UNIVERSIDADE FEDERAL DE PELOTAS
Faculdade de Veterinária
Programa de Pós-Graduação em Veterinária

Dissertação

Glicerol na criopreservação de sêmen de garanhões

Pablo Luis Fracaro

Pelotas, 2022

Pablo Luis Fracaro

Glicerol na criopreservação de sêmen de garanhões

Dissertação apresentada ao Programa de Pós-Graduação em Veterinária da Faculdade de Veterinária da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Mestre em Ciências (área de concentração: Sanidade Animal).

Orientador: Antonio Sergio Varela Junior

Coorientador: Carine Dahl Corcini

Pelotas, 2022

Universidade Federal de Pelotas / Sistema de Bibliotecas
Catalogação na Publicação

F798g Fracaro, Pablo Luis

Glicerol na criopreservação de sêmen de garanhões / Pablo Luis Fracaro ; Antonio Sergio Varela Junior, orientador ; Carine Dahl Corcini, coorientadora. — Pelotas, 2022.

28 f.

Dissertação (Mestrado) — Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, 2022.

1. Equino. 2. Criopreservação. 3. Espermatozoide. 4. Raça crioula. 5. Glicerol. I. Varela Junior, Antonio Sergio, orient. II. Corcini, Carine Dahl, coorient. III. Título.

CDD : 636.10888

Elaborada por Ubirajara Buddin Cruz CRB: 10/901

Pablo Luis Fracaro

Glicerol na criopreservação de sêmen de garanhões

Dissertação aprovada como requisito parcial para obtenção do grau de Mestre em Ciências, Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas.

Data da Defesa: 22/06/2022

Banca examinadora:

Prof. Dr. Antonio Sergio Varela Junior (Orientador)
Doutor em Aquicultura pela Universidade Federal do Rio Grande

Prof. Dr. Bruna dos Santos Suñé Moraes
Doutor em Veterinária pela Universidade Federal de Pelotas

Prof. Dr. Izani Bonel Acosta
Doutor em Veterinária pela Universidade Federal de Pelotas

Prof. Dr. Ilusca Sampaio Finger
Doutor em Biotecnologia pela Universidade Federal de Pelotas

Agradecimentos

Agradeço primeiramente a Deus por estar comigo em mais esse projeto. Agradecimento a minha família, em especial minha esposa, pelo apoio incondicional. Ao orientador, aos professores e colegas envolvidos nesse projeto, meu muito obrigado.

Resumo

FRACARO, Pablo Luis. **Glicerol na criopreservação de sêmen de garanhões.** 2022. 28F. Dissertação (Mestrado em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2022.

A espécie equina é uma das mais importantes no cenário do agronegócio atual, gerando renda para milhões de pessoas atuantes no seu entorno. A criopreservação por sua vez, expressa parcela significativa na valorização da espécie. O glicerol é um crioprotetor amplamente utilizado na técnica de congelamento de sêmen equino, porém, com a necessidade de melhoria em sua empregabilidade. Tendo isso em vista, buscou-se avaliar os resultados pós congelamento e descongelamento do sêmen equino com a utilização de glicerol nas concentrações de 2, 3, 4 e 5% em períodos de 15, 30, 45, 60, 75 e 90 minutos de exposição do mesmo às células espermáticas. Tendo em vista motilidade total e progressiva dos espermatozoides concluímos que a concentração de 5% de glicerol em 15 e 30 minutos de exposição, 4% de glicerol em 45 minutos de exposição e 3% de glicerol no período de 90 minutos de exposição, foram os protocolos testados que geraram os melhores resultados, portanto, sugerimos o uso de algum destes protocolos para uma melhor criopreservação de sêmen equino.

Palavras chave: equino, espermatozoide, glicerol, criopreservação, raça crioula.

Abstract

FRACARO, Pablo Luis. **Glycerol in stallion semen cryopreservation.** 2022. 28F. Dissertation (Master degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2022.

The equine species is one of the most important in the current agribusiness scenario, generating income for millions of people working in its surroundings. Cryopreservation, in turn, expresses a significant part in the appreciation of the species. Glycerol is a cryoprotectant widely used in the equine semen freezing technique, however, with the need to improve its employability. With this in mind, we sought to evaluate the post-freezing and thawing results of equine semen with the use of glycerol at concentrations of 2, 3, 4 and 5% in periods of 15, 30, 45, 60, 75 and 90 minutes of exposure to sperm cells. Considering the total and progressive motility of the spermatozoa, we concluded that the concentration of 5% glycerol in 15 and 30 minutes of exposure, 4% of glycerol in 45 minutes of exposure and 3% of glycerol in the period of 90 minutes of exposure, were the tested protocols that generated the best results, therefore, we suggest the use of one of these protocols for a better cryopreservation of equine semen.

Keywords: equine; sperm; glycerol; freezing; cryopreservation, Crioula race.

Lista de Tabelas

Lista de Abreviaturas e Siglas

ALH	Amplitude do Deslocamento Lateral da Cabeça
ATP	Adenosina Trifosfato
BCF	Frequência de Batimento Cruzado
CASA	Sistema de Análise Espermática Computadorizada
DAP	Distância Média Percorrida
DCF	Diacetato de Carboxifluoresceína
DCL	Distância Curvilínea
DFI	Índice de Fragmentação de DNA
DSL	Distância Retilínea
GLI []	Concentração de Glicerol
IA	Inseminação Artificial
LIN	Linearidade
LPO	Peroxidação lipídica
MAPA	Ministério de Agricultura, Pecuária e Abastecimento
M.P.	Motilidade Progressiva
M.T.	Motilidade Total
PI	Iodeto de Propídeo
PIB	Produto Interno Bruto
ROS	Espécies Reativas de Oxigênio
STR	Retilinearidade
VAP	Velocidade Média Percorrida
VCL	Velocidade Curvilínea
VSL	Velocidade Retilínea
WOB	Oscilação entre VAP/VCL (%)

Lista de Símbolos

°C	Grau Celsius
mL	Mililitros
mg	Miligrama
µL	Microlitro
%	Porcento
µm	Micrômetro

Sumário

1 Introdução

O agronegócio brasileiro apresenta constante crescimento, sendo um dos setores responsáveis pela melhora econômica do país. O montante envolvendo o setor chegou a R$ 1,55 trilhão, equivalendo 21,4% do PIB brasileiro em 2019 (CEPEA/USP). O maior rebanho de equinos e bovinos da América Latina é do Brasil, aparecendo ainda como o quarto maior rebanho de equinos a nível mundial (Relatório Animal 2018, Perfil Pecuária no Brasil). O complexo do agronegócio do cavalo foi responsável pela movimentação de R$ 16,5 bilhões e a aproximadamente 3 milhões de empregos, diretos e indiretos (MAPA, 2016).

Acompanhando esses números e esse crescimento surgiram novas formas de manejo e novas substâncias. Visando uma evolução constante na busca por melhorias, as pesquisas seguem esse mesmo caminho. A utilização de sêmen congelado, por exemplo, em programas reprodutivos de equinos está em ampla ascensão. A inseminação artificial (IA) com sêmen criopreservado é uma alternativa cada vez mais utilizada nas diversas raças que permitem sua utilização (Aurich *et al*., 2020).

A utilização de inseminação artificial é uma prática relativamente antiga, sendo relatada pela primeira vez em 1957 onde se obteve na espécie equina, a primeira gestação gerada através de espermatozoides criopreservados (Pickett e Amann, 1993). Desde então se tem a busca por substâncias que possibilitem uma melhora na qualidade de congelação e consequentemente, aumentem as taxas de concepção através do uso de sêmen congelado.

Em meados dos anos 80 a taxa de prenhez obtida através da utilização de sêmen congelado era consideravelmente inferior às obtidas nos dias atuais. Fato comprovado por *Amann e Pickett (1987)* que obtiveram taxas de prenhez 75% inferior com sêmen congelado quando comparada com a utilização de sêmen fresco. Miller, C.D. (2008) obteve taxa de prenhez de 45% utilizando sêmen congelado, amplamente superior aos dados iniciais, porém possíveis de serem superados.

Diversas são as vantagens da utilização do sêmen congelado em equinos, como por exemplo, o armazenamento do material genético de determinado garanhão por tempo indeterminado, a utilização do sêmen de animais mesmo após sua retirada reprodutiva ou morte, controle de doenças venéreas e também, a utilização do sêmen em longas distâncias, através da sua facilidade de transporte (Amann e Pickett, 1987).

Para que a criopreservação obtenha sucesso, diversos fatores são determinantes. Um desses fatores está relacionado à necessidade de o espermatozoide estar em um ambiente apropriado, obtido através dos extensores, que minimizem os danos a membrana celular e não acione prematuramente o mecanismo de capacitação espermática e reação do acrossoma (Loomis, 1992).

Os extensores usados para o congelamento de sêmen são compostos na maioria dos casos, de substâncias para estabilizar o pH, neutralizar os produtos tóxicos produzidos pelo metabolismo espermático, proteger contra o choque térmico, manter o equilíbrio eletrolítico e osmótico, inibir crescimento de bactérias e fornecer energia. Os extensores também devem conter crioprotetores para prevenir a formação de gelo intracelular e extracelular (Alvarenga et. al., 2016).

Os crioprotetores são divididos em duas classes, sendo substâncias penetrantes e não penetrantes. Os crioprotetores penetrantes desempenham seu papel no meio intracelular e extracelular, pois possuem a capacidade de ultrapassar a membrana plasmática do espermatozoide por conta do seu pequeno tamanho molecular. Os não penetrantes devido seu maior tamanho molecular não possuem a capacidade de atravessar a membrana plasmática do espermatozoide atuando apenas no meio extracelular (Amann e Pickett, 1987).

Exemplos de crioprotetores penetrantes são: glicerol, dimetilsulfóxido, propilenoglicol, acetamida e outras amidas, etilenoglicol e o 1,2 propanodiol. Por sua vez, exemplos de substâncias não penetrantes são: os açúcares como lactose, manose, frutose e trealose; os polímeros sintéticos com a polivinilpirrolidona e metilcelulose; e as proteínas presentes no leite e na gema de ovo (Amann e Pickett, 1987).

Keit (1998), afirmou ser o glicerol o crioprotetor penetrante mais utilizado nos protocolos de congelação de sêmen equino, na época em concentrações de 2 a 5%. A partir de então, diversos estudos foram desenvolvidos visando aprimorar e

especificar sua utilização, sendo ainda hoje um dos componentes mais utilizados, de forma isolada ou mesmo associado com outras substâncias.

O glicerol consiste em uma substância capaz de penetrar na membrana celular por difusão passiva e permanecer na membrana e no citoplasma celular. O glicerol é um álcool e contêm três grupos de hidroxila ligados a uma cadeia de três átomos de carbono. Sua principal atuação na criopreservação é a inibição da formação de cristais de gelo no interior da célula ou em locais adjacentes a mesma (Oliveira et. al., 2017).

Alguns estudos como por exemplo, Candeias et. al., 2012; Terracino et. al., 2008; Medeiros et. al., 2002; Nascimento et. al., 2015; Alvarenga et. al., 2003; Oliveira et. al., 2017; entre outros, demonstram a enorme variabilidade da utilização do glicerol em protocolos de congelamento de sêmen de garanhões, em diversas concentrações, como em associações com outras substâncias, obtendo uma ampla diversidade de resultados, justificando uma pesquisa específica referente a concentração e o período de exposição deste crioprotetor.

Devido a variabilidade de formas com que o glicerol foi utilizado e testado, o mesmo demonstrou alguns efeitos indesejáveis, ocorrendo no momento do equilíbrio osmótico, visto que a água realiza o processo de forma semelhante ao glicerol, porém mais rapidamente, podendo levar a desidratação celular. Outra função inadequada ocorre através de ação direta sobre a membrana celular, ligando-se a grupos de cabeças fosfolipídicas podendo reduzir a fluidez da membrana (Alvarenga et. al., 2016). Esses efeitos estão diretamente relacionados à concentração e o tempo de exposição do mesmo ao sêmen equino.

Desta forma, o objetivo desse trabalho foi avaliar diferentes concentrações de glicerol bem como os diversos períodos de exposição do mesmo ao sêmen de garanhões, buscando descobrir a melhor forma de usufruir deste crioprotetor, proporcionando melhor qualidade das células espermáticas após o processo de congelamento e descongelamento do material genético dos reprodutores.

2 Artigo

Glicerol na criopreservação de sêmen de garanhões

Pablo Luis Fracaro[1]; Norton Luis Souza Gatti[1]; Jorge Squeff Filho[1]; Izani Bonel Acosta[2]; Felipe Pires Hartwig[2]; Rodrigo Desessards Jardim[3]; Bruna Da Rosa Curcio[1-2]; Carlos Eduardo Wayne Nogueira[1-2]; Carine Dahl Corcini[1-2-3]; Antonio Sergio Varela Junior[1-3]*

[1] Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas (UFPel), Pelotas, RS, 96010-900, Brasil.
[2] Faculdade de Veterinária, Universidade Federal de Pelotas (UFPel), Pelotas, RS, 96010-900, Brasil.
[3] Reprodução Animal Comparada, Instituto de Ciência Biológicas, Universidade Federal de Rio Grande (FURG), Rio Grande, Brasil. *E-mail: varelajras@pq.cnpq.br

Foi submetido e aceito pela revista cryoletters

Glycerol and exposure time for freezing equine semen

Pablo Luis Fracaro [a], Carine Dahl Corcini[bc], Norton Luis Souza Gatti [a], Jorge Squeff Filho[a], Izani Bonel Acosta[a], Jorge Squeff Filho [a], Felipe Pires Hartwig [a], Bruna Da Rosa Curcio[a], Antonio Sergio Varela Junior[bc]

[a]Programa de pós graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, RS, Brasil.

[b]Reprodução Animal Comparada- RAC, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, Rio Grande, RS, Brasil

[c]ReproPel, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, RS, Brasil

Abstract

BACKGROUND: Glycerol is a cryoprotectant widely used in the freezing of mammalian species, but no study has demonstrated its concentration and the appropriate exposure time for equine species. **OBJECTIVE:** Different glycerol concentrations (2, 3, 4 and 5%) in exposure times of 15, 30, 45, 60, 75 and 90 minutes in semen equine. **MATERIALS AND METHODS:** The ejaculate of 12 stallions were frozen in different glycerol concentrations at different exposure times. The thawed sperm was evaluated as the kinetic parameters for Computer Assisted Semen Analysis (CASA) and cell feature parameters by flow cytometry. **RESULTS:** Considering the total and progressive motility of the spermatozoa, we concluded that the concentration of 5% glycerol in 15 and 30 minutes of exposure, 4% glycerol in 45 minutes of exposure and 3% glycerol in 90 minutes of exposure were the protocols tested that generated the best results. Therefore, we suggest the use of any of these protocols for a better cryopreservation of equine semen. **CONCLUSION:** Therefore, we suggest the use of any of these protocols for a better cryopreservation of equine semen.

Keywords: conservation; sperm; equine; motility,

INTRODUCTION

Keith (1998) stated that glycerol is the most used penetrating cryoprotectant in equine semen freezing protocols, at that time in concentrations from 2 to 5%. Since then, several studies have been developed aiming to improve and specify its use, being still today one of the most used components, alone or associated with other substances.

Glycerol is a substance capable of penetrating the cell membrane by passive diffusion and remaining in the cell membrane and cytoplasm. Glycerol is an alcohol and contains three hydroxyl groups attached to a chain of three carbon atoms. Its main action in cryopreservation is to inhibit the formation of large ice crystals inside the cell or adjacent to it (Amann et al., 1987; Miller, 2008).

Some studies, such as (Medeiros et al., 2002; Alvarenga et al., 2003); among others, have demonstrated the enormous variability in the use of glycerol in freezing protocols of stallion semen, in several concentrations, as in associations with other substances, obtaining a wide diversity of results, justifying a specific research regarding the concentration and period of exposure of this cryoprotector.

Due to the variability of the forms in which glycerol was used and tested, it has shown some undesirable effects, occurring at the moment of osmotic balance, since water performs the process similarly to glycerol, but more rapidly, and may lead to cell dehydration. Another inadequate function occurs through direct action on the cell membrane, binding to groups of phospholipidic heads, which may reduce the fluidity of the membrane (Alvarenga et al., 2016). These effects are directly related to the concentration and the time of its exposure to the equine semen.

Thus, the objective of this work was to evaluate different concentrations of glycerol as well as different periods of its exposure to the semen of stallions, trying to find the best way to make use of this cryoprotector, providing better quality of the sperm cells after the freezing and thawing process of the genetic material of reproducers.

MATERIALS AND METHODS

Animals

For this experiment, 12 stallions of the Crioula breed, aged 4 to 10 years, housed in an equine reproduction center in the city of Pelotas, Rio Grande do Sul, Brazil, were used. The stallions spent the night in stalls and were released during the day whenever possible. They received feed three times a day and were offered free forage and water. All were collected through artificial vaginal model Botucatu (Botupharma Ltda, Botucatu, São Paulo, Brazil) in artificial manikin with a mare in estrus nearby.

After collection the semen was diluted in a 1:1 ratio with laboratory diluent based on Kenney et al., (1975) in the plant itself and subsequently sent to the University laboratory, traveling a period of approximately two hours, transported in thermal boxes. Upon arrival at the laboratory a first evaluation of sperm motility was performed using AndroVision® - CASA Software with computer and monitor (Minitube), where only ejaculates presenting sperm motility above 70% were used for the study.

Cryopreservation

Semen samples with laboratory diluent were centrifuged at 600 rpm for 10 minutes, after which the supernatant was removed and egg yolk diluent without glycerol was added at a concentration of 250 million spermatozoa/mL. Semen of 500 µL with egg yolk diluent without glycerol was placed in 24 cryotubes with capacity of 1.5ml each, which were then placed in a container with 200ml of water together with the Falcon tubes with the different concentrations of glycerol (10%, 8%, 6% and 4%) and placed inside Minitube's refrigerator (Minitub® 518C). The freezing curve used was 0.5°C/minute and another 30 minutes for

stabilization. After that, 500 µL of diluent with the determined concentrations of glycerol were added in the cryotubes containing the 500 µL of semen, reaching the final concentrations of 5%, 4%, 3% and 2%, also testing different contact times between semen and diluent (15, 30, 45, 60, 75 and 90 minutes). After this process, the material was filled into 0.5ml straws and placed for 20 minutes in liquid nitrogen vapor to then be immersed in liquid nitrogen and stored at -196°C until thawing for analysis. For thawing the straws were immersed in a 37°C water bath for 30 seconds.

Sperm analysis

Software used initially (AndroVision), the post thaw semen was evaluated in total and progressive motility parameters (%), DAP (mean distance traveled, µm), DCL (curvilinear distance, µm), DSL (rectilinear distance, µm), VAP (average velocity traveled, µm/s), VCL (curvilinear velocity, µm/s), VSL (rectilinear velocity, µm/s), STR (straightness %), LIN (linearity %), WOB (oscillation, VAP/VCL, %), ALH (amplitude of lateral head displacement, µm) and BCF (cross beat frequency) (Dziewulska et al 2011).

Flow cytometry

In flow cytometry, samples were analyzed using an Attune Acoustic Focusing Cytometer® (Applied Biosystems) flow cytometer, with BL1 photodetector (530/30 filter). Sperm populations were measured by staining cells with Hoescht 33342 (B2261, Sial) and detected by VL1 photodetector (450/40 filter), eliminating non-sperm counts based on scatter plots (Martinez-Alborcia et al 2012).

Sample preparation:

In each evaluation, 10µL of treated semen was used by adding the respective fluorescent probe, leaving in a dry bath at 37°C for 10 minutes. Subsequently, an amount of 20µL of Hoescht33342 (B2261, Sial) solution (10mg/ml) was added, leaving 1 minute in contact with the sample to then be performed the analysis, determining the sperm population and the specific fluorescence colors. For the ROS and LPO analyses the contact time was 20 minutes.

Membrane integrity: The fluorescent probes used for this analysis were carboxyfluorescein diacetate (DCF) (C5041, Sigma Aldrich) and propidium iodide (PI)(P4170, Sigma Aldrich), where only cells with damaged cell membrane allow propidium iodide to enter. Carboxyfluorescein diacetate in turn penetrates the spermatozoon and is then converted into a non-permeable fluorescent compound, being retained in the cytoplasm. Thus, only DCF-labeled spermatozoa were defined as containing an intact plasma membrane (Acosta et al., 2020).

Plasma membrane fluidity: The fluorescent probes used for this analysis were hydrophobic merocyanine 540 (323756, Sigma Aldrich) and Yo-Pro 1, selecting and classifying spermatozoa into low and high membrane fluidity, where higher fluorescence consequently represented higher fluidity (Acosta et al., 2020).

DNA fragmentation index (DFI): A combination of 3 different solutions were used for this analysis. First, TNE (0.01 M Tris-Hcl, 0.15 M NaCl, 0.001 EDTA, pH 7.2) added to 10µL of treated semen. Second, Triton (10µL - Triton X - 100, 0.1%). Finally, acridine orange was added immediately before reading (Acosta et al., 2020).

Mitochondrial functionality: In this case, spermatozoa with more active mitochondria reflect higher green fluorescence and consequently, mitochondria that generated little green fluorescence were considered to have low mitochondrial activity. The fluorescent probe used in this analysis was rhodamine 123(R8004, Sigma Aldrich) (Acosta et al., 2020).

Reactive oxygen species (ROS): For this analysis it should be taken into account that only living cells produce free radicals. The fluorescent probes used were carboxyfluorescein diacetate (C5041, Sigma Aldrich) and propidium iodide (P4170, Sigma Aldrich). When oxidized, propidium iodide emits

red fluorescence and DCF emits green fluorescence, then estimating the production of reactive oxygen species by the median green fluorescence intensity used to measure only live cells, i.e., negative PI (Acosta et al., 2020)

Lipid peroxidation (LPO): This analysis seeks to evaluate the amount of sperm cells affected by the production of reactive oxygen species, using body-pi as a fluorescent probe. The result was demonstrated through the lipid peroxidation rate, obtained through a calculation made by dividing the median of the green fluorescence intensity (peroxidized lipid) by the median of the green fluorescence intensity plus the median of the red fluorescence intensity (non-peroxidized lipid), multiplied by 100 (Acosta et al., 2020) .

Statistical analysis

The variables were analyzed for normality by the Shapiro-Wilk test followed by analysis of variance (ANOVA) by Tukey test. Treatments were considered independent variables, and the kinetic spermatozoa, membrane integrity, membrane fluidity, mitochondria functionality, index DNA fragmentation, ROS, lipid peroxidation, cell disruption were considered dependent variables. All analyzes were done by Statistix® software 9.0 (Statistix 2008).

RESULTS

The most commonly used parameters for routine sperm evaluation of stallions are total and progressive cell motility. The data obtained in the present study for both parameters are described in table 1. The data evaluated for total motility showed a difference, where in the periods of 15 to 60 minutes there was superiority for the highest concentrations of glycerol tested, but in 75 and 90 minutes the best results were obtained with a concentration of 3%.

Still regarding total motility, it is worth mentioning the superiority in the periods of 30 and 45 minutes for the concentrations of 4 and 5% of glycerol, and in the period of 90 minutes, the absolute superiority in the concentration of 3%.

Considering the analysis of progressive motility also shown in table 1, there was a difference between the concentrations, and in the periods of 15 and 30 minutes the 5% concentration was superior, in 45 minutes 4%, and in 75 and 90 minutes again the 3% concentration was superior. These facts indicate that the best results, among the possibilities tested, regarding total and progressive motility, are in higher concentrations of glycerol for short periods of exposure, or a lower concentration of cryoprotectant for a longer period.

DISCUSSION

Candeias et al., (2012) obtained good results after thawing the semen of two Mangalarga Marchador stallions frozen at a 5% glycerol concentration, compatible with the data obtained in this study. The same study tested times of 35 and 60 minutes of exposure to the cryoprotectant and obtained no difference between the periods, different from what was found in this research, proving that the time of contact of the cryoprotectant with the semen interferes in the result obtained after thawing.

For Pickett & Amann, (1993) when comparing two concentrations of glycerol (2 and 5%), they highlighted higher motility and higher percentage of live spermatozoa for the group treated with 2% glycerol compared to the 5% group.

Another parameter evaluated was the average distance traveled by the cells (DAP), describing its data in table 1. For the tested parameters, the 2% glycerol concentration was lower than the other concentrations, except in the 45 minutes period, where it showed no difference. In the concentrations of 4 and 5%, superiority for the shortest times tested, 15 and 30 minutes.

Regarding the curvilinear distance traveled (Table 1), again the 2% concentration was inferior. The rectilinear distance traveled (Table 1) showed superiority for 5%, especially in the periods of 15 and 30 minutes. The average speed also showed in table 1, superiority again to 5% in the 15 minutes period.

In relation to the data evaluated for straightness described in table 1, superiority was found for the 5% concentration of glycerol in the 15-minute period, and also for the 2% concentration in the 90-minute period.

The data for linearity and oscillation between VAP/VCL, showed, differently from the other evaluations, a superiority for 2 and 4% glycerol concentration in different periods.

Facing the first table of data obtained through the AndroVision® - CASA Software system with computer and monitor (Minitube) for sperm evaluation, one can evaluate a certain variation of results in different parameters in relation to glycerol concentration and exposure time tested.

The authors (Nascimento et al., 2015) obtained lower results when comparing only glycerol as cryoprotector in relation to glycerol associated with another substance, in this specific case, methylformamide and dimethylformamide. This fact is in agreement with the low results obtained in this study, exposing a possible toxicity when glycerol alone is used as cryoprotectant, regardless of the concentration and the period exposed.

Table 2 shows the data obtained through flow cytometry. The first data exposed refer to the integrity of the spermatozoa membrane, with a positive highlight for the concentration of 5% glycerol in the period of 45 minutes mainly.

For Alvarenga et a., (2003) the parameters of membrane and acrosomal integrity with 5% glycerol were lower when compared with glycerol in other concentrations and adding other substances, differently from the data obtained in this work, showing the need to dose the concentration of glycerol, as well as the period exposed to the cells.

Subsequently we have data for plasma membrane fluidity, acrosomal integrity and for the DNA fragmentation index of the spermatozoa.

Soni et al.,(2019) observed a better membrane and acrosomal integrity for glycerol at a concentration of 2% compared to other concentrations, and regarding the parameter of DNA fragmentation index (DFI), superiority for the combination of glycerol with another cryoprotector when compared to glycerol alone at different concentrations. In this work we also found a disparity between the parameters.

Following are the data related to lipid peroxidation of the cells, mitochondrial functionality, reactive oxygen species events and sperm cell ruptures.

The mitochondria present an important need for oxygen, where through oxidative phosphorylation produces about 90% of cellular energy in the form of ATP (adenosine triphosphate) molecules, and this energy is fundamental for the spermatozoa, in processes such as motility and cellular homeostasis [22].

Reactive oxygen species are metabolites produced during oxidative phosphorylation in cellular metabolism, representing a fundamental role in physiological processes such as capacitation, interaction between spermatozoon and zona pellucida, sperm hyperactivation and acrosomal reaction (Soni et al., 2019). Studies have demonstrated a relationship among oxidative stress, DNA integrity and high mitochondrial activity, a fact that was not proven in the present study, since there was a variation among these parameters evaluated, not demonstrating a correlation among them.

CONCLUSION

Glycerol is a cryoprotectant with excellent viability in its use. Taking into consideration the total and progressive motility parameters of the spermatozoa, we concluded that 5% glycerol concentration at 15 and 30 minutes of exposure, 4% glycerol at 45 minutes of exposure and 3% glycerol concentration at 90 minutes of exposure were the protocols that generated the best results.

Acknowledgements: We would like to thank the Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES, Brasilia, DF, Brazil) by post graduate scholarships awarded. C.D. Corcini and A.S. Varela Junior are research fellow from CNPq (310003/2021-0 and 307195/2015-1). We would also like to thank the members of the Comparative Animal Reproduction (Universidade Federal do Rio Grande, Rio Grande, RS, Brazil) group for their assistance.

REFERENCES

1. Acosta IB, Corcini CD, Gheller SMM, Brito CRC, Goulart T LS & Varela Junior A S. (2020). *Cryoletters*, **41**, 1-5.
2. Alvarenga MA, Papa FO & Ramires Neto C. (2016). *Vet Clin North Am Equine Pract* **32**:521-530.
3. Amann RP & Pickett BW. (1987) *J Equine Vet Sci,* **7**, 145-173.
4. Dziewulska K, Rzemieniecki A, Czerniawski R, & Domagała J (2011) *Theriogenology* **76**: 300 –311.
5. Martınez-Alborcia MJ, Valverde A, Parrilla I, Vazquez JM, Martınez EA & Roca J (2012). *PLoS One* **7**, e36550.
6. Miller CD. (2008). *Theriogenology* **70**(3):463-8.
7. Pickett BW & Amann RP. (1993) Cryopreservation of semen. In: Mckinnon AO, Voss JL (Ed.). Equine reproduction. Philadelphia: Lea & Febiger,769-789.
8. Keith, S.(1998) Tese doutorado; Colorado State Unirversity Fort Collins, 1998.
9. Candeias ML, Alvarenga MA, Carmo MT, Ferreira HN, Souto Maior MR, Torres Filho RA, Rodrigues ALR & Brandão FZ (2012). *R. Bras. Zootec.* **41**:1989-1995.
10. Medeiros ASL, Gomes GM, Carmo MT, Papa FO & Alvarenga MA. (2002). *Theriogenology*. **58**:273-276.
11. Nascimento JN, Blume H, Oliveira FJG & Oliveira RA. (2015*). Cienc. Anim. Bras*.16:324-330.
12. Alvarenga MA, Papa FO, Landin-Alvarenga FC & Medeiros ASL (2005). *Anim Reprod Sci* **89**: 105-113.
13. Kenney RM, Berman RV & Cooper WL. (1975). In: Annual Convention American Association Equine Practioners, Boston. Proceedings, AAEP, **21**: 327–336.
14. Soni Y, Talluri TR, Kumar A, Ravi SK, Mehta JS & Tripathi B. (2019).*Cryobiology* **86**:52-5

Table 1. Mean ± SE of sperm kinetics parameters of post-thawed equine semen with different concentrations of Glycerol and different periods of exposure.

Parameters	GLI [ ]	Periods of exposure					
		15 min	30 min	45 min	60 min	75 min	90 min
Total Motility (%)	2%	20.9 ± 1.3^{Ba}	18.2 ± 1.0^{Cab}	15.4 ± 0.6^{Cc}	17.5 ± 1.5^{Bbc}	15.4 ± 0.8^{Cc}	19.6 ± 1.2^{Bab}
	3%	21.6 ± 1.5^{Bb}	23.1 ± 1.7^{Bb}	22.2 ± 1.1^{Bb}	23.0 ± 1.0^{Ab}	23.5 ± 1.3^{Ab}	28.5 ± 1.8^{Aa}
	4%	23.0 ± 0.7^{ABbc}	26.7 ± 1.4^{ABab}	29.1 ± 1.6^{Aa}	24.9 ± 1.5^{Abc}	22.7 ± 1.2^{ABc}	21.9 ± 1.2^{Bc}
	5%	25.5 ± 1.4^{Aab}	28.6 ± 1.4^{Aa}	26.6 ± 1.3^{Aa}	22.9 ± 0.9^{Abc}	19.8 ± 1.3^{Bc}	22.7 ± 0.7^{Bbc}
Progressive Motility (%)	2%	5.3 ± 0.6^{Bb}	5.3 ± 0.5^{Cb}	4.1 ± 0.3^{Cb}	6.9 ± 0.8^{Aa}	2.3 ± 0.2^{Cc}	4.7 ± 0.4^{Cb}
	3%	6.6 ± 0.7^{Bb}	6.6 ± 0.7^{BCb}	5.8 ± 0.5^{Bb}	6.8 ± 0.6^{Ab}	7.1 ± 0.5^{Ab}	10.1 ± 1.2^{Aa}
	4%	7.3 ± 0.5^{Bbc}	8.0 ± 0.6^{Bb}	10.2 ± 0.9^{Aa}	7.0 ± 0.9^{Abc}	5.9 ± 0.4^{Bc}	6.7 ± 0.5^{BCbc}
	5%	10.2 ± 1.1^{Aa}	11.3 ± 0.7^{Aa}	6.7 ± 0.6^{Bb}	7.1 ± 0.4^{Ab}	5.4 ± 0.3^{Bb}	7.4 ± 0.5^{Bb}
DAP%	2%	30.8 ± 0.7^{Ca}	32.8 ± 1.0^{Ba}	33.0 ± 0.8^{Aa}	31.4 ± 0.8^{Ba}	28.3 ± 0.7^{Cb}	31.8 ± 1.7^{Ba}
	3%	33.4 ± 0.7^{Bab}	33.6 ± 1.0^{ABab}	33.7 ± 0.8^{Aa}	34.2 ± 0.9^{Aa}	34.1 ± 0.9^{Aa}	31.2 ± 0.6^{Bb}
	4%	33.8 ± 0.9^{ABab}	35.9 ± 0.9^{Aa}	32.5 ± 0.7^{Abc}	33.2 ± 0.8^{ABbc}	31.2 ± 0.7^{Bc}	32.5 ± 0.7^{ABbc}
	5%	35.9 ± 0.8^{Aa}	35.1 ± 0.8^{ABa}	32.6 ± 0.7^{Ab}	32.0 ± 0.6^{Bb}	32.2 ± 1.5^{ABb}	34.3 ± 0.8^{Aab}
DCL%	2%	56.6 ± 1.4^{Cab}	57.5 ± 1.6^{Ca}	54.5 ± 1.3^{Cab}	57.3 ± 1.6^{Bab}	52.8 ± 1.5^{Bb}	55.4 ± 2.1^{BCab}
	3%	61.8 ± 2.1^{ABab}	60.6 ± 1.8^{BCb}	65.5 ± 1.5^{Aa}	63.1 ± 1.9^{Aab}	62.6 ± 1.6^{Aab}	53.6 ± 1.3^{Cc}
	4%	57.5 ± 1.7^{BCc}	66.3 ± 1.8^{Aa}	61.7 ± 1.6^{ABbc}	63.9 ± 1.7^{Aab}	59.2 ± 1.4^{Ac}	58.8 ± 1.5^{Bc}
	5%	64.5 ± 1.6^{Aa}	62.4 ± 1.5^{ABab}	59.9 ± 1.2^{Bbc}	59.8 ± 1.3^{ABbc}	57.4 ± 2.5^{ABc}	63.9 ± 1.6^{Aab}
DSL%	2%	24.2 ± 0.7^{Ca}	26.3 ± 0.9^{ABa}	26.0 ± 0.7^{Aa}	25.3 ± 0.8^{Aa}	21.6 ± 0.5^{Bb}	25.7 ± 0.9^{ABa}
	3%	26.5 ± 0.9^{Ba}	24.9 ± 0.7^{Bab}	25.7 ± 0.7^{ABab}	25.6 ± 0.7^{Aab}	25.2 ± 0.8^{Aab}	23.9 ± 0.6^{Bb}
	4%	26.6 ± 0.7^{Ba}	26.5 ± 0.7^{ABa}	25.2 ± 0.6^{ABab}	25.1 ± 0.8^{Aab}	22.9 ± 0.6^{Bc}	23.9 ± 0.6^{Bbc}
	5%	29.3 ± 0.8^{Aa}	27.4 ± 0.7^{Aab}	23.9 ± 0.6^{Bc}	24.1 ± 0.5^{Ac}	25.5 ± 1.4^{Abc}	26.8 ± 0.7^{Ab}
VAP%	2%	74.0 ± 2.1^{Cb}	80.2 ± 2.8^{Aab}	80.5 ± 2.4^{Aa}	75.7 ± 2.4^{Bab}	63.7 ± 1.8^{Bc}	75.3 ± 2.5^{Bab}
	3%	81.2 ± 2.6^{BCa}	80.3 ± 2.4^{Aa}	80.0 ± 2.2^{Aa}	83.8 ± 2.4^{Aa}	80.5 ± 2.5^{Aa}	78.6 ± 1.7^{ABa}
	4%	84.1 ± 2.7^{Ba}	84.5 ± 2.5^{Aa}	78.9 ± 2.0^{Aab}	79.4 ± 2.2^{ABab}	76.3 ± 1.9^{Ab}	78.8 ± 2.0^{ABab}
	5%	92.0 ± 2.6^{Aa}	85.7 ± 2.4^{Ab}	79.3 ± 2.0^{Abc}	78.6 ± 2.0^{ABc}	78.6 ± 3.6^{Abc}	81.7 ± 2.1^{Abc}
VSL%	2%	58.2 ± 1.9^{Cb}	64.7 ± 2.4^{ABa}	63.8 ± 2.1^{Aa}	61.4 ± 2.4^{Aab}	48.4 ± 1.4^{Bc}	61.3 ± 2.2^{ABab}
	3%	64.2 ± 2.3^{BCa}	59.5 ± 1.6^{Ba}	61.2 ± 1.9^{ABa}	62.9 ± 1.9^{Aa}	59.5 ± 1.9^{Aa}	60.4 ± 1.7^{ABa}
	4%	66.4 ± 2.1^{Ba}	62.1 ± 1.9^{ABab}	61.6 ± 1.8^{ABabc}	60.2 ± 2.1^{Abc}	56.6 ± 1.7^{Ac}	58.1 ± 1.7^{Bbc}
	5%	75.4 ± 2.4^{Aa}	67.1 ± 2.1^{Ab}	58.1 ± 1.6^{Bc}	59.4 ± 1.5^{Ac}	61.7 ± 3.0^{Abc}	63.9 ± 2.0^{Abc}

SRT%	**2%**	0.78 ± 0.09^{Bcd}	0.80 ± 0.09^{Aab}	0.78 ± 0.07^{Abc}	0.80 ± 0.01^{Aabc}	0.76 ± 0.07^{ABd}	0.81 ± 0.08^{Aa}
	3%	0.79 ± 0.09^{ABa}	0.75 ± 0.01^{BCb}	0.76 ± 0.08^{Bb}	0.75 ± 0.08^{Bb}	0.73 ± 0.08^{BCb}	0.76 ± 0.09^{BCb}
	4%	0.79 ± 0.09^{ABa}	0.74 ± 0.01^{Cc}	0.77 ± 0.07^{ABab}	0.75 ± 0.01^{Bbc}	0.73 ± 0.08^{Cc}	0.74 ± 0.01^{Cc}
	5%	0.81 ± 0.07^{Aa}	0.77 ± 0.06^{Bb}	0.73 ± 0.01^{Cc}	0.75 ± 0.08^{Bbc}	0.78 ± 0.01^{Ab}	0.78 ± 0.04^{Bb}
LIN%	**2%**	0.43 ± 0.01^{Bb}	0.47 ± 0.01^{Aa}	0.48 ± 0.01^{Aa}	0.45 ± 0.02^{Aab}	0.43 ± 0.008^{ABb}	0.48 ± 0.009^{Aa}
	3%	0.45 ± 0.01^{Cab}	0.42 ± 0.01^{BCbc}	0.39 ± 0.007^{Bd}	0.41 ± 0.007^{Bcd}	0.41 ± 0.009^{BCcd}	0.46 ± 0.01^{Aa}
	4%	0.48 ± 0.01^{Aa}	0.41 ± 0.01^{Cbc}	0.42 ± 0.01^{Bbc}	0.40 ± 0.01^{Bbc}	0.39 ± 0.007^{Cc}	0.43 ± 0.01^{Bb}
	5%	0.346 ± 0.009^{ABa}	0.44 ± 0.006^{Bab}	0.40 ± 0.009^{Bc}	0.41 ± 0.007^{Bc}	0.45 ± 0.01^{Aab}	0.42 ± 0.009^{Bbc}
WOB%	**2%**	0.55 ± 0.009^{Bc}	0.58 ± 0.01^{Abc}	0.61 ± 0.009^{Aa}	0.56 ± 0.01^{Abc}	0.56 ± 0.008^{ABc}	0.59 ± 0.01^{ABb}
	3%	0.56 ± 0.01^{Bb}	0.56 ± 0.01^{ABb}	0.52 ± 0.007^{Cc}	0.55 ± 0.007^{ABb}	0.55 ± 0.009^{ABb}	0.59 ± 0.008^{Aa}
	4%	0.60 ± 0.009^{Aa}	0.55 ± 0.008^{Bbc}	0.54 ± 0.01^{BCc}	0.53 ± 0.01^{Bc}	0.53 ± 0.007^{Bc}	0.57 ± 0.008^{Bb}
	5%	0.56 ± 0.008^{Ba}	0.56 ± 0.006^{ABa}	0.55 ± 0.008^{Bab}	0.54 ± 0.007^{ABb}	0.57 ± 0.01^{Aa}	0.54 ± 0.007^{Cb}
ALH%	**2%**	3.93 ± 0.1^{Bab}	3.99 ± 0.08^{Cab}	4.06 ± 0.1^{Bab}	4.32 ± 0.2^{Aa}	3.73 ± 0.1^{Cb}	4.13 ± 0.1^{ABa}
	3%	4.09 ± 0.1^{Bbc}	4.61 ± 0.1^{Aa}	4.51 ± 0.09^{Aa}	4.39 ± 0.1^{Aab}	4.14 ± 0.1^{Bb}	3.85 ± 0.1^{Bc}
	4%	3.97 ± 0.1^{Bd}	4.41 ± 0.1^{ABab}	4.11 ± 0.08^{Bbcd}	4.04 ± 0.1^{Acd}	4.51 ± 0.09^{Aa}	4.27 ± 0.1^{Aabc}
	5%	4.42 ± 0.1^{Aa}	4.21 ± 0.09^{BCab}	4.15 ± 0.08^{Bab}	4.05 ± 0.1^{Ab}	4.18 ± 0.1^{ABab}	4.07 ± 0.1^{ABb}
BCF%	**2%**	35.2 ± 0.6^{Aa}	34.7 ± 0.7^{Aa}	32.4 ± 0.6^{Cb}	36.0 ± 0.6^{Aa}	35.7 ± 0.5^{Aa}	31.8 ± 0.7^{Cb}
	3%	33.9 ± 0.6^{Aa}	34.1 ± 0.6^{Aa}	33.1 ± 0.5^{BCab}	34.1 ± 0.5^{Ba}	32.0 ± 0.4^{Bb}	33.0 ± 0.5^{BCab}
	4%	33.8 ± 0.6^{Aab}	33.0 ± 0.6^{Abc}	35.5 ± 0.7^{Aa}	35.1 ± 0.8^{ABa}	31.6 ± 0.5^{Bc}	34.1 ± 0.7^{ABab}
	5%	34.9 ± 0.6^{Aa}	34.5 ± 0.5^{Aa}	34.3 ± 0.7^{ABa}	32.1 ± 0.5^{Cb}	34.5 ± 1.2^{Aa}	34.9 ± 0.6^{Aa}

Different capital letters in the same column within the same parameter show statistical difference. Distinct lowercase letters in the same row show statistical difference ($P<0.05$)

Table 2. Mean ± SE of sperm parameters of membrane integrity, membrane fluidity, acrosomal integrity, DFI, LPO, mitochondrial functionality, ROS and sperm rupture post thawing with different concentrations of Glycerol and different exposure periods.

Parameters	GLI [ ]	Exposure periods					
		15min	30min	45min	60min	75min	90min
Membrane Integrity (%)	2%	56,91±4,01^{ABa}	59,95±4,46^{ABa}	62,32±3,87^{Aa}	68,12±2,86^{Aa}	64,52±4,37^{Aa}	64,91±4,28^{Aa}
	3%	50,02±4,30^{Bb}	51,22±5,04^{BCb}	50,94±4,28^{Bb}	65,75±3,25^{Aa}	66,17±4,33^{Aa}	60,02±3,43^{Aab}
	4%	66,19±3,08^{Aa}	46,49±4,25^{Cc}	60,84±3,80^{ABab}	65,83±4,46^{Aa}	51,87±4,31^{Bbc}	65,92±3,20^{Aa}
	5%	56,58±4,52^{ABbc}	64,77±3,54^{Aab}	69,12±3,40^{Aa}	47,55±4,35^{Bc}	63,84±3,71^{Aab}	64,05±3,12^{Aab}
Membrane Fluidity (%)	2%	54,72±6,41^{Bd}	81,44±4,00^{Aab}	81,69±1,51^{Aab}	70,52±3,45^{Bbc}	84,31±3,12^{Aa}	59,99±5,70^{Bcd}
	3%	77,18±3,27^{Abc}	82,04±1,64^{Aab}	77,34±3,46^{Abc}	77,43±2,74^{ABbc}	90,04±1,37^{Aa}	69,21±4,85^{ABc}
	4%	76,29±4,23^{Aa}	62,97±5,77^{Bb}	73,65±3,35^{ABab}	82,12±3,46^{Aa}	72,04±5,28^{Bab}	75,81±4,90^{Aa}
	5%	82,55±3,67^{Aa}	79,22±2,16^{Aa}	66,69±4,94^{Bb}	75,23±3,95^{ABab}	81,41±2,28^{Aa}	68,51±4,26^{ABb}
Acrosomal Integrity (%)	2%	53,91±4,33^{Bc}	67,95±3,40^{Aab}	72,35±3,20^{Aa}	62,51±3,87^{Aabc}	61,60±4,30^{Abc}	72,00±3,49^{Aab}
	3%	57,94±4,62^{ABb}	61,19±4,29^{Ab}	62,07±4,08^{Bab}	54,80±3,69^{Ab}	58,00±3,94^{Ab}	72,88±4,52^{Aa}
	4%	70,10±3,36^{Aa}	63,80±3,69^{Aa}	69,17±3,76^{ABa}	60,17±4,47^{Aa}	66,67±3,86^{Aa}	64,56±3,86^{Aa}
	5%	57,62±4,96^{Bbc}	66,58±3,45^{Aab}	74,83±2,74^{Aa}	55,00±3,47^{Ac}	57,27±3,65^{Abc}	64,90±4,68^{Aabc}
DFI	2%	0,88±0,01^{Bab}	0,90±0,01^{Aab}	0,88±0,01^{Aab}	0,87±0,01^{Ab}	0,91±0,01^{Aa}	0,82±0,01^{Cc}
	3%	0,92±0,01^{Aa}	0,89±0,01^{ABab}	0,87±0,01^{Ab}	0,88±0,01^{Ab}	0,89±0,01^{Aab}	0,86±0,01^{ABb}
	4%	0,88±0,02^{Bab}	0,89±0,01^{ABa}	0,87±0,01^{Aab}	0,87±0,01^{Aab}	0,89±0,01^{Aa}	0,84±0,01^{BCb}
	5%	0,90±0,01^{ABa}	0,87±0,01^{Ba}	0,89±0,01^{Aa}	0,89±0,01^{Aa}	0,88±0,01^{Aa}	0,89±0,01^{Aa}
LPO	2%	78,85±2,99^{Aa}	78,46±3,63^{Aa}	78,05±4,05^{Aa}	76,41±4,15^{Aa}	76,30±3,80^{Aa}	75,97±4,1^{Aa}
	3%	80,78±3,00^{Aa}	77,44±3,95^{Aa}	78,11±4,04^{Aa}	76,37±4,21^{Aa}	71,32±5,07^{Aa}	76,90±3,94^{Aa}
	4%	80,25±2,84^{Aa}	76,31±4,22^{Aa}	76,02±3,95^{Aa}	76,02±4,14^{Aa}	72,82±4,52^{Aa}	76,24±4,01^{Aa}
	5%	76,37±4,42^{Aa}	77,41±3,53^{Aa}	76,44±3,93^{Aa}	72,62±4,21^{Aa}	74,25±4,10^{Aa}	77,38±3,42^{Aa}
Mitochondrial Functionality (%)	2%	19,83±3,59^{Aab}	7,47±1,47^{Bc}	18,49±4,47^{Aab}	24,96±3,51^{Aa}	13,95±2,90^{Bbc}	8,82±1,17^{Bc}
	3%	14,02±3,85^{ABb}	10,49±2,20^{ABb}	15,84±3,33^{Aab}	22,44±3,27^{Aba}	14,33±2,57^{ABab}	13,04±2,37^{ABb}
	4%	15,01±3,00^{ABab}	13,62±1,72^{Ab}	12,40±2,30^{Ab}	14,72±2,68^{Bab}	22,80±4,13^{Aa}	19,38±4,37^{Aab}
	5%	8,39±1,49^{Bb}	7,23±1,27^{Bb}	10,36±1,79^{Ab}	18,74±3,33^{Aba}	12,43±2,53^{Bab}	16,94±2,87^{ABa}
ROS	2%	3130±345^{Aa}	2213±300^{Ab}	2529±242^{ABab}	2783±325^{Aab}	2352±308^{Aab}	2509±270^{ABab}
	3%	2271±379^{Aabc}	2232±333^{Abc}	3102±298^{Aa}	2150±324^{Ac}	3061±304^{Aab}	1946±225^{Bc}
	4%	768±107^{Ab}	2451±284^{Aab}	2243±294^{Bab}	2658±342^{Aab}	2953±313^{Aa}	2684±280^{Aab}
	5%	2513±548^{Aa}	2087±309^{Aa}	2372±292^{ABa}	2975±380^{Aa}	2709±338^{Aa}	2148±237^{ABa}
sperm	2%	37,14±4,69^{Cc}	70,94±3,09^{Aa}	67,16±2,92^{Aa}	56,32±3,67^{Bb}	62,15±3,32^{Aab}	62,67±3,55^{BCab}

rupture (%)	**3%**	76,34±2,56^{Aa}	69,67±3,48^{Aab}	63,13±3,14^{Ab}	68,51±4,74^{Aab}	67,58±3,01^{Aab}	68,62±3,37^{ABab}
	4%	63,48±4,68^{Bbc}	66,99±3,10^{Aabc}	58,16±3,14^{ABc}	71,92±3,86^{Aab}	68,49±4,15^{Aab}	76,18±1,81^{Aa}
	5%	70,64±3,80^{ABa}	70,17±3,34^{Aa}	49,01±4,40^{Bc}	66,02±3,71^{ABab}	62,77±3,09^{Aab}	59,53±3,81^{Cb}

Different capital letters in the same column within the same parameter show statistical difference. Distinct lowercase letters in the same row show statistical difference ($P<0.05$)

3 Considerações finais

A criopreservação de sêmen é uma técnica de extrema importância em diversas espécies, bem como na espécie equina. Diversos protocolos estão descritos e elucidados, sendo crucial em seu desenvolvimento a utilização de crioprotetores a fim de minimizar os efeitos negativos causados pelo congelamento e descongelamento sobre as células espermáticas.

Através deste trabalho podemos observar os melhores índices da utilização do glicerol como crioprotetor quando consideramos os parâmetros de motilidade total e progressiva, sendo na concentração de 3% e período de exposição de 90 minutos, na concentração de 4% e período de 45 minutos de exposição e concentração de 5% nos períodos de 15 e 30 minutos de exposição.

Sugerimos a utilização de um protocolo com alguma dessas combinações de concentração de glicerol e período de exposição do mesmo com as células para um melhor processo de criopreservação de sêmen na espécie equina.

Referências

Alvarenga *et. al.* **The use of alternative cryoprotectors for freezing stallion semen.** In: WORKSHOP ON TRANSPORTING GAMETES AND EMBRYOS, 12., 2003, Brewster, Massachusetts. Anais... New York: Havemeyer Foundation Monograph Series, 2003. p.74-76.

Amann RP, Pickett BW. **Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa**. J Equine Vet Sci, v.7, p.145-173, 1987.

Angrimani et al. **Ferramentas para avaliação da funcionalidade da mitocôndria espermática.** Rev. Bras. Reprod. Anim., Belo Horizonte, v.39, n.2, p.277-283, abr./jun. 2015.

Aurich, J. *et al*. **Efficiency of Semen Cryopreservation in Stallions**. 2020. Disponível em: https://pubmed.ncbi.nlm.nih.gov/32545785/

Alvarenga *et. al*. **Advances in Stallion Semen Cryopreservation.** 2016. https://pubmed.ncbi.nlm.nih.gov/27726989/

Alves JP., Corcini CD., Silva EF., Caldas JS., Cardoso TF, Piedras SRN, Jardim RD., Varela ASJ. **The role of amides in seminal cryopreservation of wild silverside, Odontesthes bonariensis.** http://dx.doi.org/10.1016/j.cryobiol.2016.09.001 0011-2240/© 2016 Published by Elsevier Inc.

Candeias *et. al.* **Semen cryopreservation protocols of Mangalarga Marchador stallions.** R. Bras. Zootec. 2012;41(9):1989-1995. Disponível em: http://dx.doi.org/10.1590/S1516-35982012000900004

C.D. Miller. **Optimizing the use of frozen-thawed equine semen**. 2008. Theriogenology. Disponível em: https://pubmed.ncbi.nlm.nih.gov/18550159/

Fernandez-Gago R, Domínguez JC, Martínez-Pastor F. **Seminal plasma applied post-thawing affects boar sperm physiology: a flow cytometry study.** Theriogenology 2013, 80, 400–410.

Gillan L, Evans G, Maxwell WMC. **Flow cytometric evaluation of sperm parameters in relation to fertility potential.** Theriogenology 2005, 63, 445–457.

Keith, S. **Evaluation of new cryoprotectans for the preservation of equine spermatozoa.** Tese douturado; Colorado State Unirversity Fort Collins, 1998.

Kenney RM, Berman RV, Cooper WL. **Minimal contamination techniques forbreeding mares: technique and preliminary findings.** In: ANNUAL CONVENTION AMERICAN ASSOCIATION EQUINE PRACTIONERS, Boston. Proceedings, AAEP, 21, 327–336, 1975.

Lagares et. al. **L-carnitine added to post-thawed semen acts as an antioxidant and a stimulator of equine sperm metabolism.** Andrologia. 2022 Apr;54(3): e14338. doi: 10.1111/and.14338. Epub 2021 Dec 8. PMID: 34878673.

Loomis, P.R. **Factors affecting the success of artificial insemination with cooled, transported semen.** In: AMERICAN ASSOCIATION OF EQUINE PRACTTIONERS ANNUAL CONVENTION, 38Th, 1992. Proceedings. 629-47, 1992.

Medeiros *et. al.* **Cryopreservation of stallion sperm using different amides.** Theriogenology. 2002; 58(2-4): 273-276. Disponível em: http://dx.doi.org/10.1016/S0093-691X(02)00898-1

Nascimento et. al. **Utilização de diferentes diluentes na criopreservação de espermatozoides de garanhões mangalarga marchador.** Cienc. anim. bras. v.16, n.3, p.324-330, jul./set. 2015.

Oliveira *et. al.* **Efeito da adição de glicerol em diferentes etapas do resfriamento na congelabilidade do sêmen equino.** REVISTA CIENTÍFICA DE MEDICINA VETERINÁRIA - ISSN 1679-7353 Ano XIV - Número 29 – Julho de 2017 – Periódico Semestral

Petrunkina AM, Volker G, Brandt H, Töpfer-Petersen E, Waberski D. **Functional significance of responsiveness to capacitating conditions in boar spermatozoa.** Theriogenology 64, 1766–1782, 2005.

Pickett BW, Amann RP. **Cryopreservation of semen.** In: Mckinnon AO, Voss JL (Ed.). Equine reproduction. Philadelphia: Lea & Febiger, 1993. p.769-789.

Silva, ACG. Almeida, Volcan D; Nornberg, BF; Pereira, JR; Pires, DM; Corcini, CD; Varela Junior, AS; Marins, LF. **Reproductive parameters of double transgenic zebrafish (Danio rerio) males overexpressing both the growth hormone (GH) and its receptor (GHR).** Transgenic Res. DOI 10.1007/s11248-016-9990-0. Accepted: 8 October 2016 © Springer International Publishing Switzerland 2016.

Terraciano *et. al.* **Criopreservação de espermatozoides equinos comparando duas curvas de congelamento combinadas com diluentes comerciais: uma análise laboratorial.** Cienc. Rural. 2008;38(7):1972-1977.

Y. Soni *et. al.* **Effects of different concentration and combinations of cryoprotectants on sperm quality, functional integrity in three Indian horse breeds,** *Cryobiology* (2019), doi: https://doi.org/10.1016/j.cryobiol.2018.12.005.