VETERINARSKI ARHIV 95 (1), 1-10, 2025 DOI: 10.24099/vet.arhiv.2598

Dialysis as a new method for addition of glycerol to stallion semen before freezing

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KLINC, P., V. ŽALIG, V. MAGAŠ, J. BLAGOJEVIĆ, M. SAMARDŽIJA, P. STOJIĆ, M. MALETIĆ: Dialysis as a new method for addition of glycerol to stallion semen before freezing. Vet. arhiv 95, 1-10, 2025.

ABSTRACT

The main purpose of this study was to analyse the effect of dialysis, as a method for the addition of glycerol, on the quality of frozen / thawed stallion spermatozoa. Three fertility-proven Lipizzaner stallions and 20 ejaculates were included. Each ejaculate was divided into two equal portions in order to use a split ejaculate technique. Glycerol was added either by pipetting, where the semen pellet was diluted after centrifugation with an extender containing 5% glycerol, or using dialysis in which the semen pellet was first diluted with the extender containing no glycerol, and then glycerol was added with the use of a dialysis system. All the samples were gradually cooled to 5ºC, frozen in nitrogen vapor, and kept in liquid nitrogen until analysed. The semen was thawed in a water bath at 37ºC and the samples were analysed after 10 minutes and 3 hours of incubation at 37ºC, for motility, progressive motility, and concentration, using the CASA system (Computer Assisted Sperm Analysis). The quality of the thawed spermatozoa was further analysed using the hypoosmotic swelling (HOS) test and morphological analysis. The addition of glycerol by means of dialysis significantly increased total motility ($P<0.001$), progressive motility ($P<0.001$) 10 minutes after thawing, the percentage of HOS positive spermatozoa after 10 minutes and 3 hours of incubation ($P<0.001$, $P<0.05$, respectively), and the percentage of morphologically normal spermatozoa immediately after thawing ($P<0.05$). The results of this study confirmed the hypothesis that the negative effect, caused by the fast addition of glycerol to semen by pipetting, can be reduced by the gradual addition of glycerol by dialysis.

Key words: equine reproduction; semen centrifugation; freezing; dialysis; sperm motility

Introduction

Cryopreservation of stallion spermatozoa enables the long-term conservation of valuable genetic material. Optimized conditions, including semen processing, types of semen extenders, and cryoprotectants, play an important role in processing stallion spermatozoa for cryopreservation (GHALLAB et al., 2019). After collection, the semen has to be concentrated and the excess of seminal plasma removed. Different centrifugation forces and times of centrifugation are recommended. A higher centrifu-

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ISSN 0372-5480
Printed in Croatia

gation force and longer time enable the lower loss of spermatozoa, but they also represent a higher level of stress for the spermatozoa (HOOGEWIJS et al., 2010). The use of a medium for semen centrifugation, such as Cushion-Fluid (Minitube, Germany), was recommended to prevent the negative effect of high centrifugal force (BLISS et al. 2012).

Different cryoprotective substances may be used for freezing stallion spermatozoa. Of all these substances, glycerol is most widely used. The overall highest cryosurvival rates were obtained with dimethylformamide, followed by glycerol and ethylene glycol, whereas the use of propylene glycol and dimethyl sulfoxide resulted in poor cryosurvival rates (OLDENHOF et al., 2017). Glycerol affects the fertility of equine spermatozoa as early as during the pre-freezing procedure. In consequence, the glycerol level in frozen equine semen must be low to provide good fertility. Glycerol exerted toxicity at concentrations ≥3.5% and maximum toxicity was observed at 5%. The actin cytoskeleton was especially sensitive to the presence of glycerol, inducing rapid F actin depolymerization at concentrations over 1.5%. The toxicity of glycerol is related to its osmotic and non-osmotic effects. The concentration of glycerol in the freezing media for stallion spermatozoa should not exceed 2.5% (MACIAS GARCIA et al., 2012). Results from another study suggest that using the appropriate cryoprotectant combination instead of a single cryoprotectant can improve stallion spermatozoa cryopreservation (WU et al., 2015). The glycerol concentration in a commercial freezing extender for equine spermatozoa can be successfully reduced when urea is added as a cryoprotectant additive and the glucose concentration is elevated. However, total glycerol replacement with other cryoprotectants did not give sufficient results (DE OLIVEIRA et al., 2017). There are several reports that high concentrations of glycerol added to extenders for freezing stallion spermatozoa can decrease their fertility (YÁNEZ-ORTIZ et al., 2022). However, the extenders added to semen after thawing did not improve the sperm parameters nor enhance the fertility results (SALES et al., 2019).

The process of sperm dialysis involves the gradual and controlled addition of glycerol to the sperm sample through a semi-permeable membrane, allowing for the gradual uptake of the cryoprotectant, without subjecting the sperm to osmotic shock. This technique has been shown to significantly improve sperm viability and motility post-thaw, making it a promising alternative to traditional methods of glycerol addition. Dialysis is the method that is used successfully in protocols for processing rooster semen, to reduce the concentration of glycerol after thawing (BUSS, 1993). In this species, glycerol prevents the fertilization of oocytes, and needs to be removed before insemination or in vitro fertilization (LIN et al., 2023). In other animal species dialysis is mainly used experimentally to remove toxic oxidative products before cold storage or cryopreservation of the semen, using semi-permeable dialysis bags and 12-14 kDa molecular weight cut-off (FRASER et al., 2007). Dialysis was also used successfully to remove Phospholipase A2 from buck semen before dilution with egg yolk-containing extender, and freezing (PREMROV BAJUK et al., 2018). To our knowledge, addition of cryoprotectants by dialysis (glycerol and trehalose) has only been used successfully for preparation of mouse semen before freezing (THOMPSON et al., 2001).

The purpose of this research was to study dialysis as a possible alternative method for adding glycerol to stallion semen before freezing. We hypothesized that dialyzing stallion sperm would reduce the toxic effects of glycerol, provide an approved method of cryoprotectant administration, and improve sperm viability and motility after thawing, as well as increasing the number of morphologically normal spermatozoa.

Materials and methods

All the chemicals, if not stated otherwise, were purchased from Merck, Germany.

Semen Collection, Processing and Sperm Cryopreservation. Semen samples were collected from three Lipizzaner stallions, with proven fertility, between 5 and 11 years of age. The experiment was conducted according to the positive opinion of the ethics committee of the Veterinary Faculty, University of Ljubljana. Therefore, the stallions were additionally stimulated with a light regime,

with 16 hours of illumination per day, two months before and during the experiment. The animals were maintained in standard environmental conditions, with food and water available *ad libitum*. A total of 20 ejaculates were collected using a Colorado artificial vagina (Minitube, Germany) twice a week in December. The semen was collected in collection bottles pre-heated to 37°C. Sperm motility was analyzed subjectively by microscopic (Nikon XS2-H; Japan) immediately after collection. Only samples with at least 70% total motility and 80% morphologically normal spermatozoa were used in the study. Ejaculates were diluted with a centrifugation medium for equine semen (Cushion-Fluid, Minitube, Germany) at 28°C, in a ratio of 1:3 (v/v). The ejaculates were subsequently divided into two aliquots and processed as control (C) and dialyzed (D) samples.

The extended semen was centrifuged at 530 x g, for 15 min. In control samples (C), the semen pellet was diluted with 5 mL of freezing extender (Gent, Minitube, Germany) containing 5% glycerol added by pipetting. First, the tube with the semen was inserted into a falcon tube, prefilled with 45 mL of tap water at 23°C and slowly cooled to 5°C (at 15°C for 1.5 hours and then at 5°C for another two hours). After that, the 0.5 mL straws were filled and frozen in nitrogen vapor. The other half of the ejaculate (D samples) was first diluted with 5 mL of extender (Gent, Minitube, Germany) containing no glycerol. A commercial dialysis device, Float-A-Lyzer® G2 (SpectrumLabs, USA), with a volume of 5 mL and a 300 kDa molecular weight cut-off, was used for the dialysis. The device was used according to the manufacturer's instructions. First, the tube was submerged and soaked in deionized water for 15 min to remove the glycerol. After rinsing with the dialysis buffer containing Gent extender, the device was filled with 5 mL of the semen aliquot and floated in the dialysate reservoir containing 43 mL of the Gent extender, supplemented with 2 mL of homologous seminal plasma and 5.5% (v/v) of glycerol in a 50 mL Falcon tube. The D samples was dialyzed under constant rolling of the Falcon tube at 15°C for 1.5 hours and then at 5°C for another two hours. This allowed for the same cooling curve as in the C sample. After the end of the dialysis, the D samples were poured into 0.5 mL straws and frozen in nitrogen vapor at the same time as the C samples. The success of the dialysis was verified by measurement of the osmolarity (Osmomat 030, Gonotec, Germany) in the C and D samples.

Evaluation of the semen quality variables. Apart from motility, morphology, and the concentration of the spermatozoa, which were also determined before cryopreservation, all analyses were performed of the frozen/thawed semen samples after thawing and incubation in a water bath at 37°C for 10 minutes (0 h) and 3 hours (3 h).

Concentration and motility of the spermatozoa. After dilution of the semen with distilled water in a ratio of 1:100, the concentration of the spermatozoa was determined using a Thoma counting chamber (Thoma, Brand; Germany). The final concentration represents the mean of two counts. The motility of the spermatozoa was assessed with a computer-assisted sperm analyzer (CASA, Hamilton Thorne Biosciences, Version 12.3, Beverly, MA) in a Makler chamber (Sefi-Medical Instruments, Israel) warmed to 37°C. First, the samples were diluted to 1:10 (v/v) with Gent extender and incubated at 37°C for 10 minutes. Two drops and three automatically selected fields per drop were analyzed from each sample. The following motility parameters were recorded: total motility (TM, %) and progressive motility (PM, %).

Morphology of the spermatozoa. The morphology of the spermatozoa was analyzed using a phase-contrast microscope (Olympus BH-2, Olympus Optical Co.; Japan) at x1000 magnification, after staining with Giemsa. At least 200 spermatozoa were analyzed per sample. The percentage of morphologically normal spermatozoa (MNS) and the percentage of spermatozoa with abnormal acrosomes were determined.

Viability of the spermatozoa. The viability of the spermatozoa was assessed using the hypoosmotic swelling (HOS) test. The integrity of the membranes was evaluated using the HOS test. Spermatozoa with normal plasma membranes undergo swelling and curling of their tails under hypoosmotic conditions. For the analysis, 1 mL of hypoosmotic solution (100 mOsm/L sucrose solution) was gently mixed with

100 µL of the semen sample and incubated for 50 minutes at 37°C. After incubation, the samples were smeared on slides stained with Giemsa, and evaluated under a light microscope (Olympus BH-2, Olympus Optical Co.; Japan). A total of 200 spermatozoa per smear were classified and divided into groups with coiled tails (intact membrane) and straight tails (spermatozoa with damaged membrane). The final percentage of spermatozoa with intact membranes was determined after subtraction of the spermatozoa with morphologically coiled tails.

Statistical analysis. Statistical analyses were performed using Sigma Stat 4.0. (Starcom Information Technology Ltd., Bangalore, India). Semen quality variables and the differences between the groups were analyzed by one-way ANOVA. When the data significantly deviated from normal distribution, they were analyzed with Friedman repeated measures analyses of variance. The differences in pairwise comparison were tested using the Bonferroni method for normally distributed data, and the Tukey test when the distribution of the data significantly deviated from normal distribution. Differences were considered to be statistically significant at $P<0.05$.

Results

Spermatozoa concentration and motion variables. The concentration of spermatozoa in the samples was determined using a Thoma counting chamber after centrifugation and dilution with Gent extender. The concentration in ejaculates before aliquoting to C and D samples was 69.9±20.8 x10^6/1 mL.

The data on the motion variables for semen after thawing are presented in Fig. 1 and 2. The motility and progressive motility of spermatozoa were significantly affected immediately after thawing. Group D had a higher percentage of motile and progressive motile spermatozoa immediately after thawing. After incubation of spermatozoa at 37°C for 3h the motility and progressive motility of the spermatozoa did not differ between the two groups. A similar difference was found for progressive motility, with significantly more progressive motile spermatozoa in group D immediately after thawing, and no significant difference after 3h incubation at 37°C.

Membrane integrity and viability. Fig. 3 shows data on the effect of dialysis on membrane integrity and the viability of spermatozoa after cryopreserva-

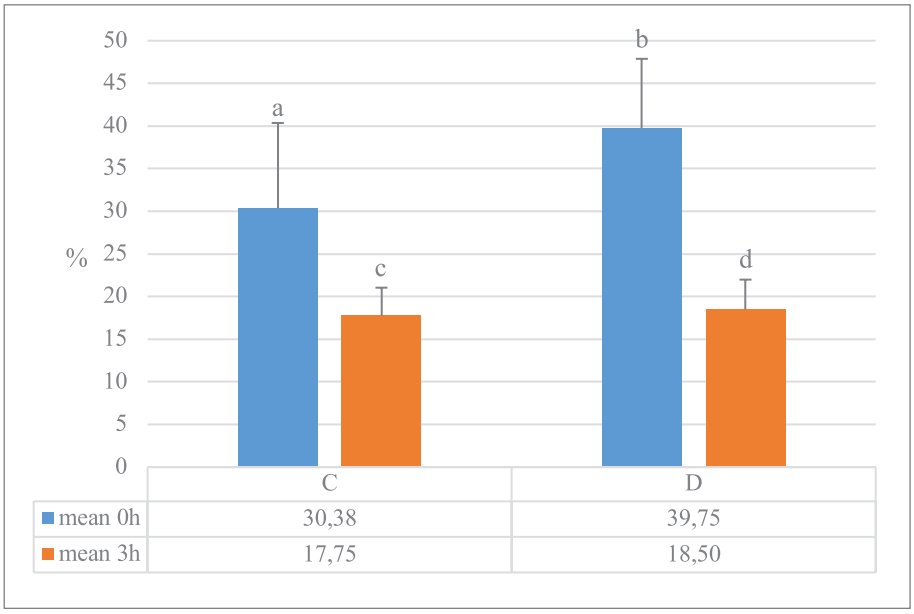

Fig. 1. Total motility of spermatozoa after thawing in the control samples (C) and in dialyzed samples (D) immediately after thawing and after 3 hours incubation at 37°C, a:b = $P<0.001$

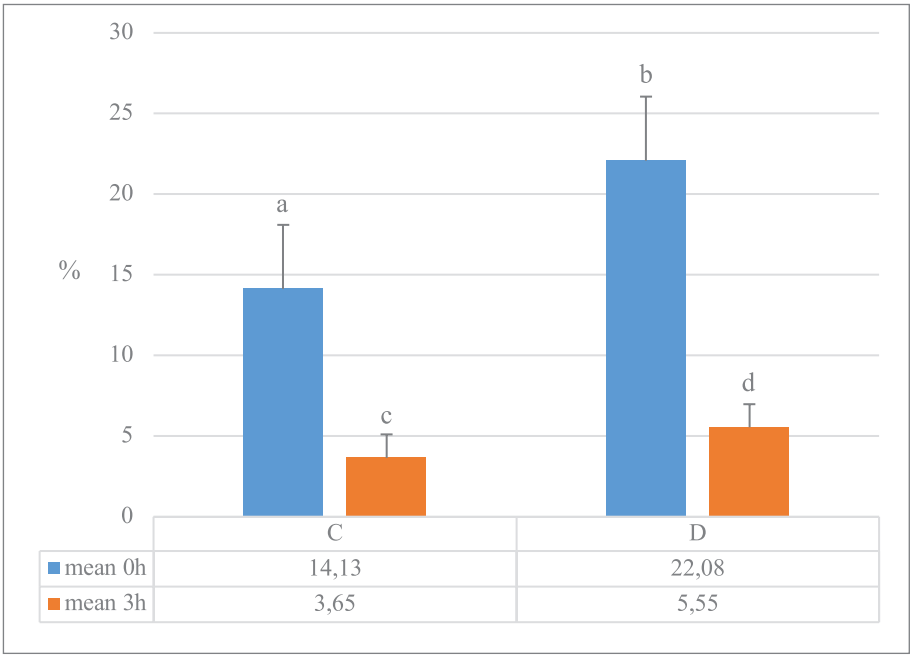

Fig. 2. Progressive motility of spermatozoa after thawing in the control samples (C) and in dialyzed samples (D) immediately after thawing and after 3 hours incubation at 37°C, a:b = P<0.001

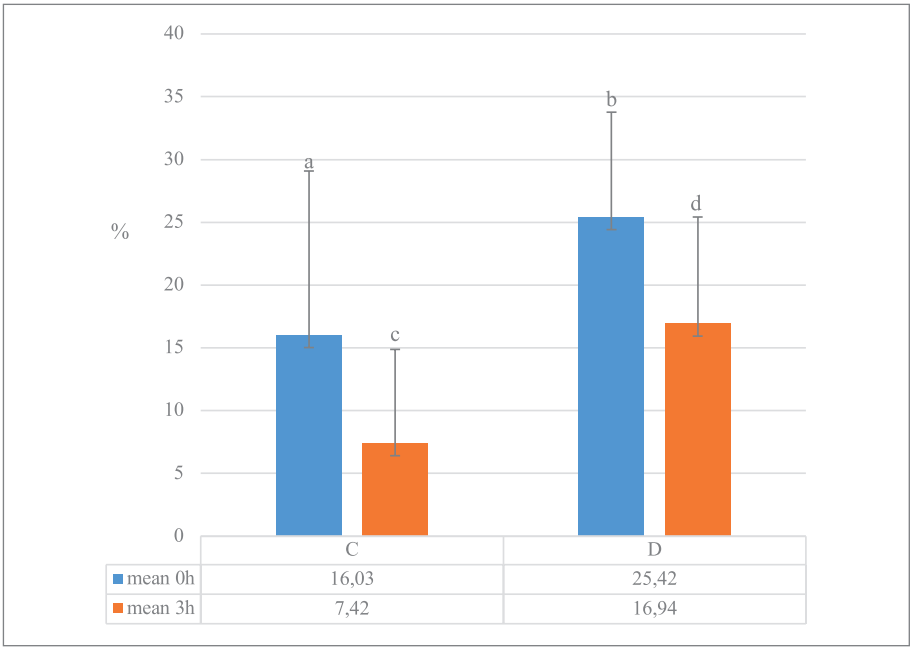

Fig. 3. Percentage of HOS positive spermatozoa, a:b = P<0.001; c:d = P≤0.05

tion. Immediately after thawing and after incubation at 37°C for 3h the percentage of viable spermatozoa was significantly higher in the dialyzed group than in the control group.

Morphology. The percentages of MNS (morphologically normal spermatozoa) and spermatozoa with coiled tails after thawing and incubation for 3 h at 37°C are shown in Fig. 4 and 5. In general, the

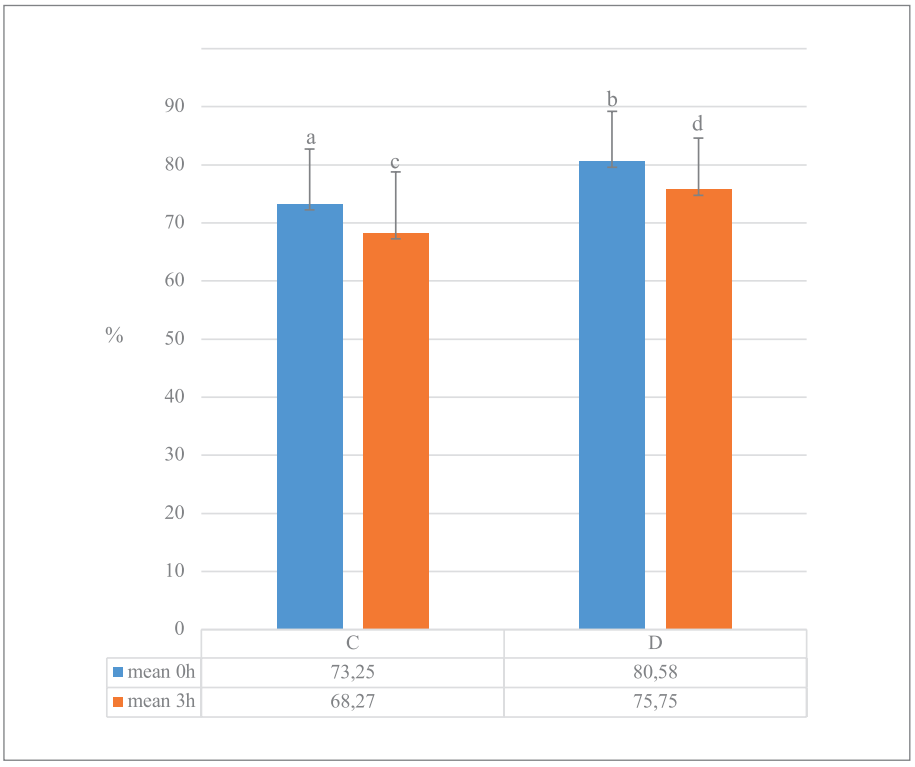

Fig. 4. Percentage of morphological normal spermatozoa (MNS), a:b = P<0.05

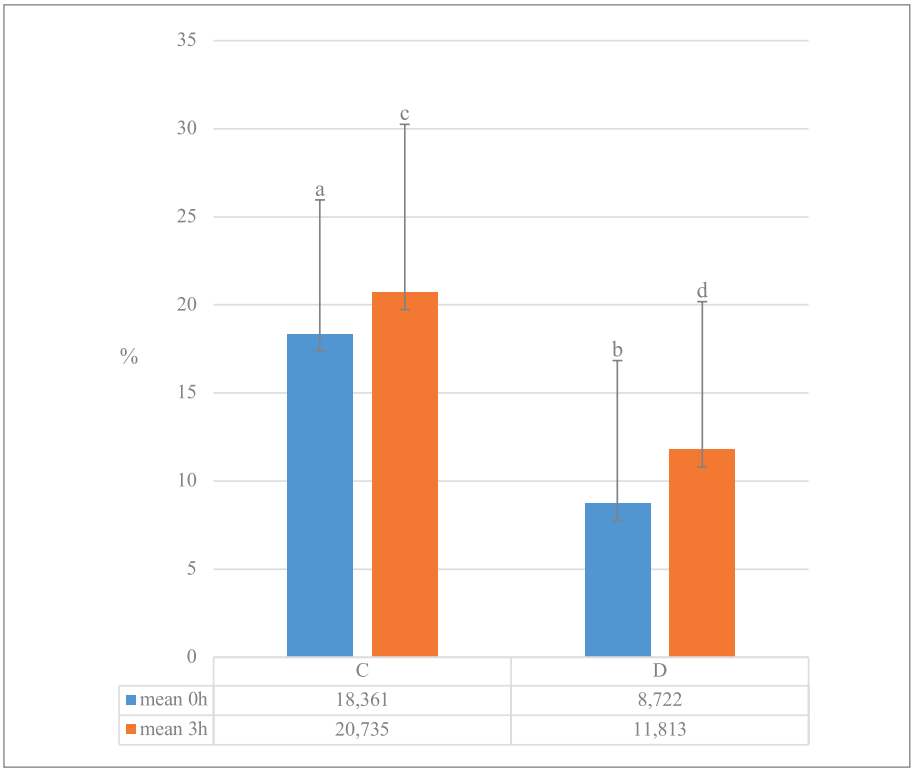

Fig. 5. Percentage of spermatozoa with coiled tails, a:b = P<0.001 c:d = P≤0.05

percentage of MNS was significantly higher in the dialyzed samples immediately after thawing, but the difference was not maintained after further incubation at 37°C. The percentage of spermatozoa with coiled tails was significantly lower in the dialyzed group compared to the control group immediately after thawing and after further incubation at 37°C for 3h.

Discussion

Stallion sperm cryopreservation is a crucial tool in the equine breeding industry, allowing for the preservation of valuable genetics, and facilitating the transportation of semen over long distances. Therefore, the correct collection and processing of sperm, in addition to the insemination technique, are the most important factors affecting the success of fertilization with cryopreserved semen. One of the key components in the cryopreservation process is the addition of cryoprotectants, such as glycerol, to protect the spermatozoa from damage during freezing and thawing. However, the traditional methods of glycerol addition can result in substantial loss of sperm viability and motility. To address this issue, we investigated the innovative technique of stallion sperm dialysis for glycerol addition. In our research, glycerol was added gradually during cooling to 5°C over 3 hours, and the effect was compared with control samples where glycerol was added in one step by pipetting.

The stallions included in our experiment corresponded to all the parameters on the basis of which we can expect good reproductive performance (age, constitution, condition). However, the experiment was performed in the off-season, which, according to some authors, is the most important factor for the production of quality sperm (CRESPO et al., 2020).

In the native semen, the motility of spermatozoa met the minimum criterion, which is over 30% for frozen / thawed (F/T) semen (KATILA, 2001). Motility is an important parameter that shows the quality and, to a certain degree, the fertilizing ability of spermatozoa, but it should not be used as the only parameter to evaluate the quality of stallion sperm (CONTRERAS et al., 2020). Immediately after thawing, we determined a significantly higher percentage of total motile spermatozoa in group D compared to group C, as well as a higher percentage of progressively motile spermatozoa. After 3 hours of incubation at 37°C, the difference between the groups remained, but was not significant. Although sperm motility is not the best predictor of fertility (GRIFFIN et al., 2019), we believe that higher motility and progressive motility are a clear sign of the superior dialysate quality compared to the control sperm (KIRK et al., 2005).

The addition of glycerol by dialysis also had a positive effect on decreasing morphological defects, and especially on the percentage of morphologically coiled tails. Given that coiled tails may be due to osmotic stress, we believe this is valuable evidence of the importance of adding glycerol slowly. From these results, it may be concluded that the addition of glycerol by dialysis has a positive effect on the quality of F/T stallion semen. This indicates less osmotic stress in sperm where glycerol was added gradually through dialysis. Our results showing the success of glycerol supplementation by dialysis are consistent with the positive effects of glycerol supplementation by dialysis in mouse sperm (THOMPSON et al., 2001), but also in goat (PREMROV BAJUK et al., 2018), and rooster sperm (LONG and KULKARNI, 2004).

The percentage of coiled tails, according to the HOS test (minus the percentage of previously morphologically coiled tails), was also higher in group D. The higher percentage of spermatozoa with intact membranes according to the HOS test indicates the superior quality of sperm processed by the dialysis system. Although the HOS test was not correlated with the percentage of pregnant mares, it showed a tendency to correlate with the number of services per pregnancy, so it could be an additional method for evaluating stallion fertility (NEILD et al., 2000). Further studies are needed to confirm this observation.

One of the key advantages of the stallion sperm dialysis method is its ability to minimize the toxic effects of glycerol on sperm cells. Glycerol toxicity is most likely the cause of lower semen quality after thawing, and other cryoprotectants have been tested as alternatives (SQUIRES et al., 2004). A

study by MACIAS GARCIA et al. (2012) suggests two modes of glycerol toxicity: biochemical and osmosis effects. The sperm membrane permeability for glycerol is low, which controls the damage that glycerol causes by passing into cells. First, sperm shrink due to dehydration, and after more than an hour of incubation in glycerol, the volume of spermatozoa increases due to the entry of glycerol into the cells, which also draws in water. The explanation for better motility in the case of group D would be that allowing for the gradual uptake of glycerol through dialysis enables the sperm cells to acclimate better to the cryoprotectant, resulting in improved post-thaw sperm quality.

In addition, the study by MACIAS GARCIA et al. (2012) suggests that extenders for stallion semen should not contain more than 3.5% glycerol due to its toxicity, but we successfully used higher concentrations in the present study. The use of lower glycerol concentrations in dialysis supplementation remains the subject of future research. Furthermore, stallion sperm dialysis offers a more controlled and reproducible method of glycerol addition compared to traditional techniques. The semi-permeable membrane used in the dialysis process allows for precise regulation of glycerol concentration in the sperm sample, leading to more consistent results across different samples. This consistency is essential in the field of equine breeding, where reliable sperm quality is paramount for successful artificial insemination and embryo transfer procedures.

Conclusions

In conclusion, our research has proven the benefits of glycerol supplementation by dialysis over the conventional glycerol addition by the pipetting method. By minimizing the toxic effects of glycerol and providing a controlled method of cryoprotectant addition, this innovative approach has the potential to improve sperm viability and motility post-thaw. Prospective research and validation of the stallion sperm dialysis method are needed to fully assess its efficacy and potential applications in the field of equine reproduction.

Declaration of competing interest

The authors report no conflict of interests. The authors are responsible for the content and writing of the manuscript.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Acknowledgements

This research was supported by the Slovenian Research Agency, and was completed within the program group 'Endocrine, immune, nervous and enzyme responses in healthy and sick animals' [P4-0053]. The study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract number 451-03-66/2024-03/200143).

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Received: 31 January 2024
Accepted: 2 May 2024
Online publication: 31 October 2024

KLINC, P., V. ŽALIG, V. MAGAŠ, J. BLAGOJEVIĆ, M. SAMARDŽIJA, M. MALETIĆ: Dijaliza kao nova metoda za dodavanje glicerola sjemenu pastuha prije zamrzavanja. Vet. arhiv 95, 1-10, 2025.

SAŽETAK

Glavna svrha istraživanja bila je analizirati učinak dijalize, kao metode dodavanja glicerola, na kvalitetu smrznutih/odmrznutih spermija pastuha.Od tri pastuha lipicanske pasmine s dokazanom plodnošću prikupljeno je 20 ejakulata. Svaki je ejakulat podijeljen na dva jednaka dijela kako bi se primijenila tehnika podijeljenog ejakulata. Glicerol je dodan ili pipetiranjem, pri čemu je talog sjemena razrijeđen nakon centrifugiranja s ekstenderom koji je sadržavao 5% glicerola, ili primjenom dijalize u kojoj je talog sjemena prvo razrijeđen ekstenderom koji ne sadržava glicerol, a zatim je dodan glicerol upotrebom sustava za dijalizu. Svi su uzorci postupno ohlađeni na 5ºC, zamrznuti u dušikovim parama i držani u tekućem dušiku do analize. Sjeme je otopljeno u vodenoj kupki na 37ºC. Uporabom CASA sustava (*Computer Assisted Sperm Analysis*) uzorci sjemena su analizirani 10 minuta i 3 sata nakon inkubacije pri prethodno navedenoj temperaturi s obzirom na obilježja pokretljivosti, progresivne pokretljivosti i koncentacije. Kvaliteta odmrznutih spermija dodatno je analizirana testom hipoosmotskog bubrenja (HOS) i morfološkom analizom. Dodatak glicerola pomoću dijalize znakovito je povećao ukupnu pokretljivost ($P<0,001$), progresivnu pokretljivost ($P<0,001$) 10 minuta nakon odmrzavanja, postotak HOS pozitivnih spermija nakon 10 minuta i 3 sata inkubacije ($P<0,001$; $P<0,05$) i postotak morfološki normalnih spermija neposredno nakon odmrzavanja ($P<0,05$). Rezultati istraživanja potvrdili su hipotezu da se negativni učinak, uzrokovan brzim dodavanjem glicerola u sjeme pastuha pipetiranjem, može smanjiti postupnim dodavanjem glicerola dijalizom.

Ključne riječi: reprodukcija konja; centrifugiranje sjemena; smrzavanje; dijaliza; pokretljivost spermija