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Archives of Veterinary Science
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Sodium caseinate as a strategy equine semen cryopreservation

Wallyson Rafael Machado Santos[1], Geraldo Francisco dos Santos Júnior[1], Eneiva Carla Carvalho Celeghini[2], Luiz Ernandes Kozicki[3], Eduarda Stankiwich Vaz[4], Natália Santana Siquiera de Lara[4], Mayara Silvestri[4], Lucas Luz Emerick[5], Fernando Andrade Souza[4]

Submitted: *26/10/2023*
Accepted:*23/04/2024*

[1]*Universidade Federal do Acre - Rio Branco, Acre, 0000-0002-7657-6300, 0000-0002-2865-8926.*
[2]*Universidade de São Paulo – Pirassununga, São Paulo, 0000-0003-1531-5497.*
[3]*Pontifícia Universidade Católica- Curitiba, Paraná, 0000-0002-3700-1811.*
[4]*Universidade Federal do Paraná – Curitiba, Paraná, 0000-0001-9373-4262; 0000-0001-8977-9740, 0000-0003-0246-8742; 0000-0002-9474-9404*
[5]*Universidade Federal Rural da Amazônia – Parauapebas, Pará, 0000-0003-1934-5183.*

*Corresponding author: Fernando Andrade Souza, fernando.andrade@ufpr.br

Abstract: The milk added to the semen extender media acts by binding to seminal plasma proteins, which may protect the sperm cell from undergoing the capacitation process or acrosomal reaction in advance. Among these proteins, sodium caseinate stands out, which represents 80% of all proteins present in milk. Caseins are also able to protect the sperm cell by decreasing the loss of lipids through the plasma membrane, decreasing the binding of seminal plasma proteins to the plasma membrane. The objective of this study was to determine whether the addition of sodium caseinate in different concentrations to the freezing extender medium favors sperm viability after a long period of refrigeration. For this, three concentrations of sodium caseinate were used, 0.0, 1.0, and 2.0%, in the cryopreservation medium. In addition to caseinate concentrations, three refrigeration times were tested at 5 ºC, 12 and 24 hours. So, the ejaculates were divided into nine groups 0H/0%, 0H/1%, 0H/2%, 12H/0%, 12H/1%, 12H/2%, 24H/0%, 24H/1% and 24H/2%. Ten stallions underwent reproductive evaluation, being included only those with sperm motility ≥ 70%. Fluorescent probes, hypoosmotic (HOST), and thermoresistance (TTR) tests were used to evaluate the functionality of the plasma membrane and the longevity of sperm cells, respectively. There was no stat. difference ($p>0.05$) between groups for membrane integrity. However, for the TTR, it was possible to observe a stat. difference ($p<0.05$) for the 24H/2% group with a lower response. The integrity of the plasma membrane, compared to the refrigeration time and the concentration of sodium caseinate, did not change. Therefore, it was concluded that sperm longevity was impaired with the use of a 2% concentration of sodium caseinate, and its use is not recommended for sending refrigerated semen, for insemination or freezing, that exceeds 12 hours.
Keywords: Antioxidant, FITC-PSA, HOST, Proteins, TTR.

1. Introduction

In horses, artificial insemination is the basis for other reproductive biotechnologies. It is considered the most important and used biotechnology (Amann and Graham, 1993), with semen refrigeration and transport being routine procedures since the late 1980s (Heckenbichler et al., 2011). The refrigeration technique allows the semen to be stored for 48 hours, allowing its transport to various places and with good fertilizing capacity (Dell'Aqua Jr et al., 2018).

However, refrigeration and/or cryopreservation of equine semen still face some barriers due to the reduction of sperm viability (Brinsko et al., 2003; Al-Mutary, 2021), which occurs due to cryoinjuries that occur during the process (Watson, 1981). These changes lead to disorders in permeability and metabolic activity, impairing the survival time of cells in the female reproductive tract (Pickett and Amann, 1993; Aurich et al. 2016).

When going through the refrigeration processes, spermatozoa suffer oxidative damage caused by the accumulation of reactive oxygen species (ROS) from sperm metabolism (Ball, 2008; Al-Mutary, 2021). These factors can lead to a subsequent reduction in the longevity of long-term refrigerated or cryopreserved sperm (Neild et al., 2005).

Thus, seeking to prevent injuries suffered by sperm cells during the cooling and cryopreservation process, extenders and cryoprotectants are added to the semen (Oliveira et al., 2013; Banday et al., 2017). Skim milk and/or egg yolk are the main agents added to these media, as they contain lipoproteins, which can stabilize protein elements in the sperm membrane (Watson, 1981).

Milk acts by binding to seminal plasma proteins, which may protect the sperm cell from undergoing the capacitation process or acrosomal reaction in advance. In milk there is about 3.5% of proteins, with caseins being the largest amount, representing 80% (Fox and Brodkorb, 2008). Casein-based extenders increase the amount of sperm that binds to the zona pellucida, which is about four times more when compared to TALP extenders (Da Silva et al., 2012). Caseins are also able to protect the sperm cell by decreasing the loss of lipids through the plasma membrane, decreasing the binding of seminal plasma proteins to the plasma membrane (Bergeron et al., 2007; Ferrer et al., 2020). The objective of this study was to evaluate the structural and functional conditions of semen cryopreserved with different concentrations of sodium caseinate, after being submitted to refrigeration at 5 ºC for 12 or 24 hours in a medium containing caseinate.

2. Materials and Methods

This study was approved by the Ethical Committee on Animal Use (CEUA) at the Federal University of Acre, under registration number 56/2018.

2.1. Location and animals

The experiment was conducted in the city of Rio Branco – Acre (Latitude: -9.974, Longitude: -67.8076; 9° 58′ 26″ South, 67° 48′ 27″ West). Ten Quarter-horse stallions were used, carrying out seminal collection through an artificial vagina using a mare in estrus. The stallions were previously selected by andrological examination, according to the Brazilian College of Animal Reproduction (Henry and Neves, 2013).

2.2. Previous assessments

One ejaculate was collected from each stallion. In case the animal did not present the standards stipulated in this study, the collection was repeated after 48 hours. Once there was no improvement in the ejaculate, the animal was replaced.

The ejaculates selected for freezing met the standards mentioned by Alvarenga et al. (2005), where they presented at least 70% of progressive motility with a minimum concentration of 60 million spermatozoa/ml. The initial analyses of motility and concentration were conducted as recommended by Henry and Neves (2013). For this purpose, a binocular biological microscope LED N 107 (Coleman, São Paulo, Brazil) with a magnification of 100x was used to estimate the number of mobile cells (0 - 100%) and vigor (0 - 5). Sperm concentration was performed using a Neubauer chamber, with 10 µL of semen diluted in 1000 µL of saline formaldehyde (1:100) being aliquoted, and then taken to optical microscopy at 400x magnification.

To analyze the sperm morphology, an aliquot of the ejaculate was separated and added into a microtube of 1000 µl of saline formaldehyde until the sample became cloudy.

Once the initial values of motility and concentration were determined, the ejaculates were diluted in a ratio of 2:1, being 2 parts of extender and one of the ejaculates, which was diluted in a transport medium based on skimmed milk and glucose (Kenney et al., 1975), replacing 2% skim milk with 2% sodium caseinate.

2.3. Experimental design

After dilution, the ejaculate was divided into three groups: H0 (cryopreservation after collection); H12 (Cryopreservation 12 hours after refrigeration); and H24 (cryopreservation after 24 hours of refrigeration). For refrigeration, specific transport boxes for semen, Botuflex (Biotech Botucatu/ME Ltda., Botucatu, Brazil) were used, capable of keeping the sample at 5 °C for up to 48 h.

All samples, regardless of storage time (0, 12, and 24 h), had their seminal plasma removed by centrifugation at 600 x *g* for 10 min (Celm LS-3 Plus; Marzano et al. 2020). After centrifugation, the supernatant was discarded and the pellet formed was resuspended with the extenders for cryopreservation to be tested: T1 - Control (TRIS extender - 36.05 g of tris-hydroxymethyl-aminomethane, 20.24 g of citric acid, 14.88 g of fructose, 500.000 IU of Penicillin G, 500.000 µg of streptomycin, 2% of glycerol and 3% of dimethylformamide in sufficient quantity of milliq water for 1000 ml); T2 and T3, sodium caseinate at concentrations of 1 and 2%, respectively, in the TRIS extender. Control treatments followed what is recommended in the literature (Costa et al., 2014; Nikitkina et al., 2020) as a standard model for seminal cryopreservation of stallions. Nine treatments were conducted in a 3 x 3 factorial (3 concentrations of sodium caseinate x 3 refrigeration times at 5 ºC; Fig. 1). After the dilutions, according to each group, the semen was manually packaged in 0.5 mL straws at a concentration of 50 x 10^6 cells per straw.

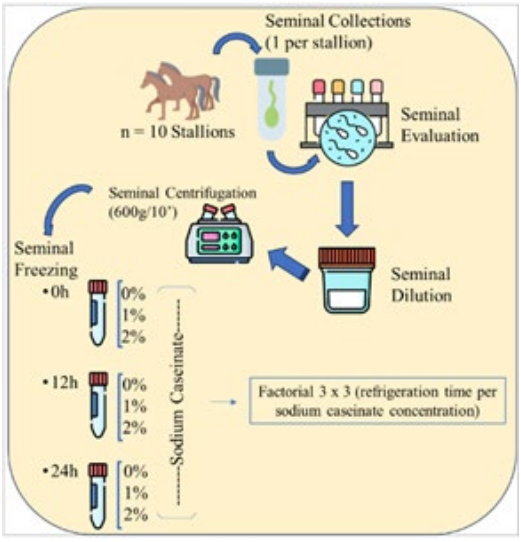

Figure 1 – Schematic protocol of study design. Icon made by flaticon (https://www.flaticon.com/br/).The straws were placed in the Botutainer transport box (Biotech Botucatu, ME Ltda., Botucatu, Brazil) for 90 minutes at T1, for the first stage of the cryopreservation process. Afterward, transferred to an isothermal box of 37 liters containing 5 cm of liquid nitrogen (N2), the straws were placed horizontally at 4 cm above the N2 level for 15 minutes and, finally, immersed in liquid nitrogen (Pugliesi et al., 2014). The freezing rates in this process were -46.8 ºC/min from 5 to -120 ºC and -9.86 ºC/min from -120 to -196 ºC (Pickett and Amann, 1993). The procedure was repeated for each treatment, respecting their cooling times, 12 and 24 hours for T2 and T3, respectively.

2.4. Seminal analyzes after cryopreservation

A 0.5 mL semen straw of each treatment and each batch was thawed at 46 °C for 20 seconds (Dell'Aqua Jr., 2000), and placed in a microtube, being kept in a water bath at 37 °C analyze motility, vigor, and morphological alterations.

Sperm motility and vigor were evaluated by optical microscopy, with two evaluators in double-blind (Binocular Biological Microscope LED N 107 Coleman) at 100x magnification. For morphological alterations, the fresh semen was diluted in saline formol. The reading was performed in wet preparations, under phase contrast microscopy (Eclipse E200, Nikon, New York, U.S.A.) with a 1000x magnification, counting the head, midpiece, and tail major and minor defects (Henry and Neves, 2013) of two hundred sperm cells per sample. The thermoresistance test (TTR) was performed, where the straws were thawed in a water bath, first at 46 °C for 20 seconds, then kept at 37 °C, evaluating the initial motility and then being evaluated every 10 minutes for 60 minutes, aiming to determine sperm motility under the imposed conditions.

The Hyposmotic Test (HOST) was performed according to Snoeck et al. (2014) using distilled water at a dilution of 1:15 (semen: distilled water), incubated for 10 minutes, and subsequently fixed in saline formalin. For counting, 10 µL of each sample were placed between the slide and coverslip to count 100 sperm, under optical microscopy (400x magnification). The calculation of the number of reactive spermatozoa to the Hyposmotic test was done using the formula cited by Melo (1999), namely: HO% = (% of changes in the tail region after HOST test) – (% of changes in the tail region before the HOST test).

To evaluate the integrity of the plasma membrane, acrosomal and mitochondrial potential, 150 µL of semen diluted in TALP-sperm medium (1:3), at a concentration of 10 x 10^6 sptz/mL, were used in a microcentrifuge tube and 3 µL of propidium iodide (PI 0.5 mg/ml) (plasma membrane), 2 µl of Hoescht 33342 (0.5 mg/mL) (plasma membrane), 6 µL of 5.5', 6,6'-tetrachloro- 1,1,3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1 153 µM) (mitochondrial potential) and 80 µL of Pisum sativum agglutinin conjugated to fluorescein isothiocyanate (FITC-PSA 100 µg/mL) (Evaluation of acrosome). Then, the samples were incubated for 8 min at 37 °C in the dark, a technique adapted from Celeghini et al., (2010).

Evaluations were performed by placing a 4 ul aliquot of the sample between the slide and coverslip preheated to 37 °C. Readings were performed at 1000x magnification using epifluorescence microscopy (Eclipse 80i, Nikon) in a triple filter (D/F/R, C58420) presenting the UV-2E/C sets (340-380 nm excitation and 435- 485 nm), B-2E/C (excitation 465-495 nm and emission 515-555 nm) and G-2E/C (excitation 540-525 nm and emission 605-655 nm), with a magnification of 1000x. Finally, two hundred cells were counted and classified into eight categories according to the fluorescence emitted by each probe, according to Celeghini et al. (2008), and the results expressed in percentage. From this evaluation, the percentages of plasma membrane integrity, acrosome integrity, and mitochondrial membrane potential were considered, as well as the percentage of spermatozoa presenting simultaneously acrosomal plasma membrane integrity and high mitochondrial membrane potential.

2.5. Statistical analysis

The design used was in randomized blocks in a 3 x 3 factorial (three concentrations of caseinate x three waiting times at 5 ºC), with the parametric variables evaluated by ANOVA using the Tukey test and the non-parametric variables using the Friedman test. All responses were evaluated with a significance of 5%. All variables passed the Lilliefors normality test. The variables that did not lie within the normal range transformed, and that did not respond to normality after the procedures, were analyzed as non-parametric. Data were presented as mean ± standard error of the mean.

3. Results

The average volume and physical characteristics of fresh and chilled semen, both with 12 and 24 hours of refrigeration, were within the standards considered normal for horses, according to Henry and Neves (2013). The values of sperm motility (%) and vigor (0-5) of samples refrigerated for 0, 12, and 24 hours were 82.0 ± 2.21, 3.7 ± 0.15, 71 ± 3.10 and 3.3 ± 1.51, 52.0 ± 3.59 and 2.6 ± 0.16, respectively. Up to 12 hours of refrigeration, the samples maintained the minimum value required by the Brazilian College of Animal Reproduction (Henry and Neves, 2013), with the indication for cryopreservation. Thus, seeking to evaluate the sperm longevity of the samples after the different periods of refrigeration, the thermo-resistance test was performed (Table 1).

	H0			H12			H24		
Time after thawing	T0%	T1%	T2%	T0%	T1%	T2%	T0%	T1%	T2%
0'	45±3.79ab	49±4.11a	42±4.68abd	45±3.79ab	42±2.65abd	32±3.92	31±2.84	21±2.43b	21±2.43bc
10'	57±4.3a	59±4.17a	50±4.43	53±5.37ac	50±3.16ac	42±4.55	39±2.97	31±4.11	27±2.63b
20'	63±4.3a	58±3,29ab	46±3.16	56±5.37abd	51±3.48	41±5.44	42±3.48	33±4.43b	30±4.11bc
30'	59±3.54a	49±2.97	40±5.06	50±5.06	47±3.79	33,5±5.44	38±4.11	31±4.74	27±4.11b
40'	48±2.15a	50±3.16ab	31±3.60	37±3.79	40±3.16	27,5±3.16b	34±3.48	24±3.79	20±2.75bc
50'	43±3.48a	41±2.91	26±3.29	33±4.43	37±4.11	25±3.16	30±3.48	24±4.43	18±3.79b
60'	42±3.16a	34±3.16	23±3.86	32±4.11	33±3.03	22±3.41	25±3.79	19±3.48	16±3.16b
Δ	3	15	19b	13	9	10	6	2a	5

Different letters on the same line differ by Friedman's test ($p<0.05$). Δ: difference between initial and final motility. H0 = immediate cryopreservation; H12 = cryopreservation after 12 hours of refrigeration; H24 = cryopreservation after 24 hours of refrigeration; T0 = no caseinate addition; T1 = 1% caseinate; T2 = 2% caseinate.

Table 1 – Mean and standard error of the mean of total motility during the thermo-resistance test of cryopreserved horse semen 0, 12 and 24 hours after collection at different concentrations of sodium caseinate.

At time zero, just after thawing, the samples refrigerated for 24 hours stood out, regardless of the treatment. As they presented values below those commended by the Brazilian College of Animal Reproduction, 30% of motility for samples considered suitable for insemination. The Delta values (difference between initial and final motility values) were compared (Figure 2).

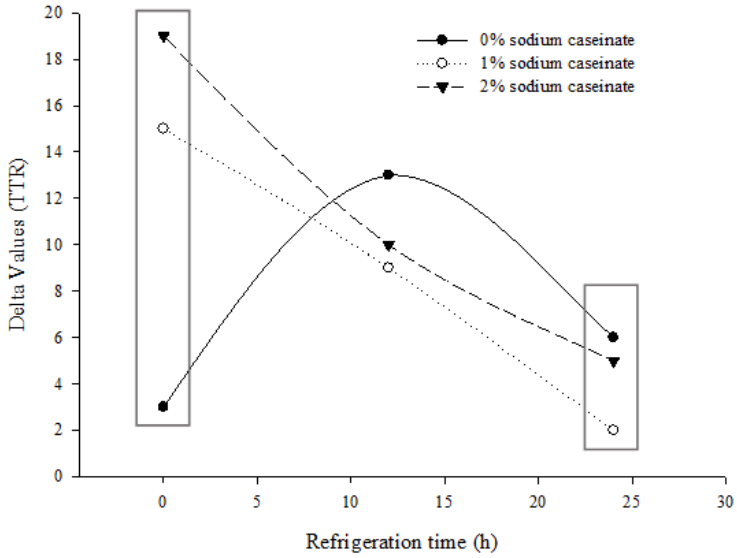

Figure 2 – Differences in the values, initial and final of each treatment evaluated by the term resistance test (TTR), means compared by the Friedman test ($p<0.05$) of horse sperm.

The values of Δ showed that the non-addition of caseinate, in the control group, showed a drop in motility over time with the greatest difference being at 12 hours of refrigeration.

Different from the treatments with 1 and 2% caseinate, which showed a greater difference at the beginning, 0 hours, with a drop in the final motility percentage, but with an emphasis on 24 hours, where these treatments showed less difference, ensuring their functionality within the time.

The membrane integrity of horse sperm frozen samples 0, 12, and 24 hours after collection are presented in Fig 3.

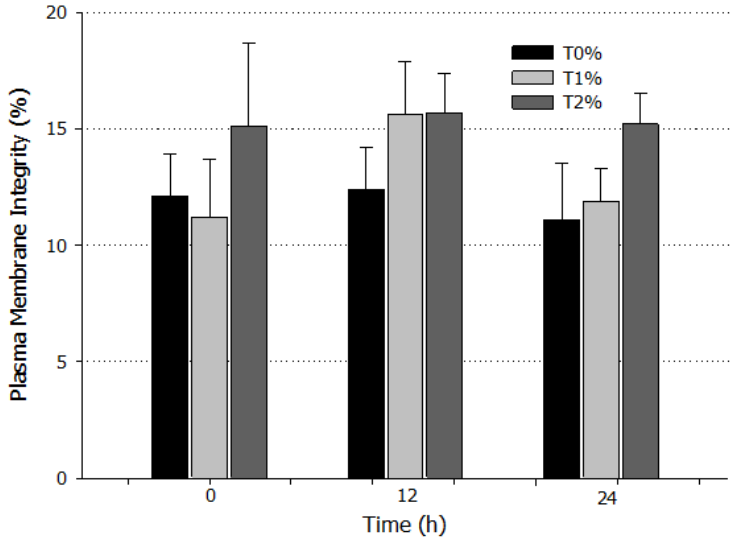

Figure 3 – Mean and standard error of the mean of plasma membrane integrity of horse sperm (HOESCHT-33342/PI) at 0, 12, and 24 hours with concentrations of 0.0, 1.0, and 2.0% sodium caseinate.

There was no difference ($p>0.05$) between treatments, regardless of caseinate concentrations and refrigeration time before cryopreservation. In this way, the responses regarding refrigeration time were grouped, contrasting only the concentrations of sodium caseinate. In this way, the responses regarding refrigeration time were grouped, contrasting only the concentrations of sodium caseinate, being evaluated for mitochondrial potential, as shown in Fig. 4.

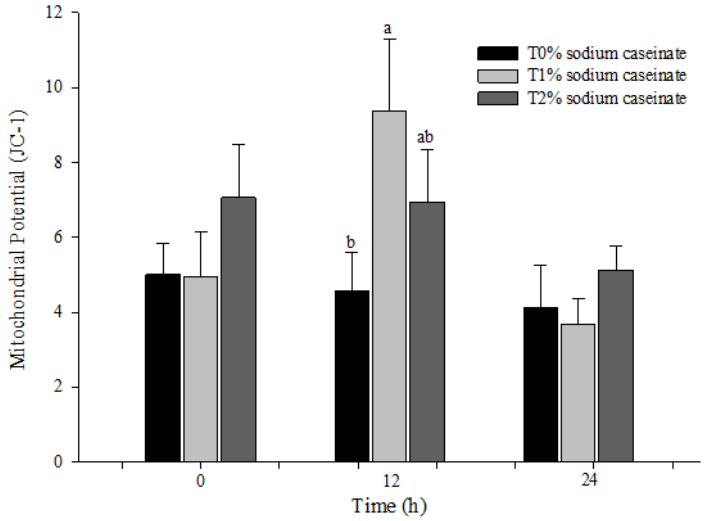

Figure 4 – Mean and standard error of the mean of the mitochondrial potential of horse samples cryopreserved 0, 12, and 24 hours after refrigeration using different concentrations of sodium caseinate. Different letters differ statisat $p<0.05$. JC-1: 5,5', 6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazolyl-carbocyanine iodide.

The mitochondrial potential showed a stat. difference ($p<0.05$) between treatments only 12 hours of refrigeration, where the addition of caseinate, regardless of concentration, provided a greater response compared to the control group.

The results of the acrosomal integrity of the cryopreserved samples, when compared between treatments (T0, T1, and T2), showed no difference ($p>0.05$), and were then grouped by refrigeration time (0, 12, and 24 hours, Figure 5).

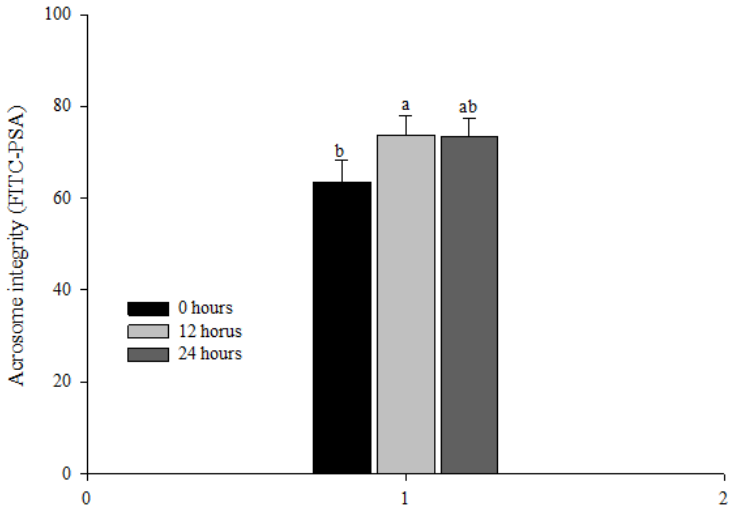

Figure 5 – Mean and standard error of the mean of acrosome integrity (FITC-PSA) of samples cryopreserved 0, 12, and 24 hours after refrigeration. Different letters differ by Tukey's test ($p<0.05$). FITC-PSA: Pisum sativum conjugated to fluorescein isothiocyanate.

Greater acrosome damage was expected within the refrigeration time for the samples from the control group, where the addition of caseinate could provide greater protection to the acrosomes within the refrigeration times. However, this was not seen, with acrosome integrity response only within time and not within the caseinate concentration used.

4. Discussion

The motility values obtained at all refrigeration times are within the desirable standards according to Henry and Neves (2013). There was a significant difference ($p<0.05$) in motility between groups H0 and H24, with a decline of 30 percentage points from time 0 to time 24 hours. Despite the anticipated decrease in semen motility under refrigeration for 24 hours, it was expected that the addition of 2% caseinate to the seminal transport medium could reduce this result, which was not confirmed.

In buffaloes, the addition of 2% sodium caseinate to Botu-Bov media® did not prevent the appearance of cryocapacitation in post-thawed samples, and no additional cryoprotective effect of its use was detected, however, the addition did not cause damage to sperm quality (Da Silva et al, 2020). In sheep semen, the addition of 2% sodium caseinate in the diluting medium showed favorable sperm kinetic parameters, as well as in the fertility test (Salgado, 2020). Peréz et al (2021) concluded in their study that supplementing freezing diluent with sodium caseinate can improve the post-thaw quality of bovine semen when added at a concentration of 3%, as it has been shown to have a positive impact on motility and sperm kinetics.

In a study carried out by Lagares et al (2012) in equine semen, there was no significant difference in motility in relation to the control group, but the values were significantly higher in sperm velocity variables with the addition of 1.35% sodium caseinate in the extender.

Costa et al. (2020), substituting 2% of skim milk for 2% of sodium caseinate, and obtained within 24 hours, a total motility of 68,6 ± 4.3%. In the present study, the values observed were 82.0 ± 6.4, 71.0 ± 7.2, and 52.0 ± 8.8% for motility at 0, 12, and 24 hours.

However, despite the present study presenting a lower response (52.0 ± 8.8%) than those found by Costa et al. (2020) within 24 hours of refrigeration, they could be used without harming the insemination, since the minimum stipulated by Henry and Neves (2013) is ≥30% of motility.

The values Δ demonstrate that the non-addition of caseinate, the control group, showed a drop in motility within time, with the greatest difference being within 12 hours of refrigeration, maintaining the pattern within 24 hours. Thus, it should be noted that: 1. caseinate, regardless of the concentration used, at the zero moment of stability (H0), showed a greater difference, while the control group remained stable; 2. after 24 hours of refrigeration, the concentration of 1% sodium caseinate showed the smallest difference. In the comparisons performed, there was a difference ($p<0.05$) between the Δ values of the treatment H0T2% and H24T1% (Fig. 01).

Lower results than those shown in this work were found by other authors using the same test (Snoeck et al., 2014), after cryopreservation of semen from stallions of different breeds.

Part of this distinct response may be due to the stallion, its breed, or the method used. One of the main implications in the response to artificial insemination using frozen semen in equine species is the time between insemination and ovulation of the mare. Thus, an attempt is made to bring these two points closer together. However, conducting the insemination closer to the time of ovulation of the mare causes wear and tear on the professional, who is often forced to perform the procedure at dawn. In trying to

overcome the need for insemination close to ovulation, one can focus on increasing sperm viability, which can be achieved through additives to the freezing medium, which in the present study used sodium caseinate.

It was expected that the addition of this protein to the refrigeration and freezing medium could, through less contact with the seminal plasma proteins, increase the viability time of the sperm cell. However, in the TTR it was observed that there was a lower response ($p<0.05$) for the H24T2% group (Fig. 01), with two factors linked to this difference: 1. the refrigeration time before freezing, according to Alvarenga and Carmo (2007), after 24 hours of refrigeration, many metabolites are accumulated in the refrigeration medium, significantly reducing cell motility; 2. the concentration of sodium caseinate where the treatment with 2%, although not statistically inferior in the other groups, showed a tendency of inferiority to the control group and the T1% group, both at times 0 and 12, as well as at 24 hours.

Results obtained in this study at the time H0T0% were superior to those found by Costa et al. (2014), when they evaluated two different cryopreservation extenders (Botucrio® and Tris-Yolk) in Quarter-horse stallions, with a motility of 19.59±10.34 (Botucrio) and 15.21±8.75 (Tris-Yolk group).

The motility results observed during the TTR of the H12 group were like the results obtained by Resende et al. (2013) when they froze seminal samples after 12 hours of refrigeration, with and without seminal plasma, where they observed total motility of 50.5±25.9% for the group refrigerated with seminal plasma. Resende et al. (2013) also concluded that refrigeration for 12 hours before cryopreservation became effective and that it allowed better use of the ejaculate, which can be transported for up to 12 hours to a specialized center for freezing.

According to the Brazilian College of Animal Reproduction (Henry and Neves, 2013), the parameters for motility of cryopreserved semen observed in this research are within the normal range, only the H24T2% group had a lower result ($p<0.05$) than the other groups. This fact can be explained by the relationship between the caseinate concentration, 2%, and the prolonged period of refrigeration to which this sample was submitted, which was exposed to several elements capable of injuring sperm cells, leading to a decline in total motility.

The drop in motility over time is partially explained by the membrane integrity results which tracked the motility relationship. The reduction in sperm motility observed in this study can be attributed to a delay in sperm capacitation caused by caseins. Caseins can cause a delay in sperm capacitation, and this can be attributed to the binding of sodium caseinate with proteins derived from seminal plasma. Authors justified that the use of casein can delay sperm capacitation through the competitive action of casein micelles with seminal plasma proteins. Caseins sequester the ligand of sperm proteins (BSP), which participate in the modifications of sperm membrane lipids that occur during capacitation (Manjunath, 2012; Manjunath and Thérien, 2002; Manjunath et al, 2007; Plante et al, 2015). Reducing BSP binding to sperm prevents the loss of membrane lipids and, consequently, maintains sperm viability (Bergeron et al, 2007)

Free radicals and lactic acid from the cellular metabolism itself can decrease the pH of the sample causing irreversible cell damage by peroxidation of membrane lipids (Aurich et al., 2007). It was expected that the higher concentration of caseinate would show a result contrary to what was observed in this study.

It is suggested that sodium caseinate may delay the sperm capacitation process at the beginning compared to skimmed milk. This is possibly associated with the control of metabolic activity carried out by sodium caseins, which promote the stability of the plasma membrane through interactions with Ca+2 ions, and these ions play an important role in tyrosine phosphorylation, involved in sperm capacitation (Bergeron et al, 2007; Pagl et al, 2006; Coutinho da Silva et al, 2012).

The results of the mitochondrial potential showed a decrease within the refrigeration time, regardless of the period analyzed, 0, 12, or 24 hours. However, only the time of 12 hours showed a difference ($p<0.05$) between the caseinate concentrations used (1 and 2%) and the control group. According to a review by Angrimani et al. (2015), mitochondria are the main source of energy for sperm motility and homeostasis, being implicated in oxidative phosphorylation, which results in the production of reactive oxygen species (ROS), which plays a fundamental role in physiological processes.

However, mitochondrial dysfunctions can cause an imbalance between ROS production and antioxidant mechanisms, causing oxidative stress, which is lethal to the sperm cell (Angrimani et al., 2015). Both refrigeration and freezing of semen promote a great loss of sperm cell functionality due to the decrease in mitochondrial potential (Amann and Pickett, 1987). However, from the data presented, the addition of sodium caseinate may decrease this response, at least for a brief period.

In semen storage, BSPs become harmful as, in the time/concentration relationship, they stimulate the efflux of cholesterol and phospholipids from the sperm membrane (Manjunath, 2012). Therefore, the addition of sodium caseinate to the cooling and/or freezing dilution medium may increase sperm viability, directly impacting the increase in mitochondrial potential, which confirms the findings of this study, within 12 hours.

The equality of mitochondrial potential responses over the 24 hours may indicate that casein binding to BSPs may be time dependent.

5. Conclusion

The conclusion is that the plasma membrane's integrity, in relation to the refrigeration time and the sodium caseinate concentration, did not change.

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