Animal Science Papers and Reports vol. 43 (2025) no. 1, 71-84
DOI: 10.2478/aspr-2025-0006
Institute of Genetics and Animal Biotechnology
of the Polish Academy of Sciences, Jastrzębiec, Poland

sciendo

The impacts of cryopreservation and lyophilization methods on the expression of selected acrosomal and mitochondrial proteins in boar spermatozoa*

Monika Trzcińska[1], Iwona Rajska[1], Magdalena Bryła[1], Lechosław Gajda[1], Małgorzata Kralka-Tabak[1], Patrycja Kurowska[2], Agnieszka Rak[2], Marcin Samiec[1]**

[1] Department of Reproductive Biotechnology and Cryoconservation, National Research Institute of Animal Production, 32-083 Balice near Kraków, Poland

[2] Laboratory of Physiology and Toxicology of Reproduction, Institute of Zoology and Biomedical Research, Jagiellonian University, 30-387 Kraków, Poland

(Accepted February 24, 2025)

At this stage of research, the primary focus in species-specific *ex situ* conservation is on long-term preservation strategies for germplasm, particularly cryopreservation and freeze-drying of male gametes (spermatozoa). Therefore, the primary objective of the present study was to determine the influence of boar semen cryopreservation and freeze-drying techniques on the molecular quality of spermatozoa as assessed by the proteomic profile and the degree of nuclear chromatin disintegration (SCSA). Semen samples collected from 4 Polish Landrace boars (3 ejaculates/boar) were used for experiments. The extent of inter-specimen differences in cryopreserved and freeze-dried boar spermatozoa was assessed based on semi-quantitative expression levels of selected acrosomal (SPACA1, AFAF) and mitochondrial (UQCRC2) proteins, which may also be valuable predictors of cytophysiological status, metabolic activity and fertilizing capacity of male gametes. The present study has confirmed that the cryopreservation and freeze-drying procedures used did not impair sperm quality, as verified by the degree of sperm nuclear chromatin integrity. Furthermore,

*The present study was financially supported by statutory grant No. 01-19-10-21 from the National Research Institute of Animal Production in Balice near Kraków, to Monika Trzcińska
**Corresponding author: monika.trzcinska@iz.edu.pl

considerable individual variability was identified between semi-quantitative proteomic profiles associated with the relative expression of AFAF, SPACA1 and UQCRC2 proteins, depending on the applied method of boar semen preservation.

KEY WORDS: boar / sperm / protein expression / long-term preservation

Ex situ conservation of genetic resources involves the collection of biological material in the form of semen, embryos and oocytes. The accumulation of biological material obtained especially from representatives of endangered or extinct breeds of farm animals and the implementation of assisted reproduction technologies (ARTs) in genetic and biotechnological conservation programs, may make it possible to restore the original size of subpopulations or to recreate extinct breeds in the future [Trzcińska and Samiec 2021]. Therefore, long-term cryopreservation or lyophilization of germplasm-carrying biological materials, including sperm and oocytes, may result in the use of genetic resources secured from extant individuals, in programs aimed to restore, sustainably restore and perpetuate previously lost biodiversity in currently existing subpopulations of livestock species and breeds [Trzcińska and Bryła 2020]. The use of preserved genetic resources can accelerate breeding progress in breeds undergoing intensive zootechnical improvement [Mara *et al*. 2013, Comizzoli and Holt 2014].

The main *ex situ* method for the conservation of livestock genetic resources is the cryopreservation of biological material. Regarding boar semen, the use of this technology is limited, which stems from the higher sensitivity of boar spermatozoa to factors associated with freezing compared to spermatozoa from other livestock species. Osmotic stress, cryogenic shock and ice crystal formation cause damage to the plasma membranes and other cell organelles of boar spermatozoa. An important factor causing the increased sensitivity of boar spermatozoa to freezing is the high proportion of polyunsaturated fatty acids in the lipids and their specific composition. In boar sperm, polyunsaturated fatty acids account for about 65% of the total content of all fatty acids. The high proportion of unsaturated acids poses a threat to the structure of sperm plasma membranes due to the susceptibility of these acids to peroxidation processes [Gadea *et al*. 2005]. An excess of reactive oxygen species or the exhaustion of the compensatory capacity of the antioxidant system in the sperm, leads to oxidative stress. Long-term exposure of sperm to oxidative stress causes peroxidative changes in sperm cell membrane lipids and compromises cell membrane structure [Trzcińska *et al*. 2015].

Another method that allows long-term storage of semen is freeze-drying (lyophilization), which, unlike cryopreservation, offers the possibility of preserving biological material at temperatures above 0ºC [Keskintepe and Eroglu 2021, Comizzoli *et al*. 2022ab]. While previous studies have shown that freeze-dried spermatozoa can undergo destruction processes within the plasma membrane and/or genomic DNA due to the occurrence of oxidative stress and impaired antioxidant processes, it is worth emphasizing that deviations in semen quality parameters such as viability,

motility and even DNA stability do not rule out the suitability of lyophilized and subsequently hydrated sperm for intracytoplasmic sperm injection (ICSI) procedures, performed for *in vitro* fertilization of metaphase II (MII) oocytes in various livestock species [Keskintepe and Eroglu 2021, Thiangthientham *et al.* 2023]. To date, *in vitro* fertilization (IVF) via ICSI of meiotically mature oocytes using lyophilized/rehydrated spermatozoa has resulted in progeny in several mammalian species, including farm animals (hamsters, mice, rats, rabbits, and horses). [Muneto and Horiuchi 2011, Kaneko *et al.* 2003, Hirabayashi *et al*. 2005, Li *et al*. 2009, Choi *et al.* 2011]. Attempts to obtain progeny using the ICSI technique with freeze-dried/rehydrated semen have, however, been unsuccessful in cattle [Hara *et al.* 2014], sheep [Palazzese *et al*. 2018] and pigs [Olaciregui *et al.* 2017]. In the latter, they only resulted in the *in vitro* development of embryos to the blastocyst stage.

The main factors determining the fertilizing capacity of sperm in *in vivo* and *in vitro* fertilization processes (including the IVF procedure by ICSI) are the structural-functional parameters and biomarkers of sperm molecular quality responsible for the correct course of the capacitation and acrosomal reaction processes. Therefore, at the current stage of research, correlations are being sought between the expression levels of marker proteins providing spermatozoa with high molecular quality and the fertilization capacity of male gametes [Pang *et al*. 2020, 2022]. One biomarker of the fertilizing capacity of sperm is the acrosome formation-associated factor (AFAF), also referred to as eqTN (EQTN) [Li *et al.* 2006]. Another biomarker determining the molecular quality of spermatozoa and their ability to counteract oxidative stress is core protein 2, which is a major integral part of the ubiquinol-cytochrome c reductase core protein 2 (UQCRC2; ubiquinol-cytochrome c reductase core protein 2) enzyme and is one of the primary polypeptide subunits of the mitochondrial respiratory chain (complex III) [Kwon *et al*. 2015]. The sperm acrosome membrane-associated protein 1 (SPACA1) also belongs to the group of biomarkers that determine the molecular quality of sperm, their capacity for proper acrosomal vesicle biogenesis and fertilization efficiency [Fujihara *et al*. 2010].

Taking this into account, and due to individual variability between boars in susceptibility to long-term preservation, the aim of this study was to evaluate the proteomic profile associated with identifying the expression levels of AFAF, UQCRC2 and SPACA1 proteins and to assess the degree of DNA fragmentation of sperm subjected to cryopreservation and freeze-drying procedures.

Material and methods

Collection of semen samples and their long-term conservation by cryogenic or lyophilizogenic protection

The experimental material consisted of semen collected from 4 Polish Landrace boars at 2.3±0.3 years of age (3 ejaculates per each boar) at the Boar AI Station in Czermin. The semen (sperm rich-fraction) was diluted (1:1) in Biosolwens Plus

(BP) extender (Biochefa, Sosnowiec, Poland) and transported to the Department of Reproductive Biotechnology and Cryoconservation of the National Research Institute of Animal Production. Semen samples were subjected to cryopreservation and freeze-drying procedures after sperm motility assessment. The ejaculates containing at least 80% motile sperm and 80% morphologically normal sperm were qualified for cryopreservation and freeze-drying.

Cryopreservation of spermatozoa was performed according to an original method (patent PL 228192 B1). The diluted semen was transferred to 50 mL centrifuge tubes, equilibrated at 15°C for 60 min, and centrifuged at 800 × g for 25 min. The supernatant was discarded, and the sperm pellet was resuspended with extender A (80 mL of 11% lactose solution and 20 mL egg yolk) to a concentration of 1.5×10^9 spermatozoa/mL. The diluted semen was cooled to 5°C for 120 min. Two parts of semen were mixed with one part of extender B (89.5% extender A with 9% glycerol and 1.5% Equex-STM paste whose active ingredient is sodium dodecyl sulphate, Nova Chemical Sales, Scituate Inc, MA, USA). The final concentration of semen was 1.0×10^9 spermatozoa/mL and 3% glycerol. The diluted and cooled semen was loaded into 0.5 mL straws (Minitüb). The straws were sealed with polyvinyl chloride powder before being placed in contact with nitrogen vapor for 15 min in a polystyrene box. After that, the straws were plunged into liquid nitrogen (-196°C) for storage.

For lyophilization, the diluted semen was centrifuged for 18 min, 800 × g at 17°C, and then diluted again in Tyrode-Hepes-PVA medium (THP; Sigma-Aldrich, Merck) to a concentration of 0.5×10^6 spermatozoa/mL. After dilution, the semen was equilibrated for 2 h at 4°C and frozen on dry ice for 10 min as 0.2 mL pellets and then deposited in liquid nitrogen containers. Then, the semen frozen in pellets was placed in chilled tubes and lyophilized in an Alpha 1-2 LD plus apparatus for 18 hours at 0.37 mBar. After lyophilization, the tightly sealed freeze-dried tubes were stored at 4°C .

Cytometric and proteomic assessment of cryopreserved and lyophilized spermatozoa

Both fresh, cryopreserved and freeze-dried spermatozoa were subjected to cytometric evaluation of the degree of nuclear chromatin integrity and a proteomic profile analysis to determine the expression levels of the following functional proteins: SPACA1, AFAF and UQCRC2 that play an important role in the processes of sperm capacitation and acrosomal reaction that together determine the efficiency of *in vitro* fertilization of oocytes.

The sperm chromatin structure assay (SCSA) was applied to assess the extent of sperm nuclear chromatin integrity. The latter was measured as a percentage of spermatozoa with nuclear DNA fragmentation, referred to as the DNA fragmentation index (DFI). The sperm samples were thawed at 37°C in a water bath and then placed on crushed liquid ice. DNA fragmentation was identified by staining the acid-treated spermatozoa with acridine orange. Each spermatozoon emitted a fluorescent light following excitation by a blue laser (λ_{ex} = 488 nm); the spermatozoa without detectable levels of DNA fragmentation emitted a green fluorescence (λ_{ab} =525 nm;

detector 525/40 BP) and those with DNA fragmentation emitted red fluorescence (λ_{ab} = 690 nm; detector 690/50 BP). For each sample, 1×10^4 individual spermatozoa were quantified to determine the DFI (%DFI = red fluorescence/(red fluorescence + green fluorescence × 100) . The flow cytometry data were analysed using the software CytoExpert version 2.4.0.28 (Beckman Coulter, Inc.) and the analysis of the data was carried out using the software WinList 3D; version 9.0.1.

In turn, to ascertain the proteomic profile of the spermatozoa, the determination of isolated total protein content, gel electrophoretic, migration, protein transfer on PVDF membrane, and Western blot analysis were performed. The protein content of the samples was measured using the micro-BCA method, the samples with a concentration of 60 µg protein were sonicated and then boiled with loading buffer at 95°C for 5 min. Ready-to-use BioRad 4-20% gels were used for electrophoretic separation at 200V and 250mA. After electrophoresis, the proteins were transferred from gel to a PVDF membrane (BioRad Laboratories) using a BIO-RAD Mini Trans-Blot apparatus at 1A, 25V for 7 min. After the transfer, the membranes were transferred to 5% bovine serum albumin (BSA; Sigma-Aldrich, Merck) for 1 h to block non-specific antibody binding sites. The membranes were washed sequentially in TBST solution (3×5 min), followed by overnight incubation with primary antibodies at 4°C (concentrations according to manufacturer's instructions; diluted in BSA). Subsequently, the membranes were washed in TBST (3×5 min) and the specific secondary antibodies (according to the manufacturer's instructions) were applied at a concentration of 1:1000 for 1 h at room temperature. After incubation, the membranes were washed in TBS (3×5 min), incubated with chemiluminescence reagent (Advansta Inc.) and chemiluminescence was measured using a ChemiDoc instrument (BioRad Laboratories). The reference protein was α-tubulin. Densitometric analysis of appropriate proteins bands selected with respect to the size marker (no. 26619, ThermoFisher), 32 kDa in case of SPACA, 33 kDA for AFAF, 48 kDa for UQCRC2, 55 kDa for tubulin, was performed using ImageJ software (US NIH).

Statistical analysis

The statistical significance of differences at a probability levels of $p\leq0.05$ and $p\leq0.01$ between experimental groups was determined using one-way ANOVA followed by Tukey's *post-hoc* test. The computations were performed using Statistica 13 package programs (TIBCO Software Inc.).

Results and discussion

This study is the first one to assess the effects of cryopreservation and freeze-drying on the molecular quality of boar spermatozoa, focusing on DNA fragmentation and proteomic profile.

In total, semen from 4 boars was evaluated. Using the SCA® Motility and Concentration module, the percentage of spermatozoa displaying total motility

and progressive motility was assessed in fresh semen, which was 87.5%±3.4 (mean±standard deviation) and 81.1%±4.1, respectively. Based on the cytometric evaluation of the degree of nuclear chromatin integrity (SCSA), the mean percentage of sperm DNA fragmentation (DFI) was distributed as follows: 1.6%±0.5 – in fresh samples, 1.7%±0.3 – in freeze-dried samples, and 1.7%±0.6 – in cryopreserved samples. There were no significant differences ($p \geq 0.05$) not only in the percentage of spermatozoa with DFI between ejaculates derived from the same boar, but also between ejaculates originating from 4 individual boars, either for fresh, freeze-dried or cryopreserved sperm samples.

Although lyophilization of spermatozoa results in the loss of their motility after rehydration, the freeze-dried sperm cells can retain genetic stability, i.e., do not exhibit chromatin disintegration. Such a condition, predestining the suitability of lyophilized/rehydrated spermatozoa for ARTs based on IVF of oocytes by ICSI has been confirmed in the investigations focused on pigs in the studies by Garcia *et al*. [2014], Men *et al.* [2013] and Nakai *et al.* [2007] as well as the research focused on sheep [Palazzese *et al*. 2018]. Analogously, as has been reported in the study by Iuso *et al.* [2013], the genome stability of lyophilized ovine lymphocytes (providing a source of nuclear donors for somatic cell cloning) has been calculated at the level of approximately 60%, while the incidence of DNA disintegration in these subpopulations of freeze-dried/rehydrated nuclear donor leukocytes oscillated around 40%. Similarly, the absence of genetic instability in lyophilized and subsequently rehydrated boar spermatozoa has been proven in the research by Garcia *et al.* [2014]. Interestingly, the abilities of lyophilized boar sperm cells to maintain their chromatin integrity have been shown to be dependent on the formulation/composition of freeze-drying buffers.The spermatozoa that had been freeze-dried in EDTA buffer enriched with lactose were characterized by much higher genetic stability than their counterparts lyophilized in EDTA buffers supplemented either with sucrose and lactose or with trehalose and lactose [Garcia *et al*. 2014]. In turn, the extent of DNA damage and fragmentation (as assessed by single-strand and double-strand breaks) after rehydration of freeze-dried ram spermatozoa has been found to remarkably depend on specimen-specific differences [Palazzese *et al*. 2018]. Furthermore, the estimation of DNA or chromatin integrity in lyophilized and subsequently rehydrated spermatozoa varies between all the above-reported investigations, which can account for the differences in the assessed incidence of genetic stability or instability, depending on the approach used to determine the DNA or chromatin damage.

The extent of inter-specimen differences in spermatozoa subjected to different long-term preservation strategies was comparatively determined by the semi-quantitative expression levels of selected acrosomal (SPACA1, AFAF) and mitochondrial (UQCRC2) proteins, which may also be excellent predictors of the ultrastructural-functional condition and fertilizing capacity of male gametes of the domestic pig. Noteworthily such investigations have not yet been undertaken.

The representative results of Western blot analysis for the relative expression profiles of SPACA1, AFAF and UQCRC2 proteins in the samples of fresh, freeze-dried and cryopreserved spermatozoa are shown as Figs. 1 to 3, respectively.

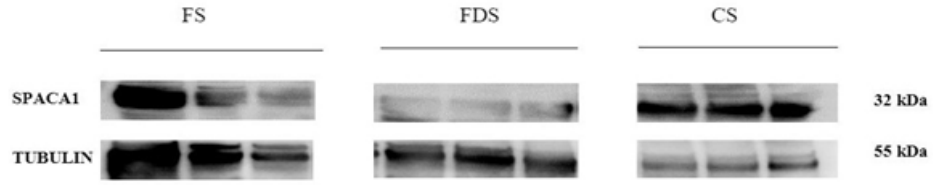

Fig. 1. Western blot analysis results of relative expression of acrosomal SPACA 1 protein in samples of fresh (FS), freeze-dried (FDS) and cryopreserved (CS) spermatozoa. Representative bands indicate the expression of SPACA1 protein in individual samples. α-Tubulin is the reference protein for all samples analyzed.

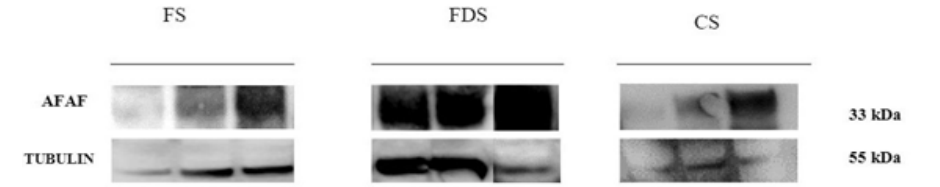

Fig. 2. Western blot analysis results of relative expression of acrosomal AFAF protein in samples of fresh (FS), freeze-dried (FDS) and cryopreserved (CS) spermatozoa. Representative bands indicate the expression of AFAF protein in individual samples. α-Tubulin is the reference protein for all samples analyzed.

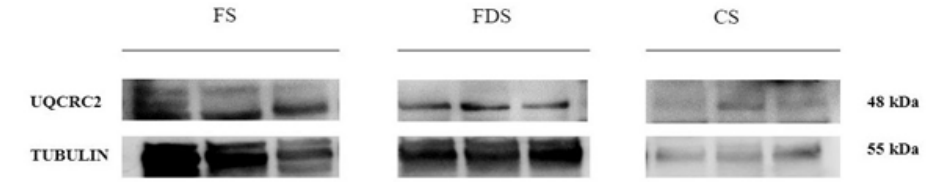

Fig. 3. Western blot analysis results of relative expression of mitochondrial UQCRC2 protein in samples of fresh (FS), freeze-dried (FDS) and cryopreserved (CS) spermatozoa. Representative bands indicate the expression of UQCRC2 protein in individual samples. α-Tubulin is the reference protein for all samples analyzed.

The results of proteomic analyses using Western blot are presented in Figs. 4 to 7, which show bar graphs depicting differences in the relative expression profiles (semi-quantitative levels) of SPACA1, AFAF, and UQCRC2 proteins in fresh, freeze-dried, and frozen spermatozoa from boars 1-4.

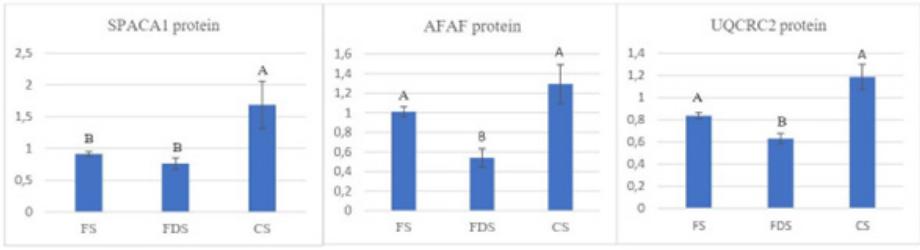

Fig. 4. Results of Western blot analysis presented as bar graphs showing differences in relative expression (semi-quantitative profile) of SPACA1, AFAF and UQCRC2 proteins in samples of fresh (FS), freeze-dried (FDS) and cryopreserved (CS) spermatozoa derived from boar 1.
The different letters A,B indicate the existence of highly significant differences between the experimental groups – with a probability of random error of $p \leq 0.01$.

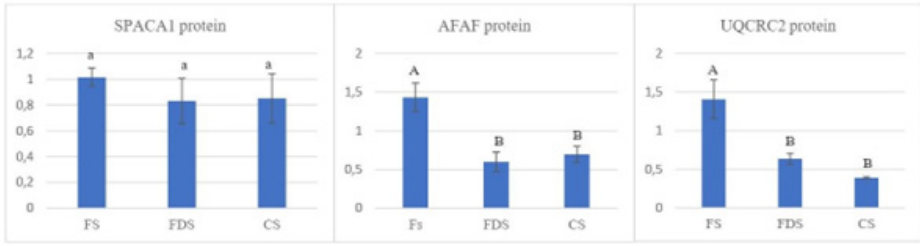

Fig. 5. Results of Western blot analysis presented as bar graphs showing differences in relative expression (semi-quantitative profile) of SPACA1, AFAF and UQCRC2 proteins in samples of fresh (FS), freeze-dried (FDS) and cryopreserved (CS) spermatozoa derived from boar 2.
The different letters A, B indicate the existence of highly significant differences between the experimental groups – with a probability of random error of $p \leq 0.01$

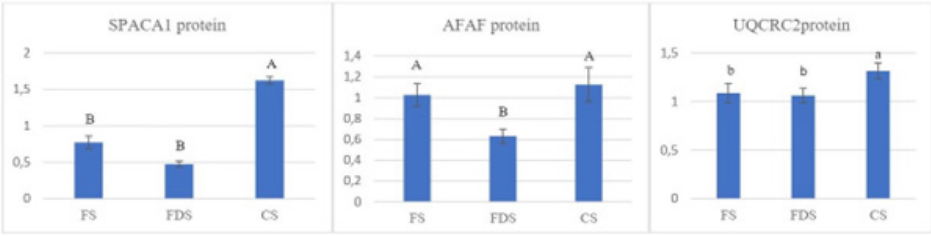

Fig. 6. Results of Western blot analysis presented as bar graphs showing differences in relative expression (semi-quantitative profile) of SPACA1, AFAF and UQCRC2 proteins in samples of fresh (FS), freeze-dried (FDS) and cryopreserved (CS) spermatozoa derived from boar 3.
The different letters a, b and A, B indicate the existence of significant and highly significant differences between the experimental groups – with a probability of random error of $p \leq 0.05$ and $p \leq 0.01$, respectively.

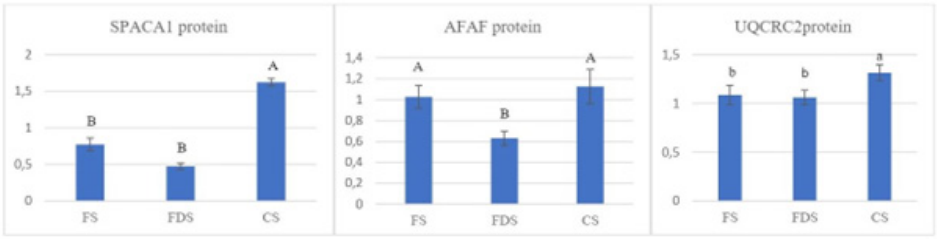

Fig. 7. Results of Western blot analysis presented as bar graphs showing differences in relative expression (semi-quantitative profile) of SPACA1, AFAF and UQCRC2 proteins in samples of fresh (FS), freeze-dried (FDS) and cryopreserved (CS) spermatozoa derived from boar 4.
The different letters a, b and A, B indicate the existence of significant and highly significant differences between the experimental groups – with a probability of random error of $p \leq 0.05$ and $p \leq 0.01$, respectively.

One of the primary predictors of sperm fertilizing capacity is the acrosomal membrane-anchored protein (AFAF, acrosome formation-associated factor), also referred to as equatorin (EQTN) [Li *et al.* 2006]. It should be emphasized that high levels of AFAF protein expression are crucial for the normal biogenesis of acrosomal vesicles in the apical segment of the sperm head during the final stages of the sperm growth and maturation processes (spermiogenesis). The presence of this protein is notable for the inner and outer acrosomal membrane domains of mature male gametes (before the processes of capacitation and acrosomal reaction take place) [Sun *et al.* 2019]. The protein is also significantly involved in the permeabilization and fusion of the inner and outer acrosomal membranes following the acrosomal reaction. This process leads to the exocytosis of the acrosomal vesicle and the externalization of protein molecules, as the inner acrosomal membrane fuses with the outer membrane and integrates into the plasma membrane within the equatorial segment of the sperm head. [Kwon *et al.* 2015, Sun *et al*. 2019]. A high expression profile of the AFAF protein prior to capacitation/acrosomal reaction processes is a prerequisite for proper sperm capacitation and acrosomal reaction processes, and thus is an excellent facilitator for predicting high efficiency of:

1) interproteomic communication accompanying sperm binding by ZP4 (zona pellucida sperm-binding protein 4) glycoprotein molecules, located in the oocyte zona pellucida, which contributes to the correct induction of the acrosomal reaction and prevents the phenomenon of polyspermia through the correct initiation of biochemical and biophysical processes of zona pellucida hardening;
2) interproteomic communication during sperm and oocyte plasma membrane interactions [Li *et al*. 2006, Sun *et al*. 2019].

The resulting high efficiency of plasma membrane fusion in the equatorial segment with the oolemma simultaneously enhances the fertilizability of capacitated spermatozoa due to their increased susceptibility to the normal course of the acrosomal

reaction. The outcome is the consumption and/or depletion of the original pool of AFAF membrane protein molecules and a reduction in the level of this protein in the equatorial segment of the sperm head due to the termination of the acrosomal reaction and the *in vivo* or *in vitro* fertilization process of the oocytes [Kwon *et al.* 2015, Sun *et al*. 2019]. Indeed, a study by Kwon *et al*. [2015] shows that high levels of AFAF protein expression persist in the equatorial segment of boar spermatozoa after capacitation and the acrosomal reaction, suggesting that the original level of this membrane protein is not consumed before capacitation. The failure of spermatozoa to deplete the pre-capacitating pool of this protein is indicative of their lower fertilizing capacity, which is reflected in the reduced fertility and prolificacy of boars and the lower number of piglets in litter observed in sows inseminated with semen from such boars.

It seems clear that long-term sperm preservation methods, such as cryoconservation or freeze-drying, may permanently impair sperm capacitation.. These procedures also may bring about the impact of decapacitating factors following the acrosomal reaction and the fertilizing capacity of sperm, which, on the one hand, may be related to a lack of capability or reduced capability to consume/exhaust the pre-capacitation pool of AFAF protein in cryopreserved or freeze-dried spermatozoa. On the other hand, this may reduce the fusogenic properties of the protein in the interaction between the acrosomal and plasma membranes of frozen/freeze-dried male gametes and the plasma membrane of female gametes [Kwon *et al*. 2015, Sun *et al*. 2019].

Considering the AFAF protein, a sizeable decrease in its expression was observed in freeze-dried sperm samples for boar 1 and boar 3. Regarding boar 2, a significant decrease in the relative expression of this protein was found in both freeze-dried and cryopreserved sperm samples. In contrast, for boar 4, no significant differences were shown between sperm samples derived from fresh semen and sperm samples subjected to long-term preservation (regardless of the preservation method used).

Sperm acrosome membrane-associated protein 1 (SPACA1) [Fujihara *et al.* 2010] is also included in the group of predictors that determine the molecular quality of spermatozoa, their capacity for normal acrosomal vesicle biogenesis and fertilization success [Fujihara *et al*. 2010]. The highest expression levels of this fusogenic protein have been reported in the inner acrosomal membrane fusing with the outer acrosomal membrane and the plasma membrane [Fujihara et al. 2012, Ogura *et al*. 2016, Minami *et al.* 2020]. This protein is responsible for the increased cryo-resistance of the acrosomal and plasma membranes of spermatozoa subjected to freezing. As a result, post-cryogenic preservation maintains SPACA1-dependent fusogenic properties of sperm acrosomal membranes, essential for proper acrosomal reaction, and preserves sperm plasma membrane fusogenicity through sequestration of SPACA1 in the equatorial segment of the sperm head. This, in turn, determines high fertilization efficiency due to successful proteomic interaction triggered by the sperm plasma membrane–oolemma fusion process during fertilization [Harayama *et al*. 2010, Ogura et al. 2016, Minami *et al*. 2020].

As part of our study, we observed a significant increase in SPACA1 protein expression in cryopreserved spermatozoa from boars 1 and 3, compared to fresh and freeze-dried spermatozoa. This seems to justify the enhanced cryogenic resilience of acrosome and plasmalemma compartments of cryopreserved/thawed spermatozoa in semen from boars 1 and 3. In contrast, frozen/thawed spermatozoa from boar 4 exhibited a decrease in relative abundance of SPACA1 protein, which might prove a diminished cryo-resistance (i.e., decreased in post-freeze survival rates) of sperm cells derived from this specimen. At the same time, for the SPACA1 protein, no differences in relative expression were observed between sperm cells in fresh semen and freeze-dried/rehydrated sperm cells. Furthermore for boar 2, no statistically significant changes in SPACA1 protein expression were identified between fresh semen-derived sperm samples and freeze-dried and cryopreserved sperm samples.

Another predictive factor characterizing the molecular quality of spermatozoa and their ability to counteract oxidative stress is the UQCRC2 protein, which is one of the key subunits of the mitochondrial respiratory chain cytochrome c ubiquinol reductase. The high expression level of this protein within the sperm midpiece guarantees an adequate antioxidant potential, resulting in a high capacity to reduce the intracellular concentration of reactive oxygen species (oxygen free radicals), which, in turn, guarantees the normal course of all high energy balance reactions in the processes of capacitation, sperm acrosomal reaction and monospermic fertilization of oocytes [Choi et al. 2008, Kwon *et al*. 2015]. Therefore, spermatozoa with high susceptibility to the processes of capacitation and acrosomal reaction are characterized by high fertilization capacity and increased UQCRC2-dependent antioxidant potential, as well as increased metabolic activity of mitochondria forming the mitochondrial sheath within the sperm midpiece [Kwon *et al*. 2015]. In turn, the diminished energy requirements of non-capacitated spermatozoa lead to reduced recruitment of the mitochondrial protein UQCRC2and a remarkable decrease in the quantitative profile of this enzymatic protein, resulting in a significant decline in sperm fertilization [Rahman *et al.* 2017]. Furthermore Kwon *et al*. [2015] confirm that an elevated UQCRC2 protein expression profile in boar sperm mitochondria sustained during such energy metabolism-enhanced processes as capacitation, acrosomal reaction and proteomic sperm-oocyte interaction during *in vivo* or *in vitro* fertilization is positively correlated with an augmentation in boar fertility and prolificacy and with an increase in litter size in farrowed sows.

The analysis of the results revealed a significant decrease in the relative abundance of UQCRC2 protein in freeze-dried sperm samples from boar 1 and boar 2 compared to fresh semen. Moreove, cryopreserved spermatozoa from boar 2 were also characterized by a significant decrease in the relative expression of the UQCRC2 protein in relation to this indicator recorded for fresh semen samples. In contrast, for boar 3 and boar 4, a significant alteration (increase and decrease, respectively) in the semi-quantitative profile of UQCRC2 protein was observed in cryopreserved sperm, correlating its relative abundance with that in fresh and freeze-dried sperm samples.

These findings suggest not only reduced antioxidant capacity but also impaired capacitation, acrosomal reaction, and fertilizability in lyophilized/rehydrated sperm from boars 1 and 2 and cryopreserved/thawed sperm from boars 2 and 4.

In conclusion, no negative effect of long-term storage methods (cryopreservation and freeze-drying) on the nuclear chromatin integrity of spermatozoa was demonstrated for boar semen. At the same time, high individual variability was found between quantitative proteomic profiles associated with the relative expression of selected acrosomal (AFAF, SPACA1) and mitochondrial (UQCRC2) proteins in spermatozoa, depending on the method of boar semen preservation used (freezing or lyophilization).

REFERENCES

1. CHOI Y.H., VARNER D.D., LOVE C.C., HARTMAN D.L., HINRICHS K., 2011 – Production of live foals via intracytoplasmic injection of lyophilized sperm and sperm extract in the horse. ***Reproduction*** 142(4), 529-538.
2. CHOI Y.J., UHM S.J., SONG S.J., SONG H., PARK J.K., KIM T., PARK C., KIM J.H., 2008 – Cytochrome c upregulation during capacitation and spontaneous acrosome reaction determines the fate of pig sperm cells: linking proteome analysis. ***Journal of Reproduction and Development*** 54 (1), 68-83.
3. COMIZZOLI P., HE X., LEE P.C., 2022a – Long-term preservation of germ cells and gonadal tissues at ambient temperatures. ***Reproduction and Fertility*** 3(2), R42-R50.
4. COMIZZOLI P., AMELKINA O., LEE P.C., 2022b – Damages and stress responses in sperm cells and other germplasms during dehydration and storage at nonfreezing temperatures for fertility preservation. ***Molecular Reproduction and Development*** 89(12), 565-578.
5. COMIZZOLI P., HOLT W.V., 2014 – Recent advances and prospects in germplasm preservation of rare and endangered species. ***Advances in Experimental Medicine and Biology*** 753, 331-356.
6. FUJIHARA Y., SATOUH Y., INOUE N., ISOTANI A., IKAWA M., OKABE M., 2012 – SPACA1-deficient male mice are infertile with abnormally shaped sperm heads reminiscent of globozoospermia. ***Development*** 139(19), 3583-3589.
7. FUJIHARA Y., SATOU Y., INOUE N., ISOTANI A., IKAWA H., OKABE M., 2010 –SPACA1, Sperm acrosome associated 1, is required for sperm-head shaping. Proceedings of the 11th International Symposium on Spermatology, P01, p. 37.
8. GADEA J., GARCÍA-VAZQUEZ F., MATÁS C., GARDÓN J.C, CÁNOVAS S., GUMBAO D., 2005 – Cooling and freezing of boar spermatozoa: supplementation of the freezing media with reduced glutathione preserves sperm function. ***Journal of Andrology*** 26(3), 396-404.
9. GARCIA A., GIL L., M C., MARTINEZ F., KERSHAW-YOUNG C., De BLAS I., 2013 – Effect of different disaccharides on the integrity and fertilising ability of freeze-dried boar spermatozoa: a preliminary study. ***Cryo Letters*** 35(4), 277-285.
10. HARA H., TAGIRI M., HWANG I.S., TAKAHASHI M., HIRABAYASHI M., HOCHI S., 2014 – Adverse effect of cake collapse on the functional integrity of freeze-dried bull spermatozoa. **Cryobiology** 68(3), 354-360.
11. HARAYAMA H., KAZUHIRO N., SAKASE M.T., MORIYUKI M.T., 2010 – Relationship of protein tyrosine phosphorylation state with tolerance to frozen storage and the potential to undergo cyclic AMP-dependent hyperactivation in the spermatozoa of Japanese Black bulls. ***Molecular Reproduction and Development*** 77(10), 910-921.
12. HIRABAYASHI M., KATO M., ITO J., HOCHI S., 2005 – Viable rat offspring derived from oocytes intracytoplasmically injected with freeze-dried sperm heads. ***Zygote*** 13(1), 79-85.

13. IUSO D., CZERNIK M., DI EGIDIO F., SAMPINO S., ZACCHINI F., BOCHENEK M., SMORAG Z., MODLINSKI J.A., PTAK G., LOI P., 2013 – Genomic stability of lyophilized sheep somatic cells before and after nuclear transfer. ***PLoS One*** 8(1), e51317.
14. KANEKO T., WHITTINGHAM D.G., YANAGIMACHI R., 2003 – Effect of pH value of freeze-drying solution on the chromosome integrity and developmental ability of mouse spermatozoa. ***Biology of Reproduction*** 68(1), 136-139.
15. KESKINTEPE L., EROGLU A., 2012 – Preservation of Mammalian Sperm by Freeze-Drying. ***Methods in Molecular Biology*** 2180, 721-730.
16. KWON WS., RAHMAN M., RYU D.Y., PARK Y.J., PANG M.G., 2015 – Increased male fertility using fertility-related biomarkers. ***Scientific Reports*** 5, 15654.
17. LI M.W., WILLIS B.J., GRIFFEY S.M., SPEAROW J.L., LLOYD K.C., 2009 – Assessment of three generations of mice derived by ICSI using freeze-dried sperm. ***Zygote*** 17(3), 239-251.
18. LI Y.C., HU X.Q., ZHANG K.Y., GUO J., HU Z.Y., TAO S.X., XIAO L.J., WANG Q.Z., HAN C.S., LIU Y.X., 2006 – Afaf, a novel vesicle membrane protein, is related to acrosome formation in murine testis. ***FEBS Letters*** 580(17), 4266-4273.
19. MARA, L., CASU S., CARTA, A., DATTENA, M., 2013 – Cryobanking of farm animal gametes and embryos as a means of conserving livestock genetics. ***Animal Reproduction Science*** 138(1-2), 25-38.
20. MEN N.T., KIKUCHI K., NAKAI M., FUKUDA A., TANIHARA F., NOGUCHI J., KANEKO H., LINH N.V., NGUYEN B.X., NAGAI T., TAJIMA A., 2013 – Effect of trehalose on DNA integrity of freeze-dried boar sperm, fertilization, and embryo development after intracytoplasmic sperm injection. ***Theriogenology*** 80(9), 1033-1044.
21. MINAMI K., ARAI-ASO M.M., OGURA-KODAMA Y., YAMADA A., KISHIDA K., SAKASE M., FUKUSHIMA M., HARAYAMA H., 2020 – Characteristics of bull sperm acrosome associated 1 proteins. ***Animal Reproduction Science*** 218, 106479.
22. MUNETO T., HORIUCHI T., 2011 – Full-term development of hamster embryos produced by injecting freeze-dried spermatozoa into oocytes. ***Journal of Mammalian Ova Research*** 28(1), 32-39.
23. NAKAI M., KASHIWAZAKI N., TAKIZAWA A., MAEDOMARI N., OZAWA M., NOGUCHI J., KANEKO H., SHINO M., KIKUCHI K., 2007 – Effects of chelating agents during freeze-drying of boar spermatozoa on DNA fragmentation and on developmental ability *in vitro* and *in vivo* after intracytoplasmic sperm head injection. ***Zygote*** 15(1), 15-24.
24. OGURA Y., TAKAGISHI Y., HARAYAMA H., 2016 – Changes in the distribution and molecular mass of boar sperm acrosome-associated 1 proteins during the acrosome reaction; their validity as indicators for occurrence of the true acrosome reaction. ***Animal Reproduction Science*** 172, 94-104.
25. OLACIREGUI M., LUÑO V., GONZÁLEZ N., DOMINGO P., DE BLAS I., GIL L., 2017 – Chelating agents in combination with rosmarinic acid for boar sperm freeze-drying. ***Reproductive Biology*** 17(3), 193-198.
26. PALAZZESE L., GOSÁLVEZ J., ANZALONE D.A., LOI P., SARAGUSTY J., 2018 – DNA fragmentation in epididymal freeze-dried ram spermatozoa impairs embryo development. ***Journal of Reproduction and Development*** 64(5), 393-400.
27. PANG W.K., AMJAD S., RYU D.Y., ADEGOKE E.O., RAHMAN M.S., PARK Y.J., PANG M.G., 2022 – Establishment of a male fertility prediction model with sperm RNA markers in pigs as a translational animal model. ***Journal of Animal Science and Biotechnology*** 13(1), 84.
28. PANG W.K., KANG S., RYU D.Y., RAHMAN M.S., PARK Y.J., PANG M.G., 2020 – Optimization of sperm RNA processing for developmental research. ***Scientific Reports*** 10(1), 11606.
29. RAHMAN M.S., KWON W.S., PANG M.G., 2017 – Prediction of male fertility using capacitation-associated proteins in spermatozoa. ***Molecular Reproduction and Development*** 84(9), 749-759.

30. SUN T.C., WANG J.H., WANG X.X., LIU X.M., ZHANG C.L., HAO C.F., MA W.Z., DENG S.L., LIU Y.X., 2019 – Effects of sperm proteins on fertilization in the female reproductive tract. ***Frontiers in Bioscience*** 24(4), 735-749.
31. THIANGTHIENTHAM P., KALLAYANATHUM W., ANAKKUL N., SUWIMONTEERABUTR J., SANTIVIPARAT S., TECHAKUMPHU M. LOI P., THARASANIT T., 2023 – Effects of freeze-drying on the quality and fertilising ability of goat sperm recovered from different parts of the epididymis. ***Theriogenology*** 195, 31-39.
32. TRZCIŃSKA M., BRYŁA M., GAJDA B., GOGOL P., 2015 – Fertility of boar semen cryopreserved in extender supplemented with butylated hydroxytoluene. ***Theriogenology*** 83(3), 307-313.
33. TRZCIŃSKA M., BRYŁA M., 2020 – The role of the National Research Institute of Animal Production in the development of cryopreservation technology of livestock biological material and its use in *ex situ* conservation (In Polish). ***Roczniki Naukowe Zootechniki*** 47(1), 29-36.
34. TRZCIŃSKA M., SAMIEC M., 2021 – *Ex situ* conservation and genetic rescue of endangered Polish cattle and pig breeds with the aid of modern reproductive biotechnology. ***Annals of Animal Science*** 21(4), 1193-1207.