A NEW SPERM SELECTION CRITERION FOR CRYOPRESERVATION OF BOAR SEMEN*

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Abstract

This study aimed to define potential markers that could determine the suitability of ejaculate for cryopreservation. Fresh semen from eleven boars (4–7 ejaculates/boar), regardless of their sperm motility, was subjected to a cryopreservation procedure. The sperm quality before and after freezing was assessed based on the sperm membrane permeability and acrosome integrity. The results showed that it was possible to effectively cryopreserve ejaculates below the accepted standards of 70–80% of fresh motile sperm and still obtain a high cryosurvival rate. Moreover, a significant correlation was found between the percentage of viable sperm with apoptotic-like changes, viable sperm with reacted acrosomes, and the cryosurvival rate. The proposed markers for assessing the quality of fresh semen could be used to predict the success of cryopreservation procedures.

Key words: boar, semen, cryopreservation, selection criterion

The initial approaches to boar semen cryopreservation gave rise to two methods in the 1970s. In the first American method created by Pursel and Johnson (1975), glucose was added to the freezing extender. The second method, in which lactose was used as the main component in the freezing extender, was developed by Westendorf et al. (1975). Over the years, the experiments mainly involved the optimization of freezing protocols (Hammit and Martin, 1989; Berger and Fischerleitner, 1992; Holt et al., 2005; Yeste et al., 2014).

Boar sperm cryopreservation has not been integrated into the current porcine AI techniques at a rate similar to that observed in other species, such as cattle (Curry, 2000). This semen has not been used under production conditions as efficiently as liquid preserved semen, due to the high susceptibility of boar spermatozoa to damage during cryopreservation and the complicated process required for deep freez-

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ing. The processes of cooling, freezing, and thawing place physical and chemical stress on the sperm membrane. It has been reported that reactive oxygen species (ROS) generation, induced by the cryopreservation process, can damage boar sperm (Buranaamnuay et al., 2011). An important indicator of the membrane damage is a change in membrane permeability, such as increased permeability to stains and a release of intracellular substances. This could be caused by lipid peroxidation because of the relatively high content of unsaturated fatty acids in the phospholipids of the boar sperm membrane. Boar sperm membrane has a low cholesterol:phospholipid ratio and the cholesterol molecules are distributed asymmetrically, so that the outer monolayer contains more cholesterol than the inner layer. This specific composition of the plasma membrane explains the high sensitivity of boar sperm to cold shock (Johnson et al., 2000; Cerolini et al., 2001). The attack of free radicals on the unsaturated fatty acid rich lipids of the sperm cell membranes leads to an irreversible reduction in membrane fluidity and damage to the cell membrane related to ATPases, which are responsible for regulating the intracellular level of ions necessary to maintain normal sperm motility (Cerolini et al., 2001).

In breeding practices in Poland, cryopreserved boar semen is not used. Routine inseminations are done with liquid extended semen, briefly stored for up to several days. However, there are many reasons for introducing frozen semen into breeding practices. One of them is the possibility of postponing the reproductive period of the boar and using it at a later time, which can contribute to the reduction of inbreeding, especially in small native breed populations. A very important aspect of cryopreservation is the possibility of maintaining a genetic reserve, allowing for the reconstruction of breeding populations subjected to liquidation for epidemiological reasons. Sperm freezing technology also allows for the possibility of transporting sperm over considerable distances and thus using it to reproduce outstanding individuals in the country and abroad.

Therefore, it is understandable that intensive research has been ongoing for years to develop an effective method for freezing boar semen. In recent years, the National Research Institute of Animal Production has undertaken a series of actions to develop an effective method of freezing boar semen. The work focused on the preparation of the composition of the freezing extender as well as the development of effective cryopreservation technology. The result of the research was the patenting of the invention number PL 228192 entitled "Extender for boar semen cryopreservation and cryopreservation procedure." An antioxidant component, such as butylated hydroxytoluene (BHT), was used in the composition of the extender. BHT, a synthetic analogue of vitamin E that checks the autooxidation reactions by converting peroxy radicals into hydroxyperoxides, was used as a component of the patented freezing extender. Semen cryopreservation using this method provides good quality semen with high fertilizing capacity after freeze-thawing (Trzcińska et al., 2015).

A separate issue in cryopreservation is related to the use of appropriate markers to allow for the evaluation of fresh sperm that would give a simple answer as to whether the ejaculate is suitable for freezing or not. Scientists have already tried to define markers that would predict the suitability of ejaculates for cryopreservation.

Thurston et al. (2002) compared Landrace boars classified as bad and good freezers through Amplified Fragment Length Polymorphisms (AFLPs). The authors found a relationship between the AFLPs profiles (genetic variation) and ejaculate freezability. However, it is not known by which mechanism genetic variation underlies differences in ejaculate freezability.

Particular attention should be paid to the fact that ejaculates are only eligible for cryopreservation when fresh semen has a minimum 70% of motile sperm. There is also individual variability in sperm freezing among boars and among ejaculates from the same boar. For this reason, boars and their ejaculates are classified as good freezers or bad freezers (Hernandez et al., 2007) and as good freezability ejaculates or poor freezability ejaculates (Yeste et al., 2013). However, to determine the freezability, it is necessary to do a time-consuming cryopreservation procedure. Accordingly, searching for markers that allow for the evaluation of fresh sperm quality to assess if the ejaculate is useful for cryopreservation is still an unsolved problem.

The aim of this study was to identify potential markers that could predict the suitability of ejaculate for cryopreservation. In the current research, all ejaculates were used for cryopreservation regardless of sperm motility. To verify the sperm quality before and after freezing, fluorescence methods were used. Sperm membrane permeability and acrosome integrity were used as potential markers for prediction of the sperm cryosurvival rate.

Material and methods

Animals

Sixty ejaculates of eleven Polish Landrace and Polish Large White boars (4–7 ejaculates/boar) aged 2 to 3 years were used in this study. The boars were kept at the Boar AI Stations in Czermin and Wet-Rol in Górká Stagniowska. All boars were housed in buildings with stable conditions of controlled temperature and humidity and were fed an adjusted commercial diet.

The procedures involving the animals were approved by the Local Ethics Board for Animal Experiments in Kraków (Poland), decision no. 126/2018.

Semen collection and cryopreservation

The sperm-rich fraction was collected by hand manipulation into water-jacketed vessels. Immediately after collection, the semen was diluted (1:1) in Biosolwens Plus extender (Biochefa, Sosnowiec, Poland). Spermatozoa were cryopreserved using the patented method (no. PL 228192). The diluted semen was transferred to 50 mL centrifuge tubes, equilibrated at 15°C for 60–80 minutes, and centrifuged at 800 g for 25 minutes. The supernatant was discarded and the sperm pellet was resuspended in LEY extender (80 mL of 11% lactose solution and 20 mL of egg yolk) at a concentration of 1.5×10^9 spermatozoa/mL. The diluted semen was cooled to 5°C for 120 minutes. Two parts of semen were mixed with one part of LEYG extender (89.5% LEY extender 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 the semen was 1.0×10^9 spermatozoa/mL, 3% glycerol and 1.0 mM butylated hydroxytoluene. 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 vapour for 15 minutes in a polystyrene box. After that, the straws were plunged into liquid nitrogen (-196°C) for storage.

Evaluation of semen quality

The assessment of sperm motility, viability and acrosome integrity was verified in both fresh semen (after dilution with Biosolwens Plus (1:1) and equilibration at 15°C for 60–80 minutes) and frozen-thawed semen.

Fluorescence analyses were performed using a Nikon Eclipse E600 compound microscope (Nikon Corp., Tokyo, Japan) equipped with PlanFluor 40 $\times$ /0.75 DIC M objective lenses (Nikon).

Sperm motility

The frozen semen was thawed in a water bath at 37°C for 40 seconds. Postthaw sperm motility was measured using computer-assisted sperm analysis (Sperm Class Analyzer, S.C.A. V5.1, Microptic, Barcelona, Spain). Before examination, a drop of semen from the thawed package was reextended (1:20) in BP extender to give a concentration of 50 to 60×10^6 spermatozoa/mL and the FT semen was incubated (38°C) for 20 minutes before 5 mL of semen was placed in a Leja counting chamber (Leja Products B.V., GNNieuw-Vennep, The Netherlands) on a heated stage (38°C). Each sample was measured twice, three fields were evaluated and at least 1000 cells per analysis were counted. Motility patterns including total sperm motility (TM%) and progressive sperm motility (PM%) were measured.

Sperm viability

The Vybrant Apoptosis Assay Kit #4 (Molecular Probes Inc., Eugene, USA) was used to detect the changes in plasma membrane permeability to YO-PRO-1 (Trzcińska et al., 2008). A total of 2×10^6 thawed sperm cells were diluted in 1 mL PBS (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 1 mL of YO-PRO-1 (100 mmol/L) was added. The tubes were gently mixed and incubated for 20 minutes at room temperature, and 2 mmol/L propidium iodide (PI) was added to each tube. After the incubation period, at least 200 spermatozoa per sample were evaluated using the appropriate filters for red (PI) and green (YO-PRO-1) fluorescence detection. The fluorescent staining was measured by microscopic observation of at least 200 cells per sample per slide in one field by one observer. The results are presented as the percentage of viable spermatozoa (YO-PRO-1/PI⁻), viable spermatozoa with apoptotic-like changes (YO-PRO-1⁺/PI⁻), and nonviable spermatozoa (YO-PRO-1⁺/PI⁺).

Acrosomal integrity

Acrosome status was assessed by fluorescein isothiocyanate conjugated with peanut agglutinin (FITC-PNA) and PI staining as described previously by Trzcińska

et al. (2015). Briefly, 100 mL of thawed spermatozoa (30×10^6 cells/mL in HEPES buffer) was diluted with 10 mL of FITC-PNA solution (1 mg/mL in double-distilled water) and 5 mL of PI solution. Samples were incubated at room temperature in the dark for 5 minutes before fluorescence analysis. The fluorescent staining was measured by microscopic observation of at least 200 cells per sample per slide in one field by one observer. The spermatozoa were classified as viable with an intact acrosome (PNA-/PI-), viable with a reacted acrosome (PNA+/PI-), or nonviable sperm (PNA+/PI+) (Trzcińska et al., 2015).

Cryosurvival rate of ejaculates

The cryosurvival rate was measured based on TM% after freeze-thawing/TM% of fresh semen $\times 100$.

Statistical analysis

The results are expressed as the means ($\pm$ SD) and the data were analysed using the Statistica 6.0 program (StatSoft, Tulsa, OK, USA). Features that had not been characterized by a normal distribution were subjected to logarithmic transformation and subsequent one-way analysis of variance (ANOVA). Afterwards, the log-transformed data were analysed by using *post hoc* Duncan's test. Sperm quality parameters were correlated with the cryosurvival rate using the Pearson correlation ($P < 0.0001$).

Results

The quality parameters of fresh semen

After assessing the fresh sperm motility, the boars were divided into two groups. The first group (Group I) included boars whose ejaculates showed over 70% progressive motility (PM%). The second group (Group II) included boars with a PM below 70%. Parameters evaluated in the fresh semen samples from all ejaculates of the 11 boars are presented in Table 1 and Table 2. In Group I, no significant differences were observed among the boars for sperm motility (TM% and PM%) as well as for the percentage of all sperm subpopulations determined by the PNA-FITC/PI fluorescent assay. Significant differences ($P \leq 0.01$) were observed in all subpopulations assessed by the Vybrant Apoptosis Assay Kit. The highest percentage of viable spermatozoa ($81.1 \pm 1.8\%$) was found for boar no. 5. For boar no. 3, the lowest percentage of viable spermatozoa with apoptotic-like changes and viable spermatozoa with damaged acrosomes was observed ($2.7 \pm 0.9\%$ and $4.5 \pm 1.1\%$, respectively).

In Group II, significant differences ($P < 0.01$) between boars were found for all sperm quality parameters (Table 2). Among all boars from group II, ejaculates from boar no. 6 were characterized by the lowest PM% ($52.1 \pm 4.0\%$). In contrast, the highest percentage of viable spermatozoa with apoptotic-like changes was found from boar no. 2 ($24.5 \pm 4.7\%$). In this case, the lowest percentage of viable spermatozoa with an integral acrosome ($49.8 \pm 2.4\%$) was noted.

Table 1. Quality parameters of fresh boar semen (Group I) with sperm progressive motility >70%

No. of boar (no. of ejaculates)	Motility		Viability			Acrosomal Integrity		
	progressive motility PM %	total motility %	viable sperm YO-PRO-1-/PI- %	viable sperm with apoptotic-like changes YO-PRO-1-/PI- %	nonviable sperm YO-PRO-1-/PI+ %	viable with acrosome integrity PNA-/PI- %	viable with re- acted acrosome PNA+/PI- %	nonviable sperm PNA-/PI+ %
1 (n=6)	82.2±2.4	87.5±2.5	80.7±2.1 AB	4.1±1.1 AB	15.2±2.0 A	81.2±2.9	6.0±1.4	12.7±2.7
2 (n=7)	81.7±2.0	86.5±3.1	80.0±2.2 AB	4.6±1.1 A	15.4±1.9 A	81.0±2.1	6.6±2.1	12.4±2.1
3 (n=6)	80.5±5.3	84.9±4.4	78.8±5.2 AB	2.7±0.9 B	18.5±4.6 AB	78.0±5.3	4.5±1.1	17.5±5.3
4 (n=6)	80.2±1.7	88.1±4.1	77.2±2.0 A	3.1±1.5 AB	19.7±2.7 B	78.8±2.4	5.3±1.3	15.8±3.1
5 (n=5)	82.3±2.4	87.0±2.5	81.1±1.8 B	3.0±1.9 AB	15.9±1.7 AB	80.7±1.2	4.9±1.4	14.4±1.5
Mean (30)	81.4±2.9	86.8±3.4	79.5±3.1	3.5±1.4	16.9±3.2	80.0±3.2	5.5±1.6	14.5±3.5

A, B – Values in columns with different letters are statistically different (P≤0.01).

Table 2. Quality parameters of fresh boar semen (Group II) with sperm progressive motility < 70%

No. boar (no. of ejaculates)	Motility		Viability			Acrosomal Integrity		
	progressive motility PM %	total motility TM %	viable sperm YO-PRO-1 ⁺ /PI ⁻ %	viable sperm with apoptotic-like changes YO-PRO-1 ⁺ /PI ⁻ %	nonviable sperm YO-PRO-1 ⁻ /PI ⁺ %	viable with acrosome integrity PNA ⁺ /PI ⁻ %	viable with reacted acrosome PNA ⁺ /PI ⁻ %	nonviable sperm PNA ⁺ /PI ⁺ %
1 (n=6)	61.9±2.8 A	68.1±2.6 A	62.4±3.0 A	4.2±2.1 A	33.4±3.9 A	60.8±2.2 A	5.9±2.1 A	33.2±3.9 A
2 (n=5)	57.8±2.7 AB	63.8±2.5 AB	50.9±1.9 B	24.5±4.7 B	24.6±5.0 BC	49.8±2.4 BC	23.2±4.8 B	27.0±4.8 AC
3 (n=5)	60.4±1.5 A	63.9±2.3 AB	63.0±2.9 A	4.1±1.3 A	32.9±3.0 AC	62.7±2.6 A	5.3±2.4 AC	32.0±3.1 A
4 (n=4)	59.0±0.9 AB	61.3±1.4 B	58.0±2.5 AC	3.9±0.6 A	38.1±2.6 AE	60.4±1.4 AD	4.9±1.3 AC	34.7±1.9 AD
5 (n=5)	56.8±2.9 AB	64.1±1.0 A	53.4±4.6 BC	23.7±3.4 B	22.9±5.4 B	55.5±5.0 ABCD	24.2±3.8 B	20.3±4.5 BC
6 (n=5)	52.1±4.0 B	58.6±3.1 B	51.6±4.0 BC	3.7±1.2 A	44.7±3.8 DE	52.9±4.5 BD	4.5±3.7 AC	42.6±5.2 D
mean (30)	58.1±4.1	63.5±3.7	56.7±5.9	10.7±9.9	32.6±8.4	57.0±5.6	11.4±9.3	31.6±7.9

A, B, C, D, E – values in columns with different letters are statistically different ($P \leq 0.01$).

Table 3. Quality parameters of frozen-thawed sperm from Group I with fresh sperm progressive motility >70%

No. boar (no. of ejaculates)	Motility		Viability			Acrosomal Integrity			Cryosurvival rate
	progressive motility PM %	total motility TM %	viable sperm YO-PRO-1+/PI- %	viable sperm with apoptotic-like changes YO-PRO-1+/PI- %	nonviable sperm YO-PRO-1+/PI+ %	viable with acrosome integrity PNA+/PI- %	viable with reacted acrosome PNA+/PI- %	nNonviable sperm PNA+/PI+ %	
1 (n=6)	73.7±1.7	77.6±1.7	74.9±2.4 A	8.2±1.6 A	16.8±2.6 A	72.4±2.3	10.6±1.3 A	17.0±3.3	88.8±3.0
2 (n=7)	71.4±3.0	76.7±2.6	70.2±3.9 AB	8.8±1.7 A	20.9±5.0 AB	70.3±3.0	10.1±2.5 AB	19.5±3.9	88.8±4.5
3 (n=6)	73.0±4.4	77.7±4.0	71.8±3.1 AB	5.0±0.9 B	23.2±3.3 B	71.2±5.0	7.9±1.0 B	20.8±5.2	91.6±2.7
4 (n=6)	72.5±1.9	76.6±1.5	68.5±3.0 B	7.5±.5 AB	24.0±3.0 B	71.0±3.1	11.1±2.9 AB	18.0±1.3	87.1±4.6
5 (n=5)	72.2±3.2	75.0±4.3	71.0±2.0 AB	8.9±1.9 A	20.1±2.3 AB	70.6±2.9	9.7±4.0 AB	19.7±3.5	86.2±3.7
Mean (30)	72.5±2.9	76.8±2.9	71.3±3.5	7.5±2.1	21.2±4.0	71.1±3.5	9.9±2.6	19.0±4.0	88.6±4.0

A, B – values in columns with different letters are statistically different (P≤0.01).

Table 4. Quality parameters of frozen-thawed sperm from Group II with fresh sperm progressive motility < 70%

No. of boar (no. of ejaculates)	Motility		Viability			Acrosomal Integrity			Cryosurvival rate
	progressive motility PM %	total motility TM %	viable sperm YO-PRO-1 ⁺ -PI ⁻ %	viable sperm with apoptotic-like changes YO-PRO-1 ⁺ /PI ⁻ %	nonviable sperm YO-PRO-1 ⁺ /PI ⁺ %	viable with acrosome integrity PNA ⁺ -PI ⁻ %	viable with reacted acrosome PNA ⁺ /PI ⁻ %	nonviable sperm PNA ⁺ /PI ⁺ %	
1 (n=6)	52.5±2.3 A	58.3±4.2 A	52.7±2.6 A	8.3±3.0 A	38.9±5.1 A	51.0±3.4 A	11.1±3.4 A	37.9±6.2 A	85.7±6.8 AC
2 (n=5)	31.9±6.4 BC	36.0±5.0 B	31.2±4.3 B	33.2±3.6 B	35.6±7.5 A	32.5±6.3 B	35.3±3.1 B	32.2±7.5 A	56.4±7.4 B
3 (n=5)	51.0±3.4 A	55.2±2.2 A	50.9±2.7 A	7.2±1.7 A	41.9±3.7 AC	49.8±3.8 A	9.1±2.2 A	41.1±5.0 AB	86.6±5.8 A
4 (n=4)	49.6±4.6 AD	52.3±4.5 AD	48.0±5.7 AC	6.7±1.7 A	45.2±5.8 AC	49.0±4.0 AC	9.5±2.3 A	41.5±6.0 AB	85.4±8.5 AC
5 (n=5)	32.0±4.0 CE	35.7±2.8 B	33.8±4.0 B	35.4±6.9 B	30.8±9.2 A	32.5±3.9 B	38.4±6.3 B	29.1±8.4 A	55.7±3.6 B
6 (n=5)	41.2±4.0 BD	45.0±2.7 CD	39.9±6.0 BC	6.7±1.2 A	53.4±6.9 BC	40.8±4.5 BC	9.2±0.9 A	50.0±4.9 B	76.7±1.1 C
Mean (30)	43.1±9.6	47.3±9.8	42.9±9.4	16.3±13.4	40.8±9.4	42.7±9.0	18.8±13.4	38.5±9.0	74.4±14.7

A, B, C—values in columns with different letters are statistically different ($P \leq 0.01$).

The quality parameters of frozen-thawed (FT) semen

The results regarding the quality parameters of frozen-thawed sperm are summarized in Table 3 and Table 4. There were no differences in the percentage of motile sperm (PM%, TM%), viable sperm with an integral acrosome and nonviable sperm among all analysed boars from Group I (Table 3). Progressive motility of the spermatozoa after freezing ranged from $71.4 \pm 3.0\%$ to $73.7 \pm 1.7\%$. Moreover, the cryosurvival rate of all ejaculates from these boars was over 80%. After freezing-thawing, the percentage of viable sperm with apoptotic-like changes did not exceed 9% and the lowest percentage was observed in ejaculates from boar no. 3.

The lowest percentage of motile sperm (TM%, PM%) and viable spermatozoa was from boar no. 2 and boar no. 5 from Group II (Table 4). Simultaneously, ejaculates of these boars had the highest percentages of viable sperm with apoptotic-like changes and viable sperm with reacted acrosomes. Moreover, the cryosurvival rate of their ejaculates was low and amounted to 56.4 ± 7.4 and 55.7 ± 3.6 for boar no. 2 and boar no. 5, respectively.

Correlation coefficient

Only in fresh semen from Group II with a PM below 70% was the percentage of live sperm with apoptotic-like changes ($r = -0.85781$), and the percentage of live spermatozoa with damaged acrosomes ($r = -0.82554$) highly negatively correlated ($P < 0.0001$) with the cryosurvival rate.

Discussion

The basic criterion for the selection of ejaculates for cryopreservation is the assessment of fresh sperm motility, which is the most important indicator of ejaculate quality. In addition, attempts are being made to determine markers of suitability for freezing ejaculates. Seminal plasma proteins and some other compounds have been used as markers of fertility in bulls (Moura et al., 2006) and semen freezability in rams (Zalazar et al., 2016) and men (Zhang and Xiong, 2013). In the case of boar semen, the protein HSP90AA1 was proposed as a molecular marker of boar sperm resistance to cold shock. Low expression of that protein was associated with high sensitivity to cold shock (Casas et al., 2010). In turn, Vilagran et al. (2014) found that the voltage-dependent anion channel 2 (VDAC2) may be used to predict the freezability of boar ejaculate before cryopreservation procedures, because the amounts of this protein in extended fresh semen differ between good and poor freezability ejaculates. It has been suggested that this protein is involved in the cryotolerance of boar semen. However, it should be noted that only ejaculates with a minimal quality standard of 80% of total motile spermatozoa and 80% viable spermatozoa were used in these experiments. The proposed markers were used to try to explain the differences in freezability among boars, but they were not used for routine assessment of the suitability of the ejaculate for freezing.

Our present study aimed to determine other criteria for selecting ejaculates for effective cryopreservation. The main difference from other authors searching for

cryotolerance markers was that in our experiment, ejaculates that met the standard guidelines for sperm motility above 70% and ejaculates that did not meet this criterion were both used in the study. All ejaculates were intended for cryopreservation and the quality of the fresh and cryopreserved semen was assessed on the basis of sperm membrane integrity and acrosome integrity.

The stability and permeability of the sperm membrane are closely related to its functionality, and thus evaluation of membrane permeability elucidates its organization and its capability to undergo biophysical changes such as water crystallizing and the movement of cryoprotectant agents during cryopreservation (Hossain et al., 2011). YO-PRO-1 is an impermeable membrane probe that can leak in after destabilization of the membrane and it is used for detecting early membrane permeability (Trzecińska and Bryła, 2015). The structure of the sperm cell membrane and the processes involved in the acrosome reaction are significant for fertilization. Acrosomal integrity is a requirement for sperm penetration of the zona pellucida (Hossain et al., 2011). In our research, the detection of changes in acrosome integrity used PNA-FITC labelling, which binds to the outer acrosomal membrane, and it can be used to monitor acrosome integrity (Petrunkina et al., 2005).

The results of the present study showed that it is possible to effectively cryopreserve ejaculates with fresh sperm motility below the accepted standard of 70%. Therefore, the initial selection of ejaculates should not be based only on sperm motility results. We showed that fresh semen with a sperm motility below 70% can be successfully cryopreserved according to a method patented by us and a high cryosurvival rate can be obtained. In the group of boars with a low percentage of viable sperm with apoptotic-like changes and a low percentage of viable spermatozoa with damaged acrosomes, a high cryosurvival rate was noted. Only fresh semen from two boars with a very high percentage of these sperm subpopulations (>20%) had cryosurvival rates below 60%. Moreover, in the statistical analysis, a high correlation was found between the percentage of viable sperm with apoptotic-like changes and viable sperm with reacted acrosomes in fresh semen and the cryosurvival rate, but only in Group II with a PM below 70%.

Previous research by Flores et al. (2009) showed that an analysis of motile sperm subpopulations with functional tests could improve the design of predictive strategies for the freezability of boar sperm. Guimarães et al. (2017) noted that the sperm motility of fresh boar semen subjected to cryopreservation procedures showed similar values among ejaculates, but they were different after thawing. These results, along with the findings of the current study, show that ejaculates should not be qualified for cryopreservation only on the basis of fresh sperm motility. In the present study, additional tests of fresh semen provided better information about the freezing efficiency of ejaculates.

Conclusions

Overall, the results of our study confirmed that predicting the suitability of boar semen for cryopreservation based only on sperm motility seems to be insufficient, especially taking into consideration inter-specimen variability in this mammalian species. Therefore, this indicates the necessity of applying other methods of semen

evaluation, including the fluorescent approaches used in the current study to assess the spermatozoon plasmalemma and acrosome integrities. Moreover, the applied approaches allow for rapid detection of the ultrastructural and functional changes within boar sperm cells, which, in turn, enables the prediction of successful semen freezing. Finally, this is of great importance for not only experimental and applied spermatology research, but also pig breeding practices in Poland.

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