DOI: https://doi.org/10.59913/dagr.2023.12254

Optimization of protocol for stallion semen cryopreservation

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Abstract

The aim of the preliminary study was to improve success of cryopreservation of stallion spermatozoa. The effect of different equilibration lengths and two techniques for freezing of stallion semen was evaluated, based on motility and viability parameters. Fresh semen was diluted with BotuCrio extender and divided into 3 groups equilibrated for 0.5h, 2h, and 4h. Subsequently, half straws of each group were cryopreserved in the vapours of the liquid nitrogen or in the automatic freezer. Results of stallion spermatozoa motility and progressive motility showed decreasing trend with increased time of equilibration. Moreover, a significant difference ($p < 0.05$) for the percentage of spermatozoa producing ROS was found between semen frozen in the vapours of the liquid nitrogen compared to semen cryopreserved by the automatic freezer in the same equilibration time. Nevertheless, results of this study are of preliminary character. Experiments using higher number of individuals need to be tested in order to find the best procedure for semen cryopreservation of Slovak national horse breeds.

Keywords: stallion, semen cryopreservation, ROS

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Introduction

Generally, animal gene banks play an important role in agricultural production globally for the present and the future, and in sustaining the most of production systems and community livelihoods. The situation with animal genetic resources in the Slovak Republic is not satisfactory due to the fact that livestock semen doses stored in the gene bank are originated only from several Slovak breeds (CHRENEK et al., 2017). Therefore, if there is an opportunity to obtain biological material from valuable breeds, it is desirable to optimize specific cryopreservation process. The genetic selection of horses is purely based on desired phenotypic features, pedigree, and athletic achievements and rarely based on fertility (BRITO, 2007). Semen freezing studies aim to improve the performance of stallions, and favorable conditions for transporting semen, reduce disease transmission, and allow the use of genetic material for an indeterminate amount of time without affecting reproduction (AVANZI et al., 2006; AMAN and GRAHAM, 1992). Equilibration as the total period of spermatozoa contact with a cryoprotectant prior to freezing helps keep spermatozoa membrane integrity as well as their survival. The length of equilibration as well as the freezing curve type are responsible for many important physicochemical changes leading to different degrees of spermatozoa structure damage (FORERO-GONZALEZ et al., 2012) deteriorating spermatozoa characteristics after thawing. The objective of study was to evaluate the effect of different equilibration lengths and two techniques for freezing of stallion semen, based on motility and viability parameters and to find a suitable protocol for the cryopreservation of the stallion semen of genetically endangered species.

Material and methods

Semen Collection and Processing

Ejaculates were collected from clinically healthy Holsteiner breed stallion in Slovak University of Agriculture in Nitra. The frequency of semen collection was performed one time per week. The stallions were handled carefully in accordance with the ethical guidelines of the Animal Protection Regulation of the Slovak Republic RD 377/12, complying with the European Union Regulation 2010/63. Experimental protocols were approved by the committee at Slovak University of Agriculture in Nitra, Slovak Republic. Semen was obtained on a regular collection schedule using a lubricated pre-warmed artificial vagina (Colorado type, Minitube, Tiefenbach, Germany) on a dummy. Immediately after collection, semen was extended 1:1 with commercial extender BotuSemen® (Nidacon, Sweden) and transported to the laboratory. One aliquot of fresh semen was allocated for the assessment of the following sperm variables: motility, viability, apoptosis, plasma

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membrane integrity, mitochondrial membrane potential, and ROS production.

Semen cryopreservation

The ejaculates were centrifuged at 600x g for 10 min at room temperature. The pellet was resuspended in BotuCrio (Nidacon, Sweden) and adjusted to 200 × 10 cells/mL. The samples were subsequently filled into 0.25 mL straws (Minitüb, Tiefenbach, Germany). After filling, straws were divided into 3 groups (n = 10 straws per group), immediately placed into a fridge (4–5°C), and equilibrated for 0.5h, 2h, and 4h. Subsequently, equilibrated straws were divided into 2 groups (n = 5 straws per equilibration length at least) and frozen using 2 freezing curves. One group of straws on the racks were placed 4 cm above the surface of the liquid nitrogen for 15 min and subsequently plunged directly into liquid nitrogen. Second group of straws were placed on a rack into the freezing chamber, cooled and frozen in the automatic freezer (IceCube, Minitube). The cooling ramps were −1°C/min from 20 to 5°C; temperature was maintained at 5°C for 5 min, followed by a −40°C/min ramp down to −140°C. After reaching −140°C, straws were removed from the chamber and plunged directly into liquid nitrogen. All straws were stored in liquid nitrogen until thawing and analysis.

Semen quality evaluation

The straws were thawed in a water bath at 37°C for 30 s and its content transferred to an Eppendorf tube pre-heated at 37°C. The same quality variables evaluated for fresh semen were assessed in frozen- thawed spermatozoa as described below.

Motility analyses

CASA (Computer Assisted Semen Analyzer) system with SpermVision software (MiniTüb, Tiefenbach, Gremany and the AxioScope A1 (Carl Zeiss Slovakia, Bratislava, Slovakia) were used to analyse spermatozoa motility. Five fields per sample were analysed for total motility (TM) and progressive motility (PM).

Flow cytometry analyses

Aliquots of semen samples from each group were adjusted to 10^6 cells/ml in Ca^- and Mg^-free PBS (Biosera, France) and stained using selected chemicals for specific markers to identify different physiological cell attributes. The viability of spermatozoa was assessed using SYBR-14, a membrane-permeant nucleic acid green fluorescent dye (LIVE/DEAD® Sperm Viability Kit). YO-PRO-1 nuclear green dye was used to detect apoptotic-like changes in spermatozoa. The integrity of acrosome was inspected using PNA-FITC (*peanut agglutinin*). The activity of mitochondria was assessed through the mitochondrial membrane potential (MMP) using MitoTracker® Green FM and the measurement of reactive oxygen species (ROS) production was performed with CellROX Green Reagent. All chemicals were

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purchased from Thermo Fisher Scientific (USA) unless stated otherwise. Fresh and post-thawed semen samples were incubated with the mentioned reagents either in accordance with the producer's manuals or as described previously (VAŠÍČEK et al., 2022). Reagents for staining were used at a final concentration in accordance with the producer's manuals or as follows: SYBR-14 (100 μM), YO-PRO-1 (100 μM), PNA-FITC (20 μM), MitoTracker Green FM (200 nM), and CellROX Green Reagent (2.5 μM). All samples were stained with a specific marker in combination with DRAQ7, a far-red fluorescent nucleic acid dye (BioStatus,Limited, Shepshed, UK), which stains nuclei of dead or membrane-compromised cells. Aliquots of stained samples were immediately analysed by flow cytometry using a FACSCalibur instrument (BD Biosciences, USA) and the FL1 (green) and FL3 (red) channels.

Statistical analysis

Experiments were repeated three times. Obtained results were evaluated using SigmaPlot software (Systat Software Inc., Germany) with one-way analysis of variance (Holm–Sidak method) and expressed as means ± SD. P-values at $p < 0.05$ were considered to be statistically significant.

Results and discussion

The data regarding effects of equilibration time and technique of freezing on the sperm quality parameters are included in Table 1 and Table 2. Freezing and thawing of stallion spermatozoa resulted in decreased values for sperm motion variables and viability parameters compared with fresh values (a vs. b, $p < 0.05$). No significant differences were found between time of equilibration, cryopreservation methods or their interactions with the variables as TM (total motility), PM (progressive motility), SYBR-14 (viable sperm), DRAQ7 (dead sperm), Yo-Pro-1 (apoptotic sperm), PNA (sperm with damaged acrosome), MT Green (sperm with high mitochondrial potential) of cryopreserved sperm. However, results of stallion spermatozoa motility and progressive motility showed decreasing trend with increased time of equilibration. Moreover, a significant difference ($p < 0.05$) for the percentage of spermatozoa producing ROS was found between semen frozen in the vapours of the liquid nitrogen compared to semen cryopreserved by the automatic freezer in the same equilibration time (14.02 ± 6.15 vs. 8.19 ± 4.97, 21.32 ± 5.19 vs. 9.83 ± 5.58, 26.18 ± 7.36 vs. 9.87 ± 5.32, respectively).

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Table 1 Parameters (mean ± SD) evaluated in fresh and post-thawed semen of stallions in different equilibration time (0.5h, 2h, 4h) cryopreserved in the vapors of the liquid nitrogen (VLN)

Parameter	Fresh	VLN 0.5h	VLN 2h	VLN 4h
TM (%)	$80.53^{a} \pm 1.52$	$56.93^{b} \pm 4.03$	$44.10^{b} \pm 4.46$	$40.33^{b} \pm 7.91$
PM (%)	$72.60^{a} \pm 2.77$	$48.72^{b} \pm 1.72$	$29.89^{b} \pm 3.97$	$26.07^{b} \pm 5.07$
SYBR-14 (%)	$75.02^{a} \pm 5.04$	$49.35^{b} \pm 5.87$	$32.42^{b} \pm 5.76$	$34.74^{b} \pm 15.56$
DRAQ7 (%)	$25.72^{a} \pm 6.23$	$48.62^{b} \pm 6.15$	$54.98^{b} \pm 11.13$	$60.68^{b} \pm 12.59$
Yo-Pro-1 (%)	$4.62^{a} \pm 3.07$	$30.67^{b} \pm 4.48$	$34.20^{b} \pm 10.63$	$40.24^{b} \pm 8.56$
PNA (%)	$7.72^{a} \pm 4.12$	$23.62^{b} \pm 11.03$	$46.85^{b} \pm 1.64$	$53.11^{b} \pm 12.94$
MT Green (%)	$77.19^{a} \pm 5.37$	$31.63^{b} \pm 6.36$	$24.19^{b} \pm 4.21$	$22.66^{b} \pm 8.53$
CellRox (%)	$3.02^{a} \pm 1,02$	$14.02^{b} \pm 6.15$	$21.32^{b} \pm 5.19$	$26.18^{b} \pm 7.36$

Table 2 Parameters (mean ± SD) evaluated in fresh and post-thawed semen of stallions in different equilibration time (0.5h, 2h, 4h) cryopreserved by the automatic freezer (AF)

Parameter	Fresh	AF 0.5h	AF 2h	AF 4h
TM (%)	$80.53^{a} \pm 1.52$	$64.77^{b} \pm 4.94$	$53.87^{b} \pm 10.66$	$51.61^{b} \pm 10.52$
PM (%)	$72.60^{a} \pm 2.77$	$54.24^{b} \pm 3.76$	$45.32^{b} \pm 9.73$	$44.32^{b} \pm 12.89$
SYBR-14 (%)	$75.02^{a} \pm 5.04$	$53.31^{b} \pm 4.12$	$46.35^{b} \pm 1.83$	$46.30^{b} \pm 7.59$
DRAQ7 (%)	$25.72^{a} \pm 6.23$	$46.14^{b} \pm 3.15$	$49.95^{b} \pm 3.95$	$52.95^{b} \pm 6.23$
Yo-Pro-1 (%)	$4.62^{a} \pm 3.07$	$26.71^{b} \pm 4.38$	$31.94^{b} \pm 12.17$	$34.41^{b} \pm 7.12$
PNA (%)	$7.72^{a} \pm 4.12$	$26.65^{b} \pm 5.14$	$34.96^{b} \pm 3.42$	$27.63^{b} \pm 5.69$
MT Green (%)	$77.19^{a} \pm 5.37$	$41.13^{b} \pm 6.56$	$36.19^{b} \pm 16.01$	$37.73^{b} \pm 9.56$
CellRox (%)	$3.02^{a} \pm 1.02$	$8.19^{b} \pm 4.97$	$9.83^{b} \pm 5.58$	$9.87^{b} \pm 5.32$

Stallion sperm are extremely sensitive to cell alterations generated by freezing, osmotic changes induced during the process and osmotic stress resulting from exposure to hypertonic media (DEVIREDDY et al., 2002). Results of the present study are consistent with previous studies indicating that freeze-thawing procedures damage the plasma membrane and lead to decrease motility of stallion spermatozoa (ORTEGA-FERRUSOLA et al., 2008; HOFMANN et al., 2011). Similar to other studies, results of the present study indicate that cryopreservation was associated with increased in ROS production (NEILD et al., 2003; BALL, 2008). Osmotic shock (OS) has long been associated with and a major factor in sperm damage during cryopreservation; and while this statement still holds true, newer research demonstrates it is just one potential problem. The influx of hypertonic concentrations while freezing, and the hypotonic concentrations when thawing have been shown to

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induce OS which has been shown to be detrimental to the integrity of sperm cells. Somatic cells have been well documented to show that OS is responsible for apoptosis, cell cycle arrest, DNA damage, oxidative stress as well as a variety of other actions (SALAMON and MAXWELL, 2000). This is especially true in stallions, as spermatozoa have a very limited osmotic threshold (DIETZ et al., 2007). Studies have shown that stallion sperm damaged during flash freezing and morphologically abnormal sperm generate greater amounts of ROS (PAGL et al., 2006). Present study showed that cryopreservation by automatic freezer didn´t rapidly increase ROS.

Conclusion and recommendation

Summing up the results, we concluded that automatic freezer and 0.5 h equilibration could be successfully use for cryopreservation of stallion semen. Nevertheless, results of this study are of preliminary character. Experiments using higher number of samples need to be tested in order to find the best procedure for semen cryopreservation of Slovak national horse breeds.

Acknowledgement

This study was supported by the grants of Slovak Research and Development Agency, APVV- 20-0006.

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