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Original article

Effect of boar ejaculate fraction, extender type and time of storage on quality of spermatozoa

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Abstract

The aim of this study was to investigate the effect the sperm-rich fraction (F1) and the post-F1 fraction (F2) on the quality of boar spermatozoa stored in a liquid state. Ejaculates were collected from three Polish Landrace boars. Each ejaculate fraction was diluted with BTS short-term extender and Safe-Cell Plus (SCP) long-term extender and stored for seven days (D1-D7) at 17°C. Analyses included sperm motility parameters, normal apical ridge (NAR) acrosomes and plasma membrane integrity (PMI). Prior to the dilution of fractions, marked changes ($p<0.05$) were noted between F1 and F2 in progressive motility (PMOT), velocity average pathway (VAP) and velocity straight line (VCL). After the ejaculate was diluted, the type of fraction and type of extender significantly affected ($p<0.05$) PMOT, being markedly higher ($p<0.05$) for F1 extended in BTS. No marked changes ($p<0.05$) were observed between F1 and F2 extended in SCP for any of the analyzed sperm quality parameters during seven days of storage. Significantly higher ($p<0.05$) values of sperm quality parameters were noted in F1 compared with F2 for BTS on D7 of storage. The results of the four-way ANOVA analysis indicate that boar, fraction of ejaculate, extender type and day of storage had significant effects on the quality of boar stored spermatozoa. The F1 was characterised by higher quality of spermatozoa during storage in comparison with F2 in the short-term extender. Using the long-term extender containing the proteins allowed for a better application of F2, which could be important for the pig industry.

Key words: boar sperm, fractions, extender, quality, protein

Introduction

The quality of stored spermatozoa is determined either by various factors, including the type of extender, temperature and period of time or by an individual one (Johnson et al. 2000, Thurston et al. 2001, 2002). Furthermore, the ejaculate fraction can also significantly influence the quality of cryopreserved sperm (Peña et al. 2006, Saravia et al. 2009, Siqueira et al. 2011). The influence of ejaculate fraction on the quality of liquid-stored spermatozoa has been investigated in only a few studies (García et al. 2009). Boar ejaculates contain three fractions which are easily identified based on discharge sequence and density. The first fraction, termed the pre-sperm fraction, is water without spermatozoa. The second fraction,

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referred to as the sperm-rich fraction (SRF; about 30-50 mL), is dense and contains the most spermatozoa, particularly at the beginning, and the sperm-peak portion (P1; 10-15 mL) has a high content of cauda epididymal fluid (Rodríguez-Martínez et al. 2009). The last fraction, known as the post-sperm-rich fraction (PSRF; about 120-150 mL), is characterised by significantly fewer sperm cells and a predominance of vesicular gland secretions, prostate and, by the end of ejaculation, bulbourethral glands (Mann and Lutwak-Mann 1981, Rodríguez-Martínez et al. 2009). For this reason the influence of a given ejaculate fraction on semen quality is attributed mainly to variations in the quality of seminal plasma (SP). SP contains various organic and inorganic substances, including proteins which influence the functions and fertilizing ability of spermatozoa (Mann and Lutwak-Mann 1981, Rodríguez-Martínez et al. 2005, Strzeżek et al. 2005). Sperm can undergo structural and functional changes during storage (Johnson et al. 2000, Gączarzewicz et al. 2010, 2015). SP proteins are capable of protecting spermatozoa against cold shock (Watson 2000) and destabilizations of the plasma membrane (Maxwell and Johnson 1999) or oxidative stress (Roca et al. 2005).

Previous studies have shown that SRF has better quality sperm when compared to PRSF, which was due to the quality of the SP in it (Garcia et al. 2009, Siqueira et al. 2011). During dilution of semen with appropriate extenders the influence of SP on the stored spermatozoa may be significantly limited. The presence of various components in extenders and SP of different ejaculate fractions and the interactions between these components could determine sperm survival during storage in a liquid state. We assumed that the type of extender could affect the quality of sperm from different fractions of the ejaculate, especially from PSRF. PSRF represents a significant volume of full boar ejaculate used mainly for commercial purposes in preserving semen in a liquid state.

In view of previous research findings, the aim of this study was to evaluate the influence of different ejaculate fractions on the quality of sperm stored in short-term and long-term extenders. Spermatozoa were subjected to qualitative analyses to determine their parameters of motility, acrosome integrity and plasma membrane integrity.

Materials and Methods

Animals and semen collections

Ejaculates were collected once weekly from three sexually mature Polish Landrace boars (average age of 2 years) using the gloved hand technique. The animals were kept at the laboratory of the Department of Animal Biochemistry and Biotechnology, Kortowo-Olsztyn (N: 53° 44' 35", E: 20° 27' 03"). A total of fifteen ejaculates were collected from the boars from September to December. During collection, semen was separated into fractions, and the pre-sperm-rich fraction was removed. Pre-warmed grader cylinders were used to collect the following fractions of ejaculate: fraction 1 contained the sperm-rich fraction (F1, 20 ml) and fraction 2 contained the rest of SRF and post-sperm-rich fraction (F2, 100 ml). Fractions F1 and F2 were used in analyses.

The boars were provided with unlimited access to water and were fed in accordance with standard housing protocols. Animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee.

Semen processing and preservation

Semen samples were subjected to macroscopic and microscopic analyses. Only semen samples characterised by more than 80% total sperm motility (CASA system) and less than 15% of abnormal spermatozoa were used in this study. Sperm concentration was determined using a Bürker counting chamber (Equimed-Medical Instruments, Kraków, Poland). The F1 and F2 of ejaculate were diluted in two commercial extenders: short-term BTS (Minitub, Tiefenbach, Germany) and long-term Safe Cell Plus (SCP, IMV Technologies, France) and stored for seven days at 17°C in the Thermobox (Minitüb GmbH, Tiefenbach, Germany). Analyses of sperm quality (parameters of motility, normal apical ridge acrosomes and plasma membrane integrity) were performed on the first day of storage (D1) and on days 3, 5 and 7 of storage (D3, D5 and D7).

Analyses of sperm quality

Sperm motility parameters were analysed using the CASA system (Hamilton-Thorne research, HTR, IVOS version 12.3; Beverley, MA, USA). Aliquots of semen samples (5 µl) were placed in a pre-warmed Makler counting chamber and examined at 37°C. A minimum of five fields per sample were assessed. The CASA system analyzed total motile spermatozoa (TMOT, %), progressive motile spermatozoa (PMOT, %), velocity average path (VAP, µm/s), velocity straight line (VSL, µm/s), curvilinear velocity (VCL, µm/s), mean amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF,

Table 1. Influence of ejaculate fraction on quality parameters of boar spermatozoa prior dilution of semen.

Parameters	Fraction of ejaculate	
	F1	F2
TMOT (%)	85.40 ± 1.10	81.33 ± 1.94
PMOT (%)	58.80 ± 2.76^{a}	41.67 ± 2.62^{b}
VAP (µm/s)	75.11 ± 2.85^{a}	62.39 ± 2.90^{b}
VSL (µm/s)	55.91 ± 1.98	48.53 ± 2.04
VCL (µm/s)	130.51 ± 6.85^{a}	108.57 ± 6.35^{b}
ALH (µm/s)	6.23 ± 0.21	6.35 ± 0.16
BCF (Hz)	33.25 ± 0.97	33.20 ± 0.79
STR (%)	74.01 ± 2.12	75.80 ± 1.13
LIN (%)	45.13 ± 2.11	45.27 ± 1.09
NAR (%)	96.93 ± 1.5	396.20 ± 1.04
PMI (%)	86.29 ± 1.70	82.86 ± 2.20

F1 – sperm-rich fraction, F2 – post-F1 fraction; TMOT – total motile spermatozoa, PMOT – progressive motile spermatozoa, VAP – velocity average path, VSL – velocity straight line, VCL – curvilinear velocity, ALH – mean amplitude of lateral head displacement, BCF – beat cross frequency, STR – straightness (ratio of VSL/VAP x 100%), LIN – linearity (ratio of VSL/VCL x 100%), NAR – normal apical ridge acrosomes, PMI – plasma membrane integrity. Values represent the mean (± SEM) of 15 ejaculates from 3 boars. Values with different letters (a, b) in the same row differ between F1 and F2 at $p<0.05$.

Table 2. Influence of ejaculate fraction and extender on quality parameters of boar spermatozoa on the first day (D1) of storage.

Parameters	BTS		SCP	
	F1	F2	F1	F2
TMOT (%)	86.47 ± 0.99	82.27 ± 1.07	81.47 ± 1.31	83.00 ± 1.22
PMOT (%)	57.47 ± 2.77^{ax}	42.53 ± 2.42^{bx}	43.27 ± 1.58^{by}	45.50 ± 1.38^{bx}
VAP (µm/s)	72.27 ± 2.94	63.73 ± 2.44	62.87 ± 1.88	62.09 ± 1.74
VSL (µm/s)	54.99 ± 1.98	48.67 ± 2.11	43.19 ± 0.99	44.95 ± 1.40
VCL (µm/s)	122.11 ± 6.42	110.67 ± 4.51	116.23 ± 4.11	110.46 ± 2.91
ALH (µm/s)	5.91 ± 0.21	6.27 ± 0.17	6.63 ± 0.18	6.37 ± 0.18
BCF (Hz)	32.38 ± 0.86	33.48 ± 0.69	32.14 ± 0.53	32.16 ± 0.59
STR (%)	75.53 ± 1.81	75.13 ± 1.55	67.73 ± 1.26	71.07 ± 0.82
LIN (%)	47.07 ± 1.88^{ax}	45.13 ± 1.94^{ax}	38.73 ± 1.13^{ay}	42.07 ± 0.92^{ax}
NAR (%)	94.20 ± 2.12	90.27 ± 2.1	894.80 ± 1.74	94.20 ± 1.43
PMI (%)	85.39 ± 1.39	82.08 ± 1.91	90.02 ± 2.48	89.28 ± 1.96

F1 – sperm-rich fraction, F2 – post-F1; TMOT – total motile spermatozoa, PMOT – progressive motile spermatozoa, VAP – velocity average path, VSL – velocity straight line, VCL – curvilinear velocity, ALH – mean amplitude of lateral head displacement, BCF – beat cross frequency, STR – straightness (ratio of VSL/VAP x 100%), LIN – linearity (ratio of VSL/VCL x 100%), NAR – normal apical ridge acrosomes, PMI – plasma membrane integrity. Values represent the mean (± SEM) of 15 ejaculates from 3 boars. Values with different letters (a, b) in the same row differ between F1 and F2 at $p<0.05$ for the same extender. Values with different letters (x, y) in the same row differ between BTS and SCP at $p<0.05$ for the same fraction.

Hz), straightness (STR, ratio of VSL/VAP x 100%) and linearity (LIN, ratio of VSL/VCL x 100%).

The percentage of spermatozoa with normal apical ridge (NAR) was assessed using the Giemsa staining method described by Watson (1975), with some modifications (Fraser et al. 2007). A minimum of 200 sperm cells per slide were examined at 1000 x magnification under a bright-field microscope (Olympus CH 30) and classified as spermatozoa with NAR (acrosome-intact) or damaged apical ridge acrosome.

The percentage of spermatozoa with an intact membrane (PMI) was assessed by dual fluorescent staining with SYBR-14 and PI (Live/Dead Sperm Viability Kit, Molecular Probes, Eugenie, OR, USA) according to the method described by Garner and Johnson (1995). Approximately 200 sperm cells per slide were examined at 600 x magnification under an epifluorescence microscope (Olympus CH 30, RF-200, Tokyo, Japan). Spermatozoa exhibiting green fluorescence were classified as viable cells with an intact membrane.

Statistical analysis

The one-way ANOVA assumption was evaluated using the Shapiro Wilk W test for normality for all data distribution. The data were examined by repeat-

Table 3. Influence of ejaculate fraction and extender on quality parameters of boar spermatozoa on the fifth day (D5) of storage.

Parameters	BTS		SCP	
	F1	F2	F1	F2
TMOT (%)	71.46 ± 4.24	61.93 ± 6.43	75.78 ± 2.72	73.64 ± 3.52
PMOT (%)	46.47 ± 3.59^{ax}	30.53 ± 3.58^{bx}	38.36 ± 1.94^{by}	36.85 ± 3.07^{bx}
VAP (µm/s)	76.61 ± 4.78	59.77 ± 3.10	71.30 ± 3.77	60.67 ± 3.37
VSL (µm/s)	53.27 ± 3.38	43.67 ± 2.38	40.66 ± 1.71	40.31 ± 2.06
VCL (µm/s)	142.64 ± 8.87^{ax}	104.05 ± 8.71^{bx}	138.54 ± 7.12^{ax}	115.26 ± 6.75^{ay}
ALH (µm/s)	6.78 ± 0.31^{ax}	6.15 ± 0.27^{ax}	8.44 ± 0.19^{ay}	7.59 ± 0.32^{ax}
BCF (Hz)	30.81 ± 1.01	31.95 ± 1.38	28.69 ± 0.94	30.66 ± 0.94
STR (%)	68.93 ± 2.52	72.13 ± 1.64	61.50 ± 1.78	66.43 ± 2.24
LIN (%)	39.67 ± 2.52	41.73 ± 1.80	32.78 ± 1.13	37.00 ± 2.17
NAR (%)	71.13 ± 2.77^{ax}	64.80 ± 3.91^{ax}	83.38 ± 2.06^{ay}	77.64 ± 2.83^{ay}
PMI (%)	81.34 ± 2.22	76.16 ± 3.95	81.88 ± 1.68	74.98 ± 5.44

F1 – sperm-rich fraction, F2 – post-F1; TMOT – total motile spermatozoa, PMOT – progressive motile spermatozoa, VAP – velocity average path, VSL – velocity straight line, VCL – curvilinear velocity, ALH – mean amplitude of lateral head displacement, BCF – beat cross frequency, STR – straightness (ratio of VSL/VAP x 100%), LIN – linearity (ratio of VSL/VCL x 100%), NAR – normal apical ridge acrosomes, PMI – plasma membrane integrity. Values represent the mean (± SEM) of 15 ejaculates from 3 boars. Values with different letters (a, b) in the same row differ between F1 and F2 at $p<0.05$ for the same extender. Values with different letters (x, y) in the same row differ between BTS and SCP at $p<0.05$ for the same fraction.

Table 4. Influence of ejaculate fraction and extender on quality parameters of boar spermatozoa on the seventh day (D7) of storage.

Parameters	BTS		SCP	
	F1	F2	F1	F2
TMOT (%)	44.85 ± 8.11^{ax}	32.33 ± 7.54^{ax}	78.06 ± 2.16 ^{ay}	77.40 ± 2.71^{ay}
PMOT (%)	31.78 ± 5.94^{ax}	15.80 ± 3.88^{bx}	38.93 ± 2.95^{ax}	34.27 ± 3.02^{ay}
VAP (µm/s)	70.08 ± 7.30^{ax}	49.35 ± 6.29^{bx}	82.67 ± 4.76^{ax}	69.83 ± 3.56^{ay}
VSL (µm/s)	54.05 ± 5.73^{ax}	33.68 ± 4.52^{bx}	51.55 ± 2.51^{ax}	45.38 ± 2.42^{ax}
VCL (µm/s)	118.64 ± 11.81^{ax}	97.74 ± 12.09^{ax}	155.84 ± 9.35^{ay}	126.7 ± 6.13^{ay}
ALH (µm/s)	5.61 ± 0.56^{ax}	5.49 ± 0.80^{ax}	8.42 ± 0.28^{ay}	8.00 ± 0.30^{ay}
BCF (Hz)	26.46 ± 2.57	27.11 ± 3.09	26.78 ± 0.99	28.73 ± 0.81
STR (%)	69.93 ± 5.61^{ax}	57.13 ± 6.46^{bx}	62.07 ± 1.67^{ax}	63.73 ± 1.63^{ax}
LIN (%)	43.36 ± 3.89^{ax}	32.13 ± 3.80^{bx}	34.93 ± 1.49^{ay}	35.93 ± 1.49^{ax}
NAR (%)	57.86 ± 3.77^{ax}	44.60 ± 3.69^{bx}	72.13 ± 2.38^{ay}	64.78 ± 2.65^{ay}
PMI (%)	64.04 ± 4.01^{ax}	50.13 ± 5.84^{bx}	82.44 ± 2.51^{ay}	73.89 ± 3.26^{ay}

F1 – sperm-rich fraction, F2 – post-F1; TMOT – total motile spermatozoa, PMOT – progressive motile spermatozoa, VAP – velocity average path, VSL – velocity straight line, VCL – curvilinear velocity, ALH – mean amplitude of lateral head displacement, BCF – beat cross frequency, STR – straightness (ratio of VSL/VAP x 100%), LIN – linearity (ratio of VSL/VCL x 100%), NAR – normal apical ridge acrosomes, PMI – plasma membrane integrity. Values represent the mean (± SEM) of 15 ejaculates from 3 boars. Values with different letters (a, b) in the same row differ between F1 and F2 at $p<0.05$ for the same extender. Values with different letters (x, y) in the same row differ between BTS and SCP at $p<0.05$ for the same fraction.

ed measures ANOVA, using a general linear model procedure from Statistica software package (v. 10; StatSoft Incorporation, Tulsa, OK, USA). Significant main effects were compared using the Neuman-Keuls *post hoc test*. All results were expressed as the mean ± standard error of the mean (SEM) and were considered significant at $p<0.05$. Furthermore, the main effects of the boar (1,2,3), fraction of ejaculate (F1, F2), extender type (BTS, SCP), day of storage (D1, D3, D5, D7) and their interactions on spermatozoa quality were analyzed under a four-way ANOVA statistical model (3 x 2 x 2 x 4).

Results

Effect of ejaculate fraction on the quality of spermatozoa

The effect of ejaculate fraction on the quality of boar spermatozoa prior to dilution of semen is shown in Table 1. Significant differences were observed between the two fractions of ejaculate (F1 vs. F2). F1 exhibited significantly higher ($p<0.05$) PMOT, VAP and VCL values in comparison to F2. After the dilution of ejaculate fractions, a marked variation in

PMOT for BTS extender was observed, being significantly higher ($p<0.05$) for F1 compared to F2 (Table 2). No significant differences ($p<0.05$) in the quality of spermatozoa were observed between F1 and F2 extended in SCP extender. On the following days of storage (D3 – not present, D5) similar dependences in PMOT were observed (Table 3). Additionally, on D5 of storage, statistically differences ($p<0.05$) in VCL analyses between F1 and F2 extended in BTS extender were noted. On D7 of storage, almost values of all the analyzed sperm quality parameters were higher for F1 compared to F2 for BTS extender (Table 4). Statistically significant differences ($p<0.05$) between F1 and F2 extended in BTS were noted in PMOT, VAP, VSL, STR, LIN, NAR acrosomes and PMI analyses.

Effect of extender on the quality of spermatozoa

There were significant differences ($p<0.05$) between extenders BTS and SCP, respectively of type of fraction and day of storage. In the case of F1, marked differences were observed in PMOT from stored D1 to D5 (Table 2, 3), it being significantly higher ($p<0.05$) for BTS when compared with SCP. In turn, significantly higher values ($p<0.05$) for SCP as compared with BTS were observed in ALH and NAR analyses on D5 of storage (Table 3) and in TMOT, VCL, ALH, LIN, NAR and PMI analyses on D7 of storage (Table 4). Significant differences ($p<0.05$) between extenders were observed for F2 in VCL and NAR analyses on D5 of storage (Table 3) and in TMOT, PMOT, VAP, VCL, ALH, NAR and PMI analyses on D7 of storage (Table 4).

The four-way ANOVA analysis

Analysis of variance results revealed that the boar had a significant effect on the CASA-analyzed stored sperm motility parameters: PMOT ($p<0.005$), VSL ($p<0.025$), VCL ($p<0.003$), ALH ($p<0.001$), BCF ($p<0.001$), STR ($p<0.001$) and LIN ($p<0.001$) and the fraction of ejaculate had an important effect on TMOT ($p<0.004$), PMOT ($p<0.001$), VAP ($p<0.001$), VSL ($p<0.001$), VCL ($p<0.001$) and ALH ($p<0.011$). Furthermore, the ejaculate fraction had a marked effect on NAR ($p<0.001$) and PMI ($p<0.001$), while the type of extender ($p<0.001$) had a significant effect on TMOT, ALH, STR, LIN, NAR and PMI. The day of storage ($p<0.001$) significantly influenced all the examined parameters of quality of spermatozoa except VSL.

The boar and type of extender had a marked effect on LIN ($p<0.018$), BCF ($p<0.001$) and STR ($p<0.037$), and the fraction of ejaculate and type of extender had a significant effect on TMOT ($p<0.049$), PMOT ($p<0.001$), VSL ($p<0.020$), ALH ($p<0.037$) STR ($p<0.043$) and LIN ($p<0.005$). The fraction of ejaculate and day of storage had a marked effect on VSL ($p<0.034$), STR ($p<0.011$) and LIN ($p<0.003$). Furthermore, the boar and day of storage interaction significantly influenced PMOT ($p<0.045$), VSL ($p<0.020$), VCL ($p<0.001$), ALH ($p<0.001$) and BCF ($p<0.006$). The fraction of ejaculate x day of storage interaction was found to be significant for STR ($p<0.001$), VSL ($p<0.034$) and LIN ($p<0.003$). ALH ($p<0.007$) and BCF ($p<0.001$) were significantly affected by the boar x type of extender x day of storage interactions.

Discussion

The results of the present study revealed that the type of ejaculate fraction significantly influenced the quality of sperm. Even before the dilution, fraction F1 was characterized by higher motility parameters compared to fraction F2. This is not surprising since, in our experiment, the F1 fraction contained the whole sperm-peak fraction. The F2 fraction contained only a small portion of SRF, mostly dominated by PSRF. Previous results confirmed the existence of essential differences between the sperm-peak fraction (P1) and the rest of the ejaculate, and are related to types and amounts of ions, proteins, bicarbonate and pH (Rodríguez-Martínez et al. 2009, Saravia et al. 2009). "The P1 spermatozoa are still bathing in a substantial amount of fluid from the cauda epididymides, in which they were emitted at ejaculation" (Rodríguez-Martínez et al. 2009). Thus, the F1 spermatozoa also remain mainly more exposed particulary to epididymal cauda fluid and its specific proteins i.e. various lipocalins, including the fertility-related Lipocalin-type Prostaglandin D synthase than to prostatic and initial vesicular gland secretions. Moreover, spermatozoa from F1 are hence exposed to less bicarbonate, zinc or fructose and mainly to PSP-I spermadhesin, than if they were in F2, similarly to the research presented by Rodríguez-Martínez (2009). Because of this it may be assumed that the presence of certain proteins in F1 which originated from epididymal fluid had a positive effect on sperm functioning. These assumptions also confirm our preliminary study on proteomic analysis of F1 and F2 using electrophoresis SDS-PAGE, performed in our laboratory (unpublished data) and other earlier studies (Zasiadczyk et al. 2015). Zasiadczyk et al. (2015) revealed that most

F1 proteins originated from cauda epididymal fluid, and that SP from F2 contained proteins from the vesicular gland and cauda epididymis secretions, where vesicular gland fluid was the main source of proteins. These secretions contain proteins which interact with sperm cells and exert different effects on their functions. More than 90% of boar SP proteins are known to be heparin-binding or non-heparin-binding spermadhesins (AQN-1, AQN-3, AWN, PSP-I and PSP-II) which are thought to participate in different aspects of porcine fertilization and preservation (Calvete and Sanz 2007, Rodríguez-Martínez et al. 2011). Proteins PSP-I/PSP-II were described as essential for preservation technologies for boar semen as they preserve the functionality of sperm cells *in vitro* and semen (Centurión et al. 2003). Sperm functions are also influenced by the content of the PSP-I/PSP-II heterodimer (Caballero et al. 2004, Rodríguez-Martínez et al. 2011). The PSP-I/PSP-II heterodimer is present in all SP fractions, although its concentration increases gradually with a dose of the heparin-binding spermadhesins proteins and the total proteins in the post-sperm-rich fractions. Centurion et al. (2003) demonstrated that sperm viability and motility are determined by the presence of the heparin-binding and non-heparin-binding spermadhesins in the incubation mixture. Thus, we can assume that differences between F1 and F2 regarding the sperm quality resulted from the presence of these specific proteins and their concentration in the analyzed portion of semen.

Another factor which can affect the differences in the quality of spermatozoa from individual fractions is the difference resulting from SP's total antioxidant ability (SP-TAC) observed in individual fractions. Barranco et al. (2015) showed that the first 10 mL of the SRF characterised the highest SP-TAC values among the ejaculate fractions, which would support the best technological utility of this particular fraction, particularly for preservation.

In our study, F1 immediately after dilution was characterised by higher PMOT than F2 in the BTS extender, which could be attributed to the influence of the above SP proteins and a high antioxidant ability of the F1 fraction. Moreover, the values of other sperm quality parameters (TMOT, VAP, VSL, VCL, ALH, NAR and PMI) for F1 as compared with F2, especially in the case of the BTS extender, were shown to increase during long term storage of ejaculate fraction. Interestingly, no differences between fractions were observed after the use of the SCP extender, which suggests that the choice of extender is essential. When the ejaculate fraction is diluted with an extender, the effect of seminal plasma on spermatozoa is significantly limited, and prolonged storage in an extender modifies the structural and functional characteristics of sperm cells (Garcia et al. 2009, Gączarzewicz et al. 2015). Our study demonstrated that the quality of stored spermatozoa is affected not only by ejaculate fraction but also significantly by the type of extender. Short-term and long-term extenders for boar semen differ in composition (Gadea 2003). The BTS extender has a much simpler composition than SCP, which is enhanced with proteins and antioxidants (manufacturer's data). The above could explain the significant differences in the quality of stored spermatozoa. During long-term storage, higher values of sperm motion parameters, NAR and PMI were noted in SCP than in BTS regardless of ejaculate fraction. Sperm stored in long-term extenders are known to be characterised by higher quality than those stored in short-term extenders during long-term preservation (Johnson et al. 2000, Dziekońska et al. 2013). In this study, the SCP extender more effectively protected the acrosomal and plasmatic membranes of stored sperm cells than BTS, which contributed to higher values of TMOT, PMOT and other sperm motion patterns (VCL, ALH, LIN) during long-term storage. This may be explained by the differences in the composition of SCP and BTS extenders. The results of SDS-PAGE of the SCP extender (unpublished data) indicated an abundance of proteins with a molecular weight of 60-66 kDa. Earlier studies demonstrated that BSA-supplemented extenders (molecular weight of approximately 65-66 kDa) reduced oxidative stress and improved the quality of boar spermatozoa stored in a liquid state at 17°C (Zhang et al. 2015). It should be noted, however, that BSA in the semen extender effused cholesterol in the plasma membrane of sperm cells, which increased the levels of reactive oxygen species (ROS) (Radomil et al. 2011, Lee and Park 2015). The above observation indicated that extenders benefited from the addition of antioxidants. Our results suggested that the addition of proteins and antioxidants to the SCP extender improved the quality of stored spermatozoa particularly in the F2 fraction. We did not observe any significant differences between F1 and F2 in parameters of motility and membrane integrity on D7 of storage, which was a positive phenomenon.

Upon completion of the four-way ANOVA analysis, it was shown in addition to the type of fraction, extender and storage period, the motility of the stored spermatozoa was also dependent on an individual factor. Interestingly, this relationship was not observed in sperm fractions, immediately before and after dilution, during the first day of storage (unpublished data). This may result from the fact that the individual factor is more noticeable in cold shock conditions (Peña et al. 2006, Hernández et al. 2007, Dziekońska and Strzezek 2011). Hernández et al. (2007) showed

that adding "good" boar SP to the extender used in cryopreservation positively influenced motility, viability and fertility ability after thawing, which may be traced back to SP activity in this process. About 70% of variability in boar spermatozoa cryosurvival was also confirmed to be attributed to male effects (Roca et al. 2006) where SP protein characteristics differ among boars (Flowers 2001, Hernández et al. 2007). Moreover, individual differences may result from other factors, such as genetic factors (Thurston et al. 2002), or the existence of variable subpopulations of spermatozoa in the ejaculate, in terms of their morphological characteristics (Thurston et al. 2001) or motility patterns (Cremades et al. 2005).

In conclusion, the obtained results indicate that the ejaculate fraction, the applied extender, the boar, day of storage and interactions between these factors significantly influence the quality of preserved boar spermatozoa in a liquid state. The F1 (sperm-rich fraction) was characterised by a higher quality of spermatozoa during liquid storage than F2 (remaining sperm-rich fraction plus post-sperm-rich fraction), in particular during storage in short-term extender. This could be caused by the presence of SP proteins of a different origin. Our findings indicate that the long-term protein-containing extender was more effective in protecting spermatozoa against damage than the short-term extender, regardless of the ejaculate fraction. The use of long-term extenders would allow for better usage of the remaining sperm-rich fraction plus post-sperm-rich fraction (F2) of boar ejaculate in preservation technology. This review depicts the best possible way to implement an efficient use of liquid-stored F2-sperm by the pig industry.

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Author contributions

A. Dziekońska designed the experiment and conducted "Effect of fraction of ejaculate on quality of boar spermatozoa stored in a liquid state". K. Świąder and K. Mietelska collaborated in the experimental part, M. Koziorowska-Gilun collaborated in data analysis and in drafting the manuscript, W. Kordan reviewed the manuscript and Ł. Zasiadczyk was responsible for collection of the animal material.

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