine Veterinary Science, 93*(1), 103201. doi: 10.1016/j.jevs.2020. 103201

Brazilian College of Animal Reproduction (2013). *Manual de exame andrológico e avaliação de sêmen animal* (3a ed.). BCAR.

Dalmazzo, A., Angrimani, D. S. R., Losano, J. D. A., Rocha, C. C., Sobrinho, C. A. B., Gurgel, J. R. C., Pacheco, P. I. M., Minazaki, C. K., Crusco, S. E., Nichi, M., & Barnabe, V. H. (2019). Insights into soy lecithin and egg yolk-based extenders for chilling canine spermatozoa. *Zygote, 27*(1), 17-24. doi: 10.1017/S0967199418000576

De Paz, P., Esteso, M. C., Alvarez, M., Mata, M., Chamorro, C. A., & Anel, L. (2010). Development of extender based on soybean lecithin for its application in liquid ram semen. *Theriogenology, 74*(4), 663-671. doi: 10.1016/j.theriogenology.2010.03.022

Deng, L. (2021). Current progress in the utilization of soy-based emulsifiers in food applications-a review. *Foods, 10*(6), 1354. doi: 10.3390/foods10061354

Florez-Rodriguez, S. A., Arruda, R. P., Alves, M., Affonso, F. J., Carvalho, H. F., Lemes, K. M., Lançoni, R., Andrade, A. F., & Celeghini, E. (2014). Morpho functional characterization of cooled sperm with different extenders to use in equine-assisted reproduction. *Journal of Equine Veterinary Science, 34*(7), 911-917. doi: 10.1016/j.jevs.2014.04.007

Forouzanfar, M., Sharafi, M., Hosseini, S. M., Ostadhosseini, S., Hajian, M., Hosseini, L., Abedi, P., Nili, N., Rahmani, H. R., & Nasr-Esfahani, M. H. (2010). In vitro comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. *Theriogenology, 73*(4), 480-487. doi: 10.1016/j.theriogenology.2009.10.005

Gibb, Z., Lambourne, S. R., Quadrelli, J., Smith, N. D., & Aitken, R. J. (2015). L-carnitine and pyruvate are prosurvival factors during the storage of stallion spermatozoa at room temperature. *Biology of Reproduction, 93*(4), 104. doi: 10.1095/biolreprod.115.131326

Harrison, R. A., & Vickers, S. E. (1990). Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *Journal of Reproduction and Fertility, 88*(1), 343-352. doi: 10.1530/jrf.0.0880343

John, J. V., Park, H., Lee, H. R., Suh, H., & Kim, I. (2015). Simultaneous extraction of phosphatidylcholine and phosphatidylethanolamine from soybean lecithin. *European Journal of Lipid Science and Technology, 117*(10), 1647-1654. doi: 10.1002/ejlt.201400396

Judde, A., Villeneuve, P., Rossignol-Castera, A., & Guillou, A. L. (2003). Antioxidant effect of soy lecithins on vegetable oil stability and their synergism with tocopherols. *Journal of the American Oil Chemists' Society, 80*(12), 1209-1215. doi: 10.1007/s11746-003-0844-4

Melo, M. I. V., & Henry, M. (1999). Teste hiposmótico na avaliação de sêmen equino. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 51*(1), 71-78.

Mousavi, S. M., Towhidi, A., Zhandi, M., Amoabedin, G., Mohammadi-Sangcheshmeh, A., Sharafi, M., & Hussaini, S. M. H. (2019). Comparison of two different antioxidants in a nano lecithin-based extender for bull sperm cryopreservation. *Animal Reproduction Science, 209*(1), 106171. doi: 10.1016/j.anireprosci.2019.106171

Mozafari, M. R. (2010). Nanoliposomes: preparation and analysis. *Methods in Molecular Biology, 605*(1), 29-50. doi: 10.1007/978-1-60327-360-2_2

Murphy, C., English, A. M., Holden, A. S., & Fair, S. (2014). Cholesterol-loaded-cyclodextrins improve the post-thaw quality of stallion sperm. *Animal Reproduction Science, 145*(3-4), 123-129. doi: 10.1016/j.anireprosci.2014.01.013

Nadri, T., Towhidi, A., Zeinoaldini, S., Martínez-Pastor, F., Mousavi, M., Noei, R., Tar, M., & Sangcheshmeh, A. M. (2019). Lecithin nanoparticles enhance the cryosurvival of caprine sperm. *Theriogenology, 133*(1), 38-44. doi: 10.1016/j.theriogenology.2019.04.024

Naves, C. S., Beletti, M. E., Duarte, M. B., & Vieira, R. C. (2004). Avaliação da cromatina espermática em equinos com azul de toluidina e acridine orange. *Bioscience Journal, 20*(3), 117-124.

Nguyen, V. V., Ponchunchoovong, S., Kupittayanant, S., & Kupittayanant, P. (2019). Effects of egg yolk and soybean lecithin on sperm quality determined by computer-assisted sperm analysis and confocal laser scanning microscope in chilled canine sperm. *Veterinary Medicine and Science, 5*(3), 345-360. doi: 10.1002/vms3.158

Nouri, H., Towwhidi, A., Zhandi, M., & Sadeghi, R. (2013). The effects of centrifuged egg yolk used with INRA plues soybean lecithin extender on semen quality to freeze Caspian horse semen. *Journal of Equine Veterinary Science, 33*(12), 1050-1053. doi: 10.1016/j.jevs.2013.03.184

Novello, G., Podico, G., Segabinazzi, L. G. T. M., Lima, S. F., & Canisso, I. F. (2020). Stallion semen cooling using native phosphocaseinate-based extender and sodium caseinate cholesterol-loaded cyclodextrin-based extender. *Journal of Equine Veterinary Science, 92*(1), 103104 doi: 10.1016/j.jevs.2020.103104

Oliveira, G. C., Oliveira, B. M. M., Celeghini, E. C. C., Fernandes, C. B., & Mattos, C. B. (2013). Criopreservação do sêmen equino: uma revisão. *Revista Brasileira de Reprodução Animal, 37*(1), 23-28.

Papa, F. O., Felício, G. B., Melo-Oña, C. M., Alvarenga, M. A., De Vita, B., Trinque, C., Puoli, J. N., Fº., & Dell'Aqua, J. A., Jr. (2011). Replacing egg yolk with soybean lecithin in the cryopreservation of stallion semen. *Animal Reproduction Science, 129*(1-2),73-77. doi: 10.1016/j.anireprosci.2011.10.006

Plante, G., Lusignan, M. F., Lafleur, M., & Manjunath, P. (2015). Interaction of milk proteins and Binder of Sperm (BSP) proteins from boar, stallion and ram semen. *Reproductive Biology and Endocrinology, 13*(1), 92-112. doi: 10.1186/s12958-015-0093-1

Ricker, J. V., Linfor, J. J., Delfino, W. J., Kysar, P., Scholtz, E. L., Tablin, F., Crowe, J. H., Ball, B. A., & Meyers, S. A. (2006). Equine sperm membrane phase behavior: the effects of lipid-based cryoprotectants. *Biology of Reproduction, 74*(2), 359-365. doi: 10.1095/biolreprod.105.046185

Sorrenti, G., Bagnoli, A., Miraglia, V., Crocetta, F., Vitiello, V., Ristoratore, F., Cirino, P., Sansone, G., & Sordino, P. (2014). Investigating sperm cryopreservation in a model tunicate, *Ciona intestinalis sp. A. Cryobiology, 68*(1), 43-49. doi: 10.1016/j.cryobiol.2013.11.005

Sun, L., He, M., Wu, C., Zhang, S., Dai, J., & Zhang, D. (2021). Beneficial influence of soybean lecithin nanoparticles on rooster frozen-thawed semen quality and fertility. *Animals, 1*(6), 1-12. doi: 10.3390/ani11061769

Swegen, A., Lambourne, S. R., Aitken, R. J., & Gibb, Z. (2016). Rosiglitazone improves stallion sperm motility, ATP content, and mitochondrial function. *Biology of Reproduction, 95*(5), 107, 1-12. doi: 10.1095/biolreprod.116.142687

Vidament, M., Dupere, A. M., Julienne, P., Evain, A., Noue, P., & Palmer, E. (1997). Equine frozen semen: freezability and fertility field results. *Theriogenology, 48*(6), 907-917. doi: 10.1016/s0093-691x(97)00319

Wang, G., & Wang, T. (2008). Oxidative stability of egg and soy lecithin as affected by transition metal ions and pH in emulsion. *Journal of Agricultural and Food Chemistry, 56*(23), 11424-11431. doi: 10.1021/jf 8022832

Watson, P. F., & Morris, G. J. (1987). Cold shock injury in animal cells. *Symposia of the Society for Experimental Biology, 41*(1), 311-340.

Zhao, J. Q., Xiao, G. L., Zhu, W. L., Fang, D., Li, N., Han, C. M., & Gao, Q. H. (2021). Ram semen preserved at 0 °C with soybean lecithin Tris-based extender substituted for egg yolk. *Animal Bioscience, 34*(2), 192-197. doi: 10.5713/ajas.20.0118