

SUA
Slovak University
of Agriculture
in Nitra

Preserving Genetic Diversity: The State of Sperm Cryopreservation in Slovak National Ram Breeds

J. VOZAF, A. SVORADOVÁ, J. VAŠÍČEK, A. BALÁŽI, L. KUŽELOVÁ, L. OLEXÍKOVÁ, A. V. MAKAREVICH, P. CHRENEK

INTRODUCTION

Biodiversity protection is one of our most important challenges today.

Local animal breeds form integral part of the biodiversity.

Cryopreservation of sperm is powerful tool for the conservation of animal genetic resources.

Several obstacles- possible influence of individuality and breed characteristics on resistance to cryodamage.

Appropriate methodology for sperm cryopreservation of Slovak national breeds.

Native Wallachian



Improved Wallachian



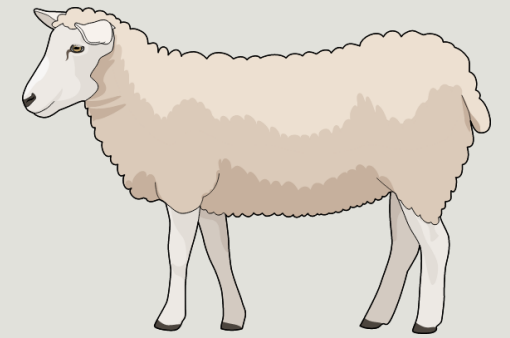
Slovak Dairy



SUBJECT OF INTEREST

TASKS

1. Suitable freezing protocol
2. Verification on different breeds
3. Storage in the gene bank



SUITABLE FREEZING PROTOCOL

- Wallachian breed rams
- Triladyl®
- Equilibration length
- Freezing techniques





Received: 22 September 2021 | Revised: 4 November 2021 | Accepted: 8 November 2021

DOI: 10.1111/asj.13670

RESEARCH ARTICLE

Animal
Science Journal  WILEY

Cryopreservation of ram semen: Manual versus programmable freezing and different lengths of equilibration

Jakub Vozaf¹  | Alexander V. Makarevich² | Andrej Balazi² | Jaromir Vasicek^{1,2} |
Andrea Svoradova^{2,3}  | Lucia Olexikova² | Peter Chrenek^{1,2}

¹Institute of Biotechnology, Faculty of Biotechnology and Food Science, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, Nitra, 949 76, Slovak Republic

²NPPC, Research Institute for Animal Production in Nitra, Hlohovecka 2, Lužianky, 951 41, Slovak Republic

³Department of Animal Morphology, Physiology and Genetics, Faculty of AgriSciences, Mendel University in Brno, Zemědělská 1/1665, Brno, 613 00, Czech Republic

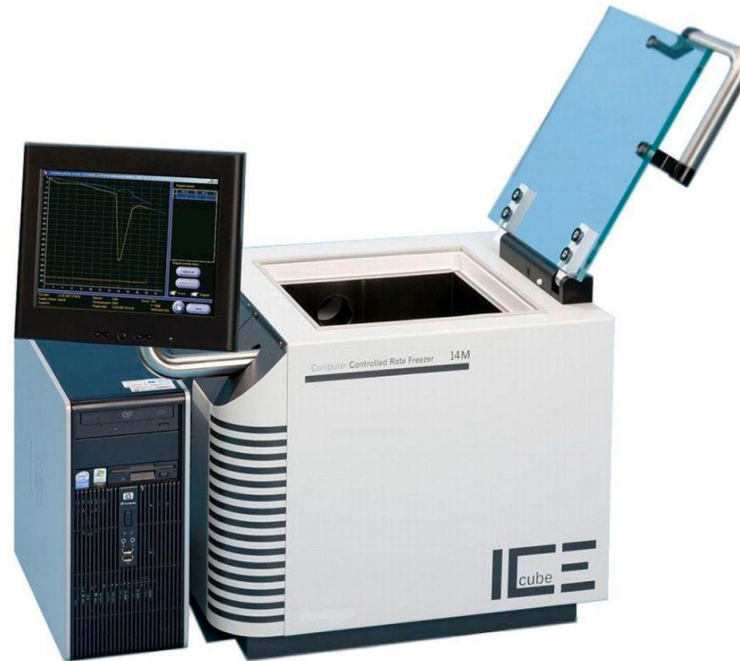
Correspondence

Peter Chrenek, Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Science, Tr. A. Hlinku 2, 949

Abstract

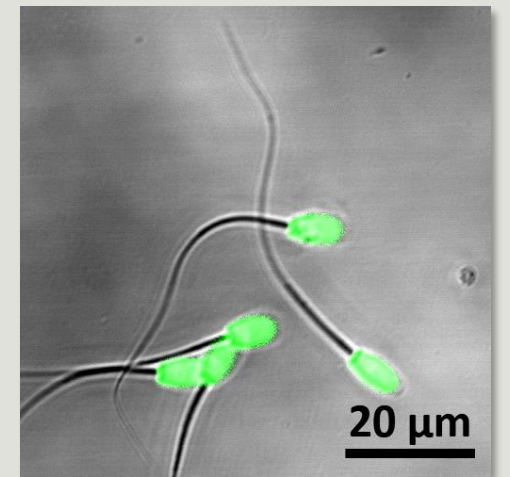
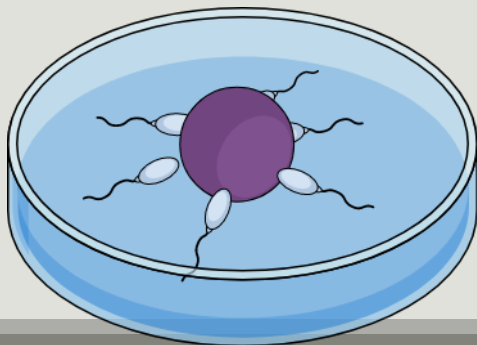
The aim of our study was to examine effects of the length of semen equilibration as well as two freezing techniques on ram sperm post-thaw quality. The ejaculates of Wallachian sheep rams ($n = 12$) were collected by an electro-ejaculation, equilibrated in a Triladyl® (0, 2, 4, 6, and 8 h) containing glycerol and egg yolk and frozen by programmable freezing (PF) or manual freezing (MF). After thawing, sperm samples were subjected to the motility (computer-assisted sperm analysis [CASA]), viability (SYBR-14/PI), and fertilizing ability (FA) (in vitro penetration/fertilization test on bovine oocytes) assays. It was found that the equilibration of 6 h (E-6) ensured higher post-thaw sperm motility and progressive movement compared with other lengths tested, irrespective of a freezing technique. The E-6 sperm viability did not differ between PF and MF but was lower ($P < 0.05$) than control. Sperm FA (E-6) was similar in PF

SUITABLE FREEZING PROTOCOL



SUITABLE FREEZING PROTOCOL

- Minimum motility requirements: TM: 90%, PM: 80%
- CASA (motility, morphological abnormalities and concentration evaluation)
- SYBR-14/PI (viability)
- IVF (fertilizing ability)



SUITABLE FREEZING PROTOCOL

- The equilibration time of 6 hours ensured higher sperm post-thaw total and progressive motility using both freezing techniques:

Programmable freezing: TM- 84.8% and PM- 74.8%

Manual freezing: TM- 76,6% and PM- 65%

- These data are also supported by the about 50% sperm viability (SYBR-14/PI) and about 60% fertilizing ability revealed using both cryopreservation techniques.






VERIFICATION ON DIFFERENT BREEDS

- Three Slovak breeds
- Vozaf et al. 2021 (prog. freezing)



Article

The Cryopreserved Sperm Traits of Various Ram Breeds: Towards Biodiversity Conservation

Jakub Vozaf ¹, Andrea Svoradová ^{2,3}, Andrej Baláži ², Jaromír Vašíček ^{1,2}, Lucia Olexiková ², Linda Dujčková ^{2,4}, Alexander V. Makarevich ², Rastislav Jurčík ², Hana Ďúranová ⁵ and Peter Chrenek ^{1,2,*}

- ¹ Institute of Biotechnology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 94901 Nitra, Slovakia; xvozaf@uniag.sk (J.V.); jaromir.vasicek@nppc.sk (J.V.)
 - ² NPPC, Research Institute for Animal Production Nitra, Hlohovecka 2, 95141 Lužianky, Slovakia; andrea.svoradova@mendelu.cz (A.S.); andrej.balazi@nppc.sk (A.B.); lucia.olexikova@nppc.sk (L.O.); linda.dujckova@nppc.sk (L.D.); alexander.makarevic@nppc.sk (A.V.M.); rastislav.jurcik@nppc.sk (R.J.)
 - ³ Department of Animal Morphology, Physiology and Genetics, Faculty of AgriSciences, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic
 - ⁴ Department of Botany and Genetics, Constantine the Philosopher University in Nitra, Trieda Andreja Hlinku 1, 94974 Nitra, Slovakia
 - ⁵ AgroBioTech Research Centre, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 94901 Nitra, Slovakia; hana.duranova@uniag.sk
- * Correspondence: peter.chrenek@uniag.sk



Citation: Vozaf, J.; Svoradová, A.; Baláži, A.; Vašíček, J.; Olexiková, L.; Dujčková, L.; Makarevich, A.V.; Jurčík, R.; Ďúranová, H.; Chrenek, P. The Cryopreserved Sperm Traits of Various Ram Breeds: Towards Biodiversity Conservation. *Animals* **2022**, *12*, 1311. <https://doi.org/10.3390/ani12101311>

Academic Editor: Maria-Jesus Palomo Peiró

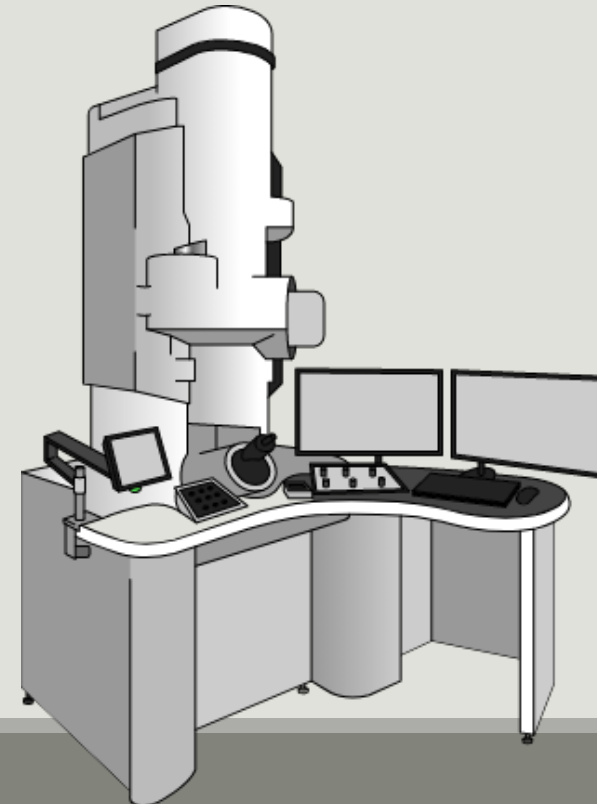
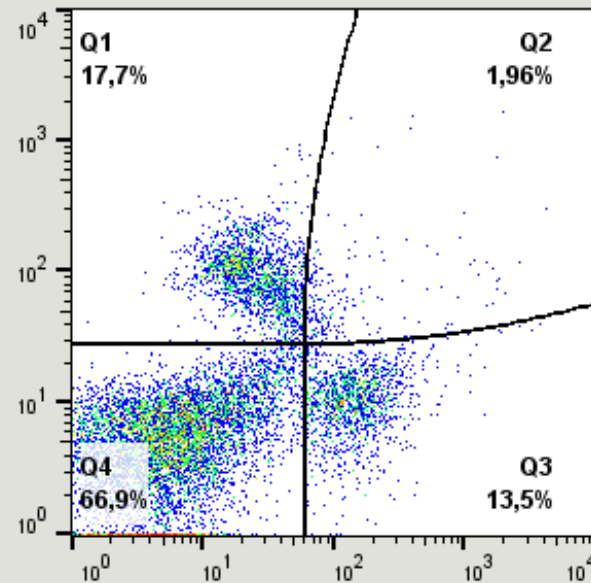
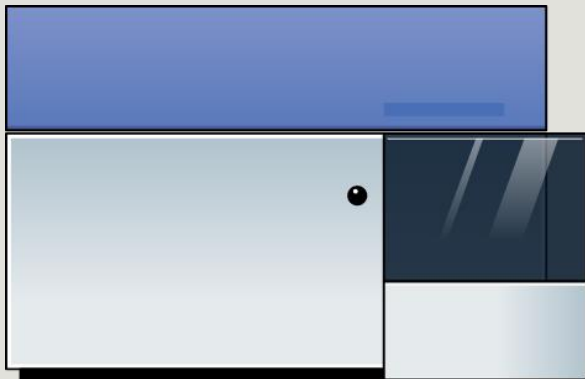
Received: 10 February 2022

Simple Summary: Biodiversity protection is one of our most important challenges today. Local animal breeds, as an essential piece of cultural heritage, form integral part of the biodiversity of individual countries and regions. Cryopreservation of sperm is one of the powerful tools for the conservation of animal genetic resources by creation of gene banks containing long-term stored genetic material. Cryopreservation of ram sperm faces several obstacles, such as the possible influence of individuality and breed characteristics on resistance to damage caused by low temperatures. In our research, we deal with the investigation of these differences between various breeds bred in Central Europe.

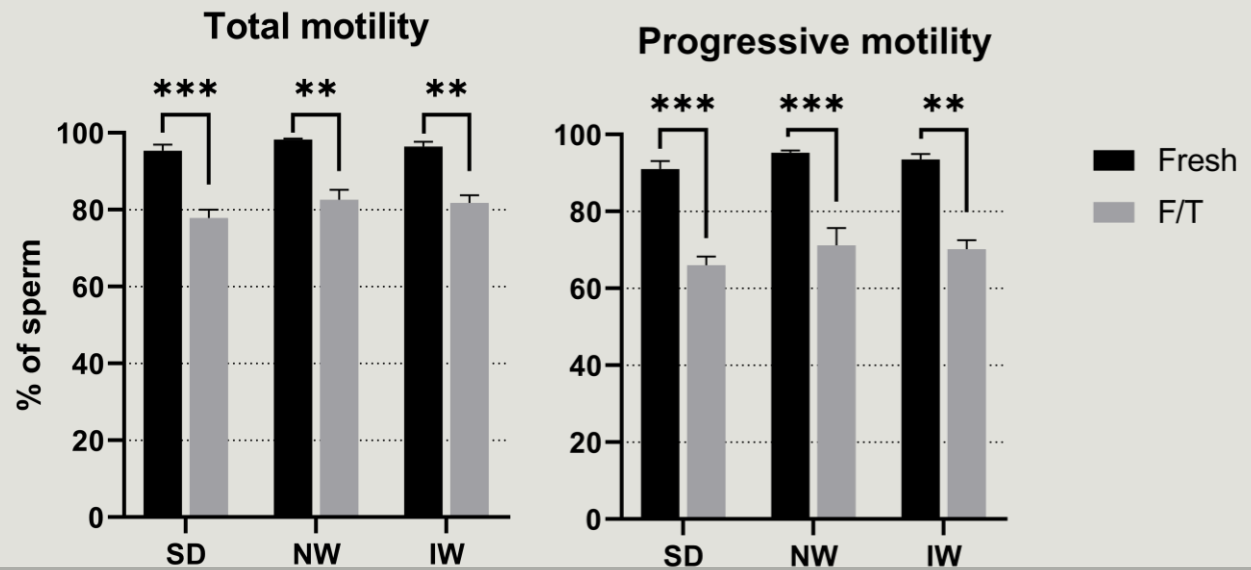
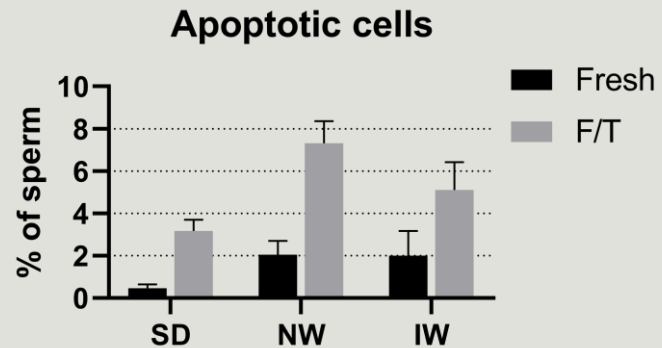
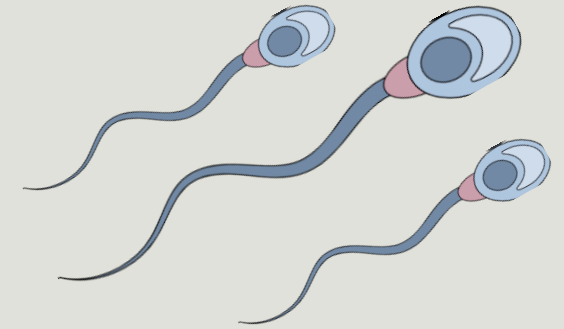
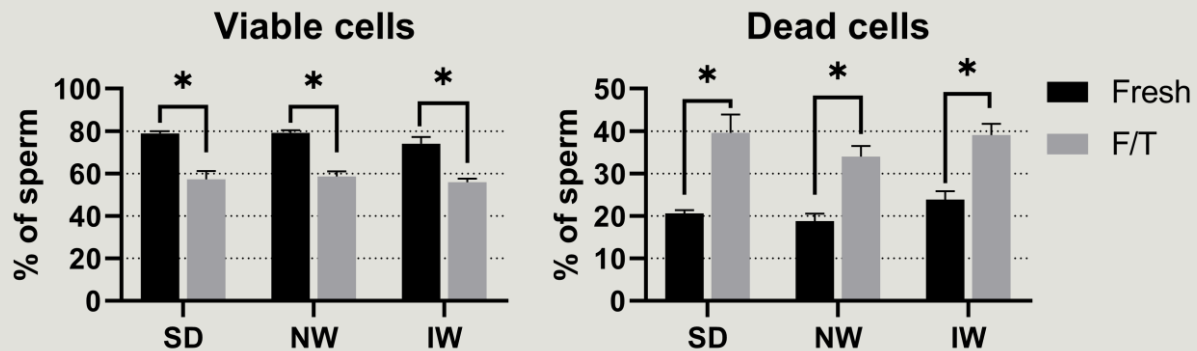
Abstract: The aim of our research was to compare three Slovak sheep breeds in the quality parameters of cryopreserved sperm. The ejaculates of Slovak Dairy (SD), Native Wallachian (NW), and Improved Wallachian (IW) sheep rams (n = 12) were collected by electro-ejaculation. Heterospermic samples were created from suitable ejaculates, separately for each breed (at least 90% of total and 80% of progressive motility). Samples were equilibrated in a Trilady[®] diluent and frozen by automated freezing. Sperm samples were subjected to the motility, morphology, (CASA), viability and apoptosis (DRAQ7/Yo-Pro-1), fertilizing capability (penetration/fertilization test (P/F) in vitro) and acrosomal

VERIFICATION ON DIFFERENT BREEDS

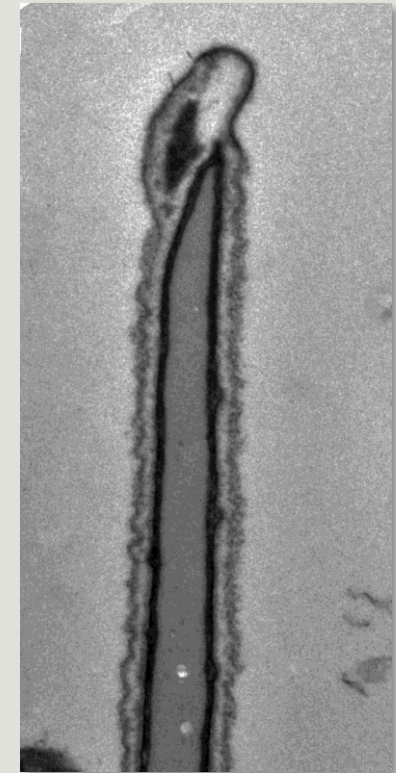
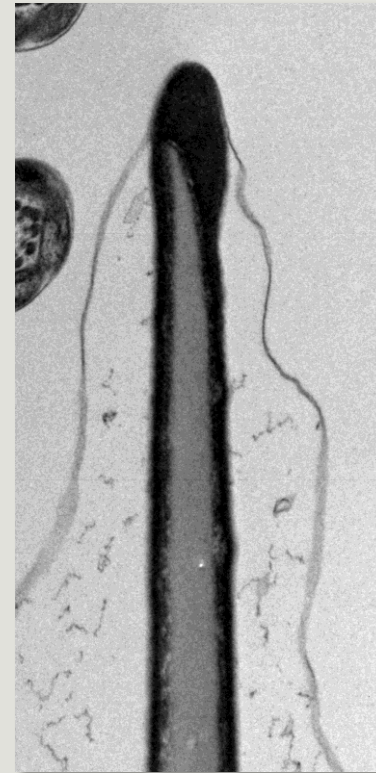
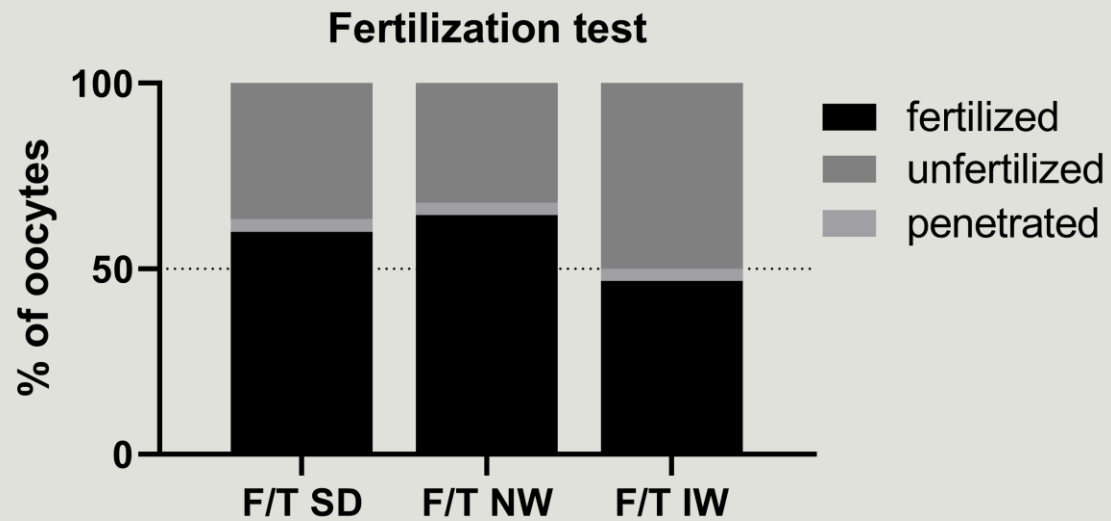
- CASA (motility, morphological abnormalities and concentration evaluation)
- Flow cytometry (DRAQ7/Yo-Pro-1; viability and apoptosis evaluation)
- Transmission electron microscopy (acrosome status)
- IVF (fertilizing ability)



VERIFICATION ON DIFFERENT BREEDS



VERIFICATION ON DIFFERENT BREEDS



VERIFICATION ON DIFFERENT BREEDS

- Results suggest that the cryopreservation protocol, established as our first task, is suitable for cryopreservation of the mentioned three Slovak sheep breeds.
- Parameters of motility, morphology, viability, and *in vitro* fertilization did not differ between individual breeds. However, significant differences among the breeds were observed in the acrosomal status.
- These findings could help to protect livestock biodiversity through possible reintroduction of native sheep breeds and long-term storage of male gametes as animal genetic resources.

PANEL OF QUALITY MARKERS

- Optimization of methods for flow cytometric analysis of standard and novel markers in ram semen.
- More than 20 biomarkers.



Article

Comprehensive Flow-Cytometric Quality Assessment of Ram Sperm Intended for Gene Banking Using Standard and Novel Fertility Biomarkers

Jaromír Vašíček^{1,2,*}, Andrej Baláži¹, Andrea Svoradová^{1,3}, Jakub Vozaf², Linda Dujíčková^{1,4}, Alexander V. Makarevich¹, Miroslav Bauer^{1,4} and Peter Chrenek^{1,2,*}

- ¹ Institute of Farm Animal Genetics and Reproduction, NPPC, Research Institute for Animal Production Nitra, Hlohovecká 2, 951 41 Lužianky, Slovakia; andrej.balazi@nppc.sk (A.B.); andrea.svoradova@nppc.sk (A.S.); linda.dujickova@nppc.sk (L.D.); alexander.makarevic@nppc.sk (A.V.M.); miroslav.bauer@nppc.sk (M.B.)
 - ² Institute of Biotechnology, Faculty of Biotechnology and Food Science, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia; xvozaf@uniag.sk
 - ³ Department of Morphology, Physiology and Animal Genetics, Faculty of Agri Sciences, Mendel University in Brno, Zemědělská 1/1665, 613 00 Brno, Czech Republic
 - ⁴ Department of Botany and Genetics, Faculty of Natural Sciences, Constantine the Philosopher University in Nitra, Nábřežie Mládeže 91, 949 74 Nitra, Slovakia
- * Correspondence: jaromir.vasicek@nppc.sk (J.V.); peter.chrenek@uniag.sk (P.C.);
Tel.: +421-37-654-6600 (J.V.); +421-37-641-4274 (P.C.)



Citation: Vašíček, J.; Baláži, A.; Svoradová, A.; Vozaf, J.; Dujíčková, L.; Makarevich, A.V.; Bauer, M.; Chrenek, P. Comprehensive Flow-Cytometric Quality Assessment of Ram Sperm Intended for Gene Banking Using Standard and Novel Fertility Biomarkers. *Int. J. Mol. Sci.* **2022**, *23*, 5920. <https://doi.org/10.3390/ijms23115920>

Academic Editors: Elisabetta Baldi, Sara Marchiani and Lara Tamburrino

Received: 30 March 2022
Accepted: 23 May 2022
Published: 25 May 2022

Abstract: Flow cytometry becomes a common method for analysis of spermatozoa quality. Standard sperm characteristics such as viability, acrosome and chromatin integrity, oxidative damage (ROS) etc. can be easily assess in any animal semen samples. Moreover, several fertility-related markers were observed in humans and some other mammals. However, these fertility biomarkers have not been previously studied in ram. The aim of this study was to optimize the flow-cytometric analysis of these standard and novel markers in ram semen. Ram semen samples from Slovak native sheep breeds were analyzed using CASA system for motility and concentration and were subsequently stained with several fluorescent dyes or specific antibodies to evaluate sperm viability (SYBR-14), apoptosis (Annexin V, YO-PRO-1, FLICA, Caspases 3/7), acrosome status (PNA, LCA, GAPDHS), capacitation (merocyanine 540, FLUO-4 AM), mitochondrial activity (MitoTracker Green, rhodamine 123, JC-1), ROS (CM-H₂DCFDA, DHE, MitoSOX Red, BODIPY), chromatin (acridine orange), leukocyte content, ubiquitination and aggresome formation, and overexpression of negative biomarkers (MKRN1, SPTRX-3, PAWP, H3K4me2). Analyzed semen samples were divided into two groups according to viability as indicators of semen quality: Group 1 (viability over 60%) and Group 2 (viability under 60%). Significant ($p < 0.05$) differences were found between these groups in sperm motility and concentration, apoptosis, acrosome integrity (only PNA), mitochondrial activity, ROS production (except for DHE), leukocyte and aggresome content, and high PAWP expression. In conclusion, several standard and novel fluorescent probes have been confirmed to be suitable for

STORAGE IN THE GENE BANK

- Gene bank of animal genetic resources in Lužianky (Nitra, Slovakia)
- Member of EUGENA (the European Genebank Network for Animal Genetic Resources) from 2021



N P
P C
NÁRODNÉ POĽNOHOSPODÁRSKE
A POTRAVINÁRSKE CENTRUM
VÝSKUMNÝ ÚSTAV ŽIVOČÍŠNEJ
VÝROBY NITRA

GÉNOVÁ BANKA
ŽIVOČÍŠNYCH GENETICKÝCH ZDROJOV

STORAGE IN THE GENE BANK

Species	Breed	Material type	Samples	Male donors	Female donors	First collected	Last collected
+ CATTLE	Slovenský pinzgauský	Semen	200	4	0	1999	2014
+ CATTLE	Slovenský strakatý	Semen	250	5	0	1999	2015
+ CATTLE	Slovenský pinzgauský	Embryos	17	0	1	2021	2021
+ CHICKEN	Oravka	Semen	348	6	0	2017	2017
+ RABBIT	Zoborský	Semen	341	5	0	2014	2017
+ RABBIT	Nitriansky	Semen	228	5	0	2014	2017
+ RABBIT	Holíčsky modrý	Semen	261	4	0	2016	2017
+ RABBIT	Slovenský sivomodrý rex	Semen	73	3	0	2016	2017
+ RABBIT	Slovenský pastelový rex	Semen	53	3	0	2016	2017
+ RABBIT	Zd. slovenský sivomodrý rex	Semen	2	1	0	2017	2017
+ RABBIT	Zoborský	Stem Cells	100	0	3	2020	2020
+ RABBIT	Nitriansky	Stem Cells	228	0	3	2020	2020
+ RABBIT	Zemplínsky	Stem Cells	11	1	0	2022	2022
+ SHEEP	Valaška	Semen	842	3	0	2018	2021
+ SHEEP	Zošľachtená valaška	Semen	355	1	0	2020	2021
+ SHEEP	Slovenská dojná ovca	Semen	469	1	0	2020	2021



THANK YOU FOR YOUR ATTENTION
