Biotechnology in Animal Husbandry 38 (1), 31-39, 2022
Publisher: Institute for Animal Husbandry, Belgrade-Zemun
ISSN 1450-9156
UDC 636.454'636.32
https://doi.org/10.2298/BAH2201031C

ANALYSIS OF VITALITY AND BIOCHEMICAL PARAMETERS IN FREEZE-THAWED SEMINAL PLASMA OF RAMS

Bogdan Cekić[1], Hristiyana Kanzova[2], Georgi Petrov[2], Nevena Maksimović[1], Ivan Ćosić[1], Aleksandar Milovanović[3]

[1]Institute for Animal Husbandry, Belgrade - Zemun, Autoput za Zagreb, 11080 Zemun, Serbia
[2]Laboratory of Free Radical Processes, Institute of Neurobiology, Bulgarian Academy of Sciences, 23, Acad. G. Bonchev str., Sofia 1113, Bulgaria
[3]Scientific Veterinary Institute Novi Sad, Rumenački put, 21000 Novi Sad, Serbia
Corresponding author: Bogdan Cekić, bcekic@istocar.bg.ac.rs
Original scientific paper

Abstract: The current study aimed to examine the percentage changes of viability sperm and the activity of the enzymes LDH, ALP, GOT / AST, GPT / ALT in the sperm plasma of Lacaune rams, before and after cryopreservation. For this purpose, five rams were examined, and two ejaculates were obtained from each ram. Ejaculates are collected by the method of artificial vagina, during the insemination campaign. All ejaculates were diluted with a 6AG extender and frozen by the Cassou's sequin method. Sperm viability was determined by eosin and nigrosine smears, and enzyme activity was examined spectrophotometrically. As a result, the percentage of vital sperm after cryopreservation decreased by 15% ($P \leq 0.001$). The freezing and thawing process also reduced the activity of the enzymes LDH, ALP, GOT / AST and GPT / ALP. In conclusion, the observed enzymes, in relation to sperm vitality, could be used as indicators to optimize the protocols for cryopreservation of ram's sperm.

Key words: ram, sperm, cryopreservation, biochemical parameters, vitality

Introduction

Mammalian sperm plasma is a composite mixture of epididymal secretions and accessory gonads (*La Falci et al., 2002*). It creates the mandatory environment for the normal functioning of sperm, and the present amount and type of enzymes and metabolites strongly affects sperm quality and freezing stability (*Asadpour, 2012; Juyena and Stelletta, 2012; Mráčková et al., 2015; Atroschenko et al., 2019*). Some of the enzymes are found in sperm, and they play an important role in fertilization, metabolic processes and the conversion of chemical energy

into mechanical, which ensures the movement of sperm (*Baychev et al., 2007*). Some authors have also established the role of various enzymes in semen. For example, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are crucial for processes of metabolism that provide energy for sperm survival, motility and fertility (*Sirat et al., 1996; Duan and Goldberg, 2003*). According to some studies in horses, γ-glutamyl transferase (GGT) correlates with sperm motility (*Pesch et al., 2006; Dogan et al., 2009*). Also, plasma aminopeptidases of sperm, such as alanyl aminopeptidase (APN), are involved in many physiological processes. Alkaline phosphatase (ALP) can be used to differentiate azoospermia (*El-Bishbishy et al., 2013*).

Some studies show that if a certain enzyme is present in total semen (that is, in both sperm and plasma), there is often a large difference in its activity (*Andreeva, 2020*). Most often, intracellular enzymes (found in sperm) enter the sperm plasma after an effusion resulting from disruption of the cell membrane during sudden cooling, after deep freezing or ultrasonic disintegration by a sonifier (*Murdoch and White, 1967; Andreeva et al., 2022*). Therefore, evaluations of these enzymes are endorsed as indicators of quality of the sperm (*Sirat et al., 1996; Tvrda et al., 2013; Tejaswi et al., 2016*). Vitality assessment is also one of the major part of analysis of sperm. This is an extremely important method of distinguishing the dead from the living sperm (*Björndahl et al., 2004*). Currently, there are numerous available techniques for staining and evaluating quality of sperm in different animal species (*Suttiyotin and Thwaites, 1992; Łącka et al., 2016; Gerzilov and Andreeva, 2021*).

The aim of our research is to study the percentage changes of viable sperm and the enzymatic activity of LDH, ALP, GOT / AST, GPT / ALT in the sperm plasma of Lacaune rams, before freezing and after thawing.

Material and Methods

Animals and sperm production

The experiment included five clinically healthy rams, aged 3-4 years of the Lacaune breed, during the insemination campaign. Two sperm samples were obtained from each ram, given that the interval between ejaculations was 1-5 minutes. All ejaculates were diluted 1:12 with a 6AG sperm extender, prepared by us and containing sodium citrate, lactose, sucrose, egg yolk and glycerin. Semen from rams were collected using the method of artificial vagina, by an experienced operator. All of the obtained ejaculates underwent a preliminary macroscopic evaluation and those that did not meet the criteria were discarded. After that, vitality and enzyme activity were examined.

Analysis

Sperm viability was determined with a solution containing the dyes eosin and nigrosine. To 1 mL of the staining solution 30 µl of diluted semen was added using a micropipette. The mixture was incubated for 10-15 minutes at 37 ° C. The total of 10 µl of the prepared mix was smeared on glass while allowing to dry at room temperature. Microscopic analysis was performed on a Boeco BM-180 binocular microscope, 100X magnification, oil immersion. 100 spermatozoa were counted from each smear. The smears were made before freezing and after thawing the ejaculate.

Sperm freezing was carried out in semen straws according to the method of *Cassou (1964)*. All samples were thawed after one week. Thawing process was carried out in water bath at 37°C for 30 seconds.

Biochemical analysis

In an Eppendorf tube with a capacity of 1 ml, the ejaculates (diluted 1:12) were centrifuged at 3500 rpm for 15 minutes. The sperm plasma obtained from each Eppendorf was gently aspirated into sterile tubes with a micropipette and the enzymatic activity was determined. The activity of enzymes was determined spectrophotometrically with a set of reagents from Via Campania - Italy, according to the manufacturer's protocol. The enzymes LDH, GOT / AST and GPT / ALT were determined at a wavelength of 340 nm, and ALP of 405 nm. The activity was determined before freezing and after thawing the ejaculate. The data obtained are presented in U / L.

Statistical analysis

Data sets were analyzed using SPSS 23 to compare characteristics of the sperm using a Paired T-test. Differences between groups were assessed for significance by Student's t-criteria, and results were considered statistically significant at $P < 0.05$.

Results and Discussion

Freezing-thawing processes can cause irreversible damage to ram semen. Some authors reported that about 40-60% of sperm retain their motility after cryopreservation, but only about 20-30% retain biological function (*Medeiros et al., 2002*). In our study, the viability of sperm decreased by nearly 15% after cryopreservation (Fig. 1). Our results are in line with the 12% obtained for the same breed by *Andreeva and Stefanov (2020a)*. *Salmon and Maxwell (1995)* also reported reduction in sperm viability after freezing-thawing.

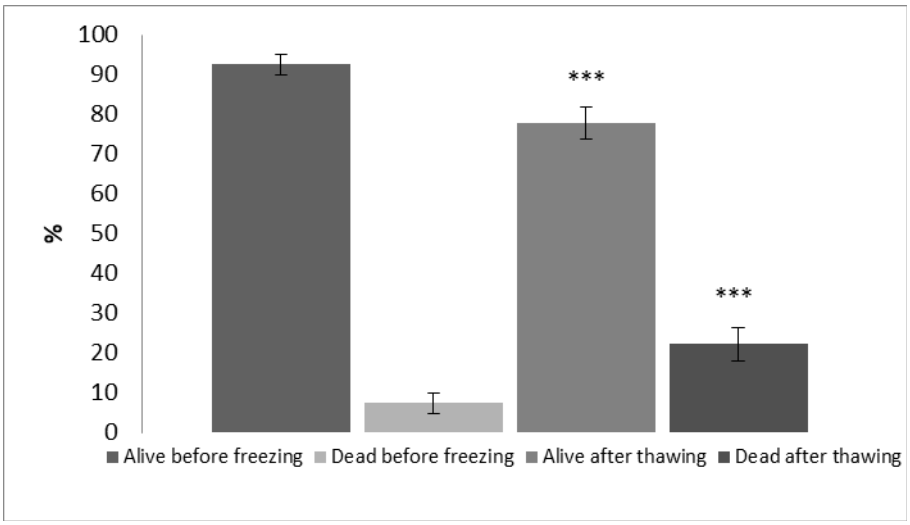

Figure 1. Sperm viability before freezing and after thawing (Mean ± SD, Significant differences *** P ≤0.001)

An important role in capacitance and fertilization has enzyme LDH (*Duan and Goldberg, 2003*). The activity we observed before freezing was lower than reported by some authors (*Zakrzewska et al.,2002; Tejaswi et al., 2016*), but significantly higher than that found by others (*Asadpour, 2012; Andreeva and Stefanov, 2020b*). After thawing of the ejaculates, the activity of the LDH enzyme in the sperm plasma decreased (P≤0.001) (Table 1). *Tejaswi et al. (2016)* obtained results different from ours. They reported an increase in enzyme activity after 24-hour refrigeration, explaining the result with damage to the membrane of the sperm and subsequent leaking of enzymes into the extracellular fluid due to cold shock. Our results are in line with those of *Fatihah et al. (2015)* who also observed decreased enzymatic activity after cryopreservation. According to *Brooks (2001)*, one of the main reasons for low fertility when using frozen-thawed ram sperm is the loss of LDH activity in most sperm after cryopreservation. An intracellular enzyme that regulates protein phosphorylation through the cAMP-dependent protein kinase pathway, which is required for sperm motility, is ALP. *Ciereszko et al. (1992)* reported a high correlation between ALP enzyme activity and sperm quality when sperm were under the stress. In our studies, its activity after cryopreservation decreased (Table 1). The leakage of ALP in the sperm plasma from the sperm can be used as a indicator to optimize the cooling and freezing steps during thawing for cryopreservation of ram semen (*Upreti et al. 1996*).

Table 1. Enzyme activity before freezing and after thawing

Parameters	Before freezing	After thawing
LDH, U/L	253.00 ± 73.97	178.88 ± 20.25***
ALP, U/L	731.13 ± 128.24	703.13 ± 164.16
GOT/AST, U/L	5.16 ± 2.03	2.25 ± 1.04**
GPT/ALT, U/L	46.13 ± 17.9	3.13 ± 1.55***

Note: Results are presented as Mean ± SD, Significant differences ** $P \leq 0.01$; *** $P \leq 0.001$

Transaminases (ALT-AST) are also intracellular enzymes used as a marker in sperm membrane damage. Leakage of these enzymes into sperm plasma is a sign of damage to sperm membranes (*Tuli and Singh, 1982; Katila, 2001; Alamaary et al., 2020*). In our studies, the activity of both enzymes after thawing decreased, and cryopreservation showed a significant depletion in ALT ($P \leq 0.001$) (Table 1). Most likely the process of cryopreservation led to inhibition of the enzyme and from there to the reporting of lower results. The values obtained for the extracellular activity of the enzyme are close to those reported by *Tejaswi et al (2016)*, who studied the activity of the enzyme in fresh samples and samples refrigerated at 4° C. *Rastegarnia et al. (2010)* obtained a different correlation than ours in studies conducted with buffalos. They report an increase in the enzyme activity of AST and ALT after cryopreservation. In our studies, the activity of enzymes in sperm plasma decreased, which is an indicator of good cryopreservation of samples and preservation of sperm fertility.

Conclusion

Cryopreservation reduces sperm viability, along with the activity of the enzymes LDH, ALP, GOT / AST and GPT / ALT. The studied enzymes can be used as indicators to optimize the protocols for cryopreservation of ram's sperm.

Analiza vitalnosti i biohemijski parametri seminalne plazme ovnova prilikom zamrzavanja i odmrzavanja

Bogdan Cekić, Hristiyana Kanzova, Georgi Petrov, Nevena Maksimović, Ivan Ćosić, Aleksandar Milovanović

Rezime

Istraživanje je imalo za cilj ispitivanje promena u procentu vijabilnih spermatozoida i promena u aktivnosti enzima LDH, ALP, GOT / AST, GPT / ALT u seminalnoj plazmi ovnova rase Lakon, pre i nakon krioprezervacije. Za tu svrhu, ispitivana je sperma pet ovnova, sa dva ejakulata po ovnu. Ejakulati su dobijeni metodom veštačke vagine, u toku sezone parenja. Svi ejakulati su razređeni razređivačem 6AG i zamrznuti po metodi Cassou sequin. Vijabilnost spermatozoida je određena na osnovu razmaza sa eozinom i nigrozinom, a aktivnost enzima je određena spektrofotometrijski. Rezultati istraživanja pokazuju da je udeo vitalnih spermatozoida nakon krioprezervacije smanjen za 15% ($P \leq 0.001$). Procesi zamrzavanja i odmrzavanja takođe smanjuju i aktivnost enzima LDH, ALP, GOT/ AST i GPT / ALP. Ispitivani enzimi, združeno sa vitalnošću spermatozoida, mogu da se koriste kao markeri za optimizaciju stope hlađenja i koraka u procesima zamrzavanja i odmrzavanja sperme ovnova.

Ključne reči: ovan, sperma, krioprezervacija, biohemijski parametri, vitalnost

Acknowledgment

This study research was funded by the Ministry of Education, Science and Technological Development, the Republic of Serbia, Agreement on the realization and financing of scientific research work of SRO no. 451-03-68/2022-14/200022.

References

ALAMAARY M.S., HARON A.W., HIEW M.V.H., ALI. M. (2020): Effects of cysteine and ascorbic acid in freezing extender on sperm characteristics and level of enzymes in post-thawed stallion semen. Veterinary Medicine and Science, 6, 4, 666-672.

ANDREEVA M. (2020): Investigation of the effect of breed characteristics in sheep on cryotolerance of sperm. Ph.D. Dissertation. Institute of Biology and Immunology of Reproduction „Akad.Kiril Bratanov", Bulgarian Academy of Sciences, Sofia, (in Bulgarian).

ANDREEVA M., KARADJOVA V., STEFANOV R. (2022): Investigation of the effect of ultra-low temperatures in cryopreservation on the activity of the enzymes lactate dehydrogenase and gamma-glutamyl transferase. Journal of Chemical Technology and Metallurgy, 57, 2, 298-301.
ANDREEVA M., STEFANOV R. (2020a): Influence of the cryopreservation on the vitality of the sperm of the different breeds of rams. Tradition and Modernity in Veterinary Medicine, 5, 2, 9, 26–30. doi: 10.5281/zenodo.4317364
ANDREEVA M., STEFANOV R. (2020b): Study of the relationship between the age of the rams and the quality of their ejaculates obtained outside the breeding season. Biotechnology in Animal Husbandry 36, 4, 437-445. doi: 10.2298/BAH2004437A
ASADPOUR R. (2012): Relationship between mineral composition of seminal plasma and semen quality in various ram breeds. Acta Scientiae Veterinariae, 40, 2,1027.
ATROSCHENKO M.M., KUDLAEVA A.M., FOMINA M.A., KALASHNIKOV V.V., ZAITCEV A.M., DENISOVA O.V., NAVASARDYANTS D.G., BELONOVSKAYA O.S., PASKOV A.A. (2019): Analysis of seminal plasma biochemical parameters and sperm cryostability in different age groups of stallios. IOP Conference Series: Earth and Environmental Science, 341.
BAYCHEV J., P. PARVANOV P., NIKOLOV I., SABEV M., SHINDARSKA Z. (2007): Artificial insemination and andrology of farm animals. Videnov and son, Sofia (in Bulgarian)
BJÖRNDAHL L., SÖDERLUND I., JOHANSSON S., MOHAMMADIEH M., POURIAN M. R., KVIST U. (2004): Why the WHO recommendations for eosin-nigrosin staining techniques for human sperm vitality Why the WHO recommendations for eosin-nigrosin staining techniques for human sperm vitaity assessment must change. Journal of Andrology, 25, 671 – 678.
BROOKS G. A. (2001): Lactate shuttles in Nature. Biochemical Society Transactions, 30, 2, 258 - 264.
CASSOU R. (1964): La method des paillettes en plastique et son adaption la generalion la generalization de la congelation. C.R. Acad/ Agric., 50, 881 – 887.
CIERESZKO A., GLOGOWSKI J., STRZEZEK J., DEMIANOWICZ W. (1992): Low stability of aspartate aminotransferase activity in boars semen. Theriogenology, 37, 1269-1281.
DOGAN I., POLAT U., NUR Z. (2009): Correlations between seminal plasma enzyme activities and semen parameters in seminal fluid of Arabian horses. Iranian Journal of Veterinary Research, 10, 2,119-124.
DUAN C., GOLDBERG E. (2003): Inhibition of lactate dehydrogenase C4 (LDHC4) blocks capacitation of mouse sperm in vitro. Cytogenetic and Genome Research, 103, 3-4, 352-359.

EL-BISHBISHY H. A., ALY H. A., EL-SHAFEY M. (2013): Lipoic acid mitigates bisphenol A-induced testicular mitochondrial toxicity in rats. Toxicol Ind Health, 29, 10, 875 – 887.
FATIHAH S.N., ABOL-MUNAFI A. B., IKHWANUDDIN M. (2015): Biochemical changes of total protein, glucose, lactate, dehydrogenase and total lipid in the cryopreserved sperm of mud spiny lobster, panulirus polyphagus. Jornal of Fisheries and Aquatic Science, 10, 1, 33 – 44.
GERZILOV V., ANDREEVA M. (2021): Effect of three extenders on the motility and morphological characteristics of spermatozoa in diluted Muscovy semen stored at 4°C up to 120 hours. Bulgarian Journal of Agricultural Science, 27, 6, 1187–1193.
JUYENA N.S, STELLETTA C. (2012): Seminal Plasma: An Essential Attribute to Spermatozoa. Journal of Andrology, 33, 536-551.
KATILA T. (2001): In vitro evaluation of frozen-thawed stallion semen: A review. Acta Veterinaria Scandinavica, 42, 2, 199–217.
LA FALCI V.S., TORTORELLA H., RODRIGUES J.L., BRANDELLI A. (2002): Seasonal variation of goat seminal plasma proteins. Theriogenology, 57, 1035-1048. doi:10.1016/S0093-691X(01)00714-2.
ŁĄCKA K., KONDRACKI S., IWANINA M., WYSOKIŃSKA A. (2016): Assessment of stallion semen morphol-ogy using two different staining methods, microscopic techniques, and sample sizes. Journal of Veterinary Research, 60, 99–104.
MEDEIROS C. M., FORELL F., OLIVEIRA A.T., RODRIGUES J.L. (2002): Current status of sperm cryopreservation: why isn't better. Theriogenology, 57, 327 - 344.
MRÁČKOVÁ M., ZAVADILOVÁ M., SEDLINSKÁ M. (2015): Assestment of the effect of selected components of equine seminal plasma on semen freezability. Macedonian Veterinary Review, 38, 1, 91–96.
MURDOCH R. N., WHITE I. G. (1967): Studies of the Distribution and source of enzymes in mammalian semen. Australian Journal of Biological Sciences, 21, 483 – 490.
PESCH S., BERGMANN M., BOSTEDT H. (2006): Determination of some enzymes and macroand microelements in stallion seminal plasma and their correlations to semen quality. Theriogenology, 66, 2, 307-313.
RASTEGARNIA A., EVIAZ SAHARA Y., SHAFIPOUR V. (2010): Studies on seminal plasma enzymes (GOT, GPT and LDH) profile and its relationship with quality of buffalo frozen semen. Journal of Veterinary Clinical Research, 1, 3, 3,189-199.
SALMON S., MAXWELL W.M.C. (1995): Frozen storage of ram semen. Part I. Processing freezing–thawing and fertility after cervical insemi–nation (review). Animal Reproduction Science, 37,185–249.

SIRAT M.P., SINHA A.K., SINGH B.K., PRASAD R.L. (1996): Effect of cryoprotectants on release of various enzymes from buck spermatozoa during freezing. Theriogenology, 45, 405-416. doi:10.1016/0093-691X(95)00377-K.
SUTTIYOTIN P., THWAITES C.J. (1992): Comparison of a swim-up technique with Hamilton Thorn Motility Analyser for measurement of sperm velocity and motility. Reproduction, fertility and development, 4, 2, 153 – 160.
TEJASWI V., SWAMY M., YATHIRAJ S., HONNAPPA T., ISLOOR S. (2016): Enzymatic activities in fresh seminal plasma and extended refrigerated semen in nari suvarna rams. Theriogenology, 6, 1, 27-33.
TULI R. K., SINGH M. (1982): Effect of Different Extenders on Glutamic Oxalacetic Transaminase (GOT) and Glutamic Pyruvic Transaminase (GPI) Release from Frozen Buffalo Semen. Theriogenology, 18, 1, 55–59.
TVRDA E., SIKELI P., LUKACOVA J., MASSANYI P., LUKAC N. (2013): Mineral nutrients and male fertility. Journal of Microbiology, Biotechnology and Food Sciences, 3, 1, 1–14.
UPRETI G.C., PAYNE S.R., DUGANZICH D.M., OLIVER J.E., SMITH J.F. (1996): Enzyme leakage during cryopreservation of ram spermatozoa. Animal Reproduction Science, 41, 27–36.
ZAKRZEWSKA H., UDALA J., BLASZCZYK B. (2002): In vitro influence of sodium fluoride on ram semen quality and enzyme activities. Fluoride, 35, 3,153-160.

Received 29 April 2022; accepted for publication 5 June 2022