TRADITION AND MODERNITY IN VETERINARY MEDICINE, 2023, vol. 8, No 1(14): 11–16

EVALUATION OF THE CRYOTOLERANCE OF SPERMATOZOA IN SOFIA (ELIN-PELIN) SHEEP BREED

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ABSTRACT

The Sofia (Elin-Pelin) breed is a local Bulgarian breed with a reduced population and poorly studied possibilities for cryotolerance. Therefore, the aim of this study was to evaluate the effect of cryopreservation on sperm parameters – total motility (TM), velocity parameters and lipid peroxidation (LPO) levels. Sperm motility and velocity parameters were examined by computer-assisted sperm analysis (CASA). LPO levels were determined spectrophotometrically. As a result, we found a decrease in sperm TM after thawing from 99.01±0.68% to 71.92±5.12%, which is acceptable for artificial insemination and a slight increase in LPO levels from 0.86±0.23 nmoles MDA/10x106 sp. to 1.44±0.37 nmoles MDA/10x106 sp. after thawing. In conclusion from the conducted studies sperm from the Sofia (Elin-Pelin) breed showed good cryotolerance with a low percentage of damage after thawing, with which we believe that cryopreservation would be a suitable method for breeding and preserving this breed.

Key words: rams, sperm, cryopreservation, CASA, LPO.

Introduction

The specific natural and climatic conditions in the local regions of Bulgaria has contributed to the creation of a significant number of breeds, adapted to them. According to Zhelev et al. (2009), Bulgaria takes one of the leading places in the world in the number of indigenous breeds of sheep. Despite the good natural and climatic conditions, the rich traditions and the great variety of indigenous breeds, the sheep breeding in Bulgaria is in a deepening crisis and there is a threat of breeds extinction (Sabkov et al., 2017). The Sofia (Elin-Pelin) breed is one of the indigenous breeds in Bulgaria, created through mass selection and currently endangered (Chervenkov et al., 2012). One of the main approach to protect and preserve the breed is through cryopreservation of male gametes. This requires detailed studies on the effect of cryopreservation on sperm. Cryopreservation is a technique that allows sperm to prolong the storage time, maintaining its fertilisation capacity (Sharma, 2011). However, during cryopreservation, spermatozoa undergo ultrastructural changes in plasma membrane structure and chromatin integrity, biochemical changes such as osmotic dehydration, metabolic retardation, and functional changes like sperm motility and morphology impairment (Salamon and Maxwell, 1995, 2000). It has been shown that during cryopreservation and thawing process oxidative stress (OS) occurs in germ cells (Liu et al., 2021). The produced large amount of reactive oxygen species (ROS) can cause oxidation of lipids, proteins and nucleic acids, leading to irreversible damages and even apoptosis (Len et al., 2019). Furthermore, the application of classic

permeable and impermeable cryoprotectants are failing to reduce oxidative damage to cells (Liu et al., 2021).

Rams sperm contain a high amount of polyunsaturated fatty acids and a relatively low percentage of cholesterol and phospholipids in the plasma membrane compared to other ruminant species (Holt, 2000). This makes the sperm membrane in rams prone to lipid peroxidation (LPO), leading to disruption of the structure and function of the sperm membrane and acrosome (Alvarez and Storey, 1993). Thus, the measurement of lipid oxidation by-products is one of the most widely used techniques for assessing oxidative stress in sperm (Simões et al., 2013; Mojica-Villegas et al., 2014).

The use of cryopreservation of ram semen can support the conservation of endangered breeds and preserve biodiversity (Andrabi and Maxwell, 2007). For this purpose, it is important to identify the changes in sperm during cryopreservation and to outline strategies to preserve their quality and fertility. Therefore, the aim of this study was to evaluate the effect of cryopreservation on Sofia (Elin-Pelin) ram breed sperm parameters – motility, velocity parameters and the levels of LPO.

Materials and methods

Animals

The experiment was conducted with eight sexually mature rams, aged 2-4 years old, with body weight 80kg, during the breeding season. The rams were kept under the same conditions of feeding, breeding and sexual use. Two ejaculates were obtained from each ram with an interval between ejaculates of 1-5 minutes using the artificial vagina method. Ejaculates were diluted 1:12 with 6A-G extender prepared by us with comprising 100.0 cm^3 distilled water, 2.8g sodium citrate, 0.4g sucrose, 0.4g lactose, 25% egg yolk and 7% glycerol. The ejaculates were tested for sperm motility, velocity parameters and lipid peroxidation (LPO) levels before freezing and after thawing.

Techniques for cryopreservation of spermatozoa

The cryopreservation was performed at the laboratory following a standard straw freezing procedure (Cassou, 1964). The semen was frozen in straws (length 133 mm, diameter 2 mm and volume 0.25 cm^3). Once filled with semen, the straws were loaded into tripods. Cryopreservation of semen was performed by rapid cooling to -80℃ for 5 minutes; the temperature was monitored in order to be adjusted the height of the tripod above the nitrogen vapor if necessary. The straws were then transferred to liquid nitrogen tank at -196℃ and placed in a canister for one-week storage at ultra-low temperatures. Thawing of the semen was done in a water bath at 37℃ for 30 s.

Sperm Analysis

The assessment of parameters for motility was carried out with a CASA system (Sperm Class Analyzer [SCA] 5.0. Microptic, Barcelona, Spain). The semen was loaded into a Leja 20 chamber (Leja Products B.V., Nieuw-Vennep, The Netherlands) and examined using a light microscope (Nikon, Tokyo, Japan) with a warm stage at 37°C. The total number of motile sperm cells, as well as, the velocity parameters – Rapid %; Medium %; Slow %; VCL (μm/s) – curvilinear sperm velocity; VSL (μm/s) straight line velocity; VAP (μm/s) average path velocity (μm/s); LIN linearity (%) LIN= (VSL/VCL) × 100; STR straightness (%) STR = (VSL/VAP) × 100, WOB wobble (%) WOB = (VAP/VCL) × 100 were measured, using the SCA software.

Lipid peroxidation assay

The lipid peroxidation of sperm was measured by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), an end products of lipid peroxidation (Hunter et al., 1963). Sperm

aliquots (1 ml) were heated at 100°C for 15 minutes after addition of 0.6 mL of a mixture, containing 2.8% trichloroacetic acid : 5N HCl : 2% TBA in 50 mM NaOH (2:1:2 v/v). The absorption of the resulting colour complex was read at λ = 532 nm. The results were expressed as nmoles MDA/10x10^6 sp., using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

Statistical analysis

Data sets were analyzed with IBM SPSS Statistics 23, USA to compare sperm characteristics using statistical tests of Paired T-test. The significance of the differences between groups was evaluated by Student's t-test. Findings were considered as statistically significant at $P \leq 0.001$.

Results and discussion

Total sperm motility (TM) after thawing decreased significantly ($P \leq 0.001$) from 99.01±0.68% to 71.92±5.12 %. However, the percentage of motile sperm after thawing allows the application of artificial insemination. The percentage of fast-moving (Rapid) sperm was also significantly reduced ($P \leq 0.001$) and the percentage of the slow-moving (Slow) sperm was increased after thawing. The velocity parameters reflecting the type of movement – VCL, VSL and VAP also decreased significantly after thawing ($P \leq 0.001$). The VCL parameter was the most strongly affected by thawing. Inverse dependence was found in the parameters – LIN, STR and WOB, and after thawing they significantly increased ($P \leq 0.001$) (Table 1).

The level of LPO before freezing was 0.86±0.23 nmoles MDA/10x10^6 sp, and it increased insignificantly after thawing of ejaculate to 1.44±0.37 nmoles MDA/10x10^6 sp (Table 1).

The cryopreservation of ejaculates from the Sofia (Elin-Pelin) breed in our studies was significantly better than the published data of Nijs et al. (2009) for other sheep breeds, as well as compared to our previous study (Andreeva et al., 2018) for domestically bred imported breeds. The percentage of immobile sperm after freezing was lower than that those, found in other breeds of rams bred in Bulgaria (Ivanova et al., 2019). In comparison to other indigenous ram breeds, in this study higher pre-freeze TM was observed than that in the research of Mahmuda et al. (2015) and Asaduzzaman et al. (2021). In the Sofia (Elin-Pelin) rams, the percentage of immobile sperm before freezing is lower than that obtained by Chervenkov et al. (2012) for the same breed. Likely the difference in the obtained data comes from the period of the sperm collection (during or beyond the insemination campaign of the breed), which may has an impact. The obtained herein results for the degree of LPO before cryopreservation showed close (Kasimanickam et al., 2006) or even lower (Asadpour et al., 2012) values than those established in other studies in the field examined fresh semen of various inbreeding rams. The last finding probably is due to the presence of better antioxidant defense system of sperm from Sofia (Elin-Pelin) breed, as we found in previous studies (Andreeva et al., 2021). Our results showed insignificant increase in comparison to the levels before freezing. In addition, the obtained results after cryopreservation showed lower levels of LPO than those reported by Kurmi et al. (2018). All these findings showed high cryotolerance of the Sofia (Elin-Pelin) breed and the possibility of successful application of artificial insemination with cryopreserved sperm. The Sofia (Elin-Pelin) breed is an old national selection, adapted to the climate and breeding conditions in Bulgaria, which in our opinion makes it more stable biochemically and genetically than many of the newly introduced breeds in the country.

Table 1: Sperm motility parameters and LPO levels before freezing and after thawing.

	SPERM PARAMETERS											
	TM, %	**Immotile, %**	**Rapid, %**	**Medium, %**	**Slow, %**	**VCL, µm/s**	**VSL, µm/s**	**VAP, µm/s**	**LIN, %**	**STR, %**	**WOB, %**	**LPO,nmol/ MDA/10x10^{6}sp**
Before freezing	99.01±0.68	0.99±0.68	76.09±9.29	13.67±3.55	9.25±5.58	96.74±3.87	31.98±0.68	57.34±1.12	27.64±0.81	53.01±0.53	53.43±0.97	0.86±0.23
After thawing	71.92±5.12^a	28.08±5.12^a	3.43±2.25^a	9.22±4.01^a	59.27±6.23^a	32.71±2.42^a	15.18±0.64^a	24.61±0.97^a	55.00±2.57^a	72.02±2.45^a	76.41±1.92^a	1.44±0.37^{ns}

Note: Means±SEM; Significant differences ^{a} at P≤0.001; ^{ns} – non significant

Conclusion

From the conducted studies sperm from the Sofia (Elin-Pelin) breed showed good cryotolerance with a low percentage of damage after thawing, with which we believe that cryopreservation would be a suitable method for breeding and preserving this breed.

Acknowledgements

The study has personal grant by the Bulgarian Ministry of Education and Science under the National Research Programme "Young scientists and postdoctoral students-2" approved by DCM 206/07.04.20.

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