Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2021) 45:

doi:10.3906/vet-2012-63

Quality and fertility of extended boar semen after prolonged transport

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Received: 21.12.2020 • **Accepted/Published Online:** 25.07.2021 • **Final Version:** 00.00.2021

Abstract: The aim of this study was to analyse semen quality parameters of fresh-extended porcine semen imported from Denmark through prolonged importing regulations and transport and to analyse fertility performance in sows inseminated four days post semen collection. Semen was analysed immediately upon the arrival (4 days post collection) using computer-assisted sperm analyses (CASA), cytomorphology and flow cytometry, and sows were inseminated later the same day. The mean sperm concentration was 1718.09 ± 100.11 × 10^6, with average of 1193.40 ± 90.58 × 10^6 motile cells (69%) and 756.04 ± 70.57 × 10^6 progressive motile spermatozoa (43.58%) in a dose, which was almost twice lower in value than producer certified-declared number of 2 billion of motile cells. Acrosome and sperm membrane integrity test indicated high percentage of total damaged acrosome. Mean values of semen samples analysed by sperm chromatin structure assay (SCSA) showed relatively high degree of chromatin damage. Membrane permeability test showed high percentage of sperm with damaged membrane. Prolonged transport negatively affected the sperm quality in terms of both motility and chromatin structure stability. Fertility of sows was affected by semen quality in terms of concentration and sperm motility parameters, as well as ratio of live sperm cells population with damaged acrosome.

Key words: Import, semen, boars, computer-assisted sperm analyses (CASA), flow cytometry

1. Introduction

Researchers and practitioners worldwide try to find solutions for the improvement of pig production in order to make this industry more profitable. The development of biotechnology methods and application of selection programs have significantly increased sows prolificacy in Western Europe, United States and Canada, improving overall fertility and production [1]. With the application of artificial insemination (AI) in swine reproduction, conditions have been created for obtaining significantly more progeny from single boar of high genetic potential in relation to natural breeding.

Semen extenders that permit the use of fresh semen for more than 5 days post-collection are largely responsible for the success of AI in pigs worldwide [2], enabling long distance transportation, diagnostic testing and semen quality assessment prior to its use for AI on the farm [3]. Prolonged storage time has adverse effects on porcine sperm viability; therefore, approximately 85% of inseminations are performed within 48 h of semen collection [3]. Farrowing rates of 80 to 85% can be achieved by using extended boar semen up to 48 h post collection, but longer storage may be linked with a significant reduction in piglet productivity of inseminated sows [4].

The improvement of genetic potential of the porcine meat industry in Serbia so far has been based mainly on the importation of breeding gilts and boars used through selection programs [5]. Recently, single swine corporation owning 7000 breeding sows imported fresh diluted semen from Denmark for their nucleus study in order to further improve genetic traits in existing animals.

Currently, Serbia is not a member of the European Union (EU), and the process of importation of boars' semen requires administrative procedures that may take up to three working days requiring approval from the Veterinary Directorate of the Ministry of Agriculture and Environment of the Republic of Serbia and Customs Administration. During this process, semen must be stored in quarantine and each produced batch must be sampled and analysed in a relevant/competent veterinary laboratory.

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Evaluation of the spermatozoa of animals has a purpose of determining semen fertilizing ability. Routine sperm evaluations are suitable to identify clear cases of infertility and, sometimes, to provide cues for potential sub-fertility. However, they can't measure (among other things) if the genomic message that spermatozoa deliver to the gamete counterpart throughout fertilization is intact enough to enable the development of the early embryo [6]. To that end, flow cytometry and the sperm chromatin structure assay (SCSA) can produce information about sperm DNA status, i.e. provide meaningful biological information on sperm nuclear DNA defects [7]. Alongside computer assisted sperm analysis, which allows more detailed and precise semen motility assessment, flow cytometry is considered as an important tool for determining fertilising capacity of semen cells. Boar fertility after AI with fresh diluted semen can be predicted based upon the evaluation of sperm morphology and chromatin integrity as the field fertility tests confirm a strong correlation between the farrowing rates with live normal sperm and stable deoxyribonucleic acid complex [8], as well as the evidence of sperm DNA fragmentation affecting litter size in sows [4].

The aim of this paper was to present results on semen quality parameters from fresh-extended porcine semen imported from Denmark through a prolonged process of importing regulations and to analyse fertility performance in sows inseminated four days post semen collection.

2. Material and methods

2.1. Experimental design

Fresh diluted semen doses from 14 production series of 11 boars were shipped five times in total (in five different transports) from Denmark to the Republic of Serbia. Semen production and quality control were performed according to manufacturer's quality standards laid down in the Danish Research Centre for pigs (DanAvl/Hatting).[1] As stated by the manufacturer, semen collection was done using the two-glove method so that contamination of the ejaculate with material from the prepuce and skin is avoided (Hatting/SEGES). Upon collection, fresh semen was diluted in Tricell diluent (Denmark standard EDTA (ethylenediaminetetraacetic acid) - based extender manufactured by a Danish pharmacy). The EDTA extender contained 112 mg of amoxicillin, 112 mg of gentamycin and 30 mg tylosin per litre of extender. Semen was packed in plastic bags, 80 mL of volume, certified by producer to contain two billion motile sperm cells and transported by air in the insulated transport box. Temperature fluctuations from 11°C to 15°C have been observed only during the first transport (50 doses from three boars) from Denmark to Serbia. Next four transports were within temperature range proposed by the manufacturer (DanAvl/Hatting). The producer's recommendation for storing fresh boar semen of 16–18 °C is set with a safety margin of 1–2 °C during transport (DanAvl/Hatting).

Sampling of imported semen was carried out in the on-farm quarantine early in the morning and immediately subjected to analyses. One dose from each boar and each produced batch was collected and analysed. The samples were transported to the Laboratory of reproduction of the Scientific Veterinary Institute Novi Sad, in a climate controlled box powered by car charger at a temperature of 17 °C–19 °C. Semen quality analysis was performed on the arrival (4 days post-collection) and test results along with a report on semen quality and usability for AI were issued. Sows were inseminated later the same day, on day 4 after semen collection.

2.2. Semen quality analysis

Semen quality assessment was carried out on all 11 samples from boars of several breeds (Yorkshire-3, Landrace-7 and Duroc-1 samples)[2]. Quality assessment analysis included following:

1) Complete assessment of motility parameters, including sperm concentration, was performed using CASA system with integrated software system for sperm analysis (ISAS D4C20, "Proiser" projectes i Serveis R + D, Valencia, Spain). Briefly, testing was performed on four-chambered microscopic ISAS disposable slides with 20 μm chambers depth and a volume of 5 mL (ISAS D4C20), which was marked by 7 visual fields provided for recording. The study included at least 1000 sperm per sample. CASA method (number and motility of spermatozoa) is laboratory method accredited by the Accreditation Body of Serbia (SOP-3-01-120); the speed range of the CASA was as follows: static < 15 μm / s < slow < 25 μm / s < medium < 50 μm / s < rapid. Progressivity was set up as 70% of the STR (straightness). The following characteristics of sperm motility were determined: sperm cell motility percentage, the progressive motility percentage, curvilinear velocity (VCL, μm / s), straight line velocity (VSL, μm / s), average path velocity (VAP, μm / s), sperm linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral sperm head displacement (ALH, μm) and beat cross frequency (BCF, Hz). Circular tracks (%) were also assessed. Also, total number of spermatozoa, total number of motile spermatozoa and total number of progressive motile sperm cells were calculated using CASA.

2) Cytomorphological analysis of supravital stained sperm smear with trypan blue-eosin-nigrosin/in one step,

[1] DanAvil/Hatting. Export - Artificial insemination with DanAvl semen from Hatting A/S [online]. Website www.hatting-as.dk [accessed 15th December 2018].

[2] Hatting/SEGES. Quality assurance program for DanAvl boar studs in Denmark [online]. Website www.hatting-as.dk [accessed 15th December 2018].

as described by Björndahl et al. [9], established a ratio of live/dead cells, acrosome defects, protoplasmic droplets and the total percentage of pathological forms of spermatozoa, under microscope magnification of 1000× (Olympus BH-40, Tokyo, Japan). Spermatozoa subpopulations detected were as follows: total live sperm (ΣL) as a sum of both intact and damaged acrosome cells, live sperm with intact acrosome (LIA), dead sperm with intact acrosome (LDA), total damaged acrosome (ΣDA) as a sum of both live and dead cells with damaged acrosome, protoplasmic droplet (ΣPPD) and total sperm abnormalities (ΣPAT) as a sum of primary and secondary abnormalities.

3) Flow cytometry (Guava Millipore, Easy Cite Mini, software Cytosoft version 4.4 beta 1; Hayward, California, USA), with built-in software for semen quality analysis (IMV Technologies, France), using 488 nm coherent sapphire blue diode laser and photomultipliers with 583 ± 30nm (yellow), 680 ± 30 nm (red) and 525 ± 30 (green) filters, was carried out with the following 3 tests:

3 a) Membrane and acrosome integrity assay (combination of fluorometric stain PNA-FITC — PNA-Fluorescein isothiocyanate (Lectin FITC labelled from Arachis Hypogea–peanut, L7381 Sigma - Aldrich, St. Louis, MO, USA) and propidium iodide (PI) from the Live/dead sperm viability kit (L7011, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA)) was used to determine the integrity of sperm membrane and acrosome. The peanut origin lectin mixed with FITC stains the sperm acrosome (the broken acrosome is more stained than the intact acrosome), and PI enters sperm with altered membrane. Therefore, the spermatozoa with an intact membrane and with an intact acrosome do not react in green and red, the spermatozoa with intact membrane but with a broken acrosome are sensitive to green but do not react in red, the spermatozoa with a broken membrane but with an intact acrosome are sensitive to red and do not react to green, and spermatozoa with broken membrane and broken acrosome are sensitive to red and green.

Briefly, PNA-FITC was diluted to 1 mg/mL in deionized water to prepare a stock solution and stored at –20 °C. For analyses, 1 mL of PNA-FITC and 2 µL of PI (from concentration of 2.4 mM dissolved in water) were added in 1.5 Eppendorf tube and diluted with 97 µL of Easy Buffer B solution (IMV Technologies, France). Subsequently, 8 µL of boar semen was added into Eppendorf tube and diluted again with additional 292 µL of Easy Buffer B to reach total volume of 400 µL. This mix was incubated for 10 min in Eppendorf thermomixer shaking block (300 rpm) at 37 °C, away from light. The study included reading of 2000 events (sperm cells) per sample. Sperm membrane and acrosome integrity assay was estimated according to official IMV-Technologies test protocol in built-in Viability Acrosome setup software (IMV Technologies, L'Aigle, Basse-Normandie, France) and in reference to Graham et al. [10].

3 b) Viability assay (sperm membrane assay to determine dead and live sperm cells) using SYBR14 and PI fluorometric stains from Live/Dead sperm viability kit (L7011, Invitrogen). Shortly, Sybr14 was diluted to 1 mM in DMSO (to prepare a stock solution) and PI to 2.4 mM in deionized water. Working solution of the Sybr14 was prepared using 1 µL of Sybr14 solution (1mM) and of 49 µL Easy Buffer B solution, resulting in Sybr14 dilution of 20 µM. For test analysis, 2 µL of PI and 2µL of SYBR14 stock solution were added into 1.5 mL Eppendorf tube and diluted with 96 µL of Easy Buffer B solution, and then 6 µL of semen was added. Next, 294 µL of Easy Buffer B was added to achieve a total volume of 400 µL. This mix was incubated for 10 min in Eppendorf thermomixer shaking block (300 rpm) at 37 °C, away from light. The study included reading of 2000 events (sperm cells) per sample. Sperm viability assay was estimated according to official IMV-Technologies test protocol in built-in Viability setup software (IMV Technologies, L'Aigle, Basse-Normandie, France).

3 c) Sperm chromatin structure assay (SCSA) was estimated according to official IMV-Technologies test protocol and in reference to Evenson et al. [7]. The SCSA technique is based on acridine orange stain, which fluoresces green when combined with double-stranded DNA, and red when combined with single-stranded DNA (denatured). Spermatozoa (6 mL) were diluted in 194 mL of TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM EDTA; pH 7.4). Then 400 mL of an acid detergent solution was added (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2). Exactly 30 s after adding the acid-detergent solution, 1.2 mL of staining solution (6 mg⁄mL of acridine orange in a buffer containing 37 mM citric acid, 126 mM Na_2HPO_4, 1.1 mM disodium EDTA and 150 mM NaCl; pH 6) was added. Samples were incubated for 3 min at 37 °C, and, subsequently, run through a flow cytometer. Sample acquisition was stopped at 2000 sperm cells per sample. DNA fragmentation index (DFI) for each spermatozoa was calculated as the ratio of red fluorescence with respect to total fluorescence (red + green), expressed as a percentage.

2.3. AI and fertility traits

Insemination was performed on a nucleus swine farm with 300 sows (2–5 parities). For oestrus induction and synchronization after weaning, one dose (2 mL) of SERGON PG 400 + 200 (lyophilized mixture of 400 IU of human chorionic gonadotropin and 200 IU of pregnant mares' serum gonadotropin, Bioveta A.S., Ivanovice na Hane, Czech Republic) was injected intramuscularly behind the ear. Oestrus was checked daily in the presence of a mature teaser boar. Sows were inseminated upon observation of standing behaviour using disposable spiral catheters for classic intracervical insemination (Magapor, Zaragoza, Spain). A total of 59 multiparous sows (two to

six pregnancies) were inseminated twice using 118 doses of semen from 11 boars, at 24 h interval. Two groups were formed: group BAS (below average semen) in which sows were inseminated with semen containing less than 756.04 million progressive sperm cells in a dose (semen from 6 boars), and group AAS (above average semen) in which sows were inseminated with semen containing more than 756.04 million progressive sperm cells/dose (semen from 5 boars). This cut off value was selected because it was the average value for all analysed sperm samples. Number of progressive sperm cell per dose was implemented as a parameter because the producer had only stated the number of motile sperm per dose in their quality declaration. Fertility was measured for every ejaculate, as well as for groups BAS and AAS, as the percentage of sows farrowing to AI, the number of dead and live piglets, and the total number of piglets born. AI results were monitored from the software program AGROSOFT WinPig (AgroSoft A/S, Tørring, Denmark).

2.3. Statistical analysis

Statistical analysis was performed using statistical software SPSS 21 for Windows (IBM Corp., Armonk, NY, USA). Homogeneity of variance was tested using Levene's test. The data obtained in the study were analysed using t-test for independent samples (for sperm quality traits and litter size) and chi square test (for farrowing rate). Spearman's rank correlation coefficients were used to examine the relationships between sperm traits and field fertility. Data were considered statistically significant if P value was less than 0.05.

Animal experimentation was conducted within standard ethical norms.

3. Results

Aggregate descriptive statistical results for CASA parameters for all 11 boars' semen of Yorkshire, Landrace and Duroc breeds, as well as for groups A and B, are shown in Table 1.

The mean sperm concentration was 1718.09 ± 110.11 × 10^6, with average of 1193.40 ± 90.58 × 10^6 motile cells (69%), and 756.04 ± 70.57 × 10^6 progressive motile spermatozoa (43.58%) in a dose, which was almost twice lower in value than producer certified-declared number of 2 billion of motile cells. Boars' semen from group AAS had significantly better motility parameters in relation to semen used in group BAS. Statistically significant difference between the two groups was found in the number of motile and progressive spermatozoa per mL/dose, as well as percentage of progressively motile spermatozoa in a dose. The values of the CASA analysed kinetic parameters as reported in Table 2 showed statistically significant difference in VSL, VAP and BCF between the two groups, with semen from group AAS having significantly higher value of these parameters.

Table 1. Descriptive statistic of the CASA parameters for motility and concentration of fresh diluted boar semen.

Groups	Total No. of sperm. (×10^6/dose)	Total No. of motile cells (×10^6/dose)	% of motile sperm cells	% progressive motile sperm cells	Total No. of progressively motile cells (×10^6/dose)
All boars N = 11					
Mean	1718.09	1193.40	69.00%	43.58%	756.04
SEM	100.11	90.58	0.02	0.03	70.57
Max	2217.5	1547.1	78.6%	56.4%	1142.1
Min.	1118.3	701.4	47.6%	26.5%	391.1
CV%	19.35	25.20	12.09	19.73	30.10
Group BAS N = 6					
Mean	1581.7	816.8	63.18%	36.86%	583.4
SEM	132.94	58.53	3.77	3.07	56.53
CV%	20.59	17.56	14.63	20.40	23.74
Group AAS N = 5					
Mean	1853.8	1391.6	74.98%	48.53%	825.3
SEM	109.42	78.48	1.53	2.94	36.74
CV%	13.22	12.63	4.57	13.59	9.98
t-test; p =	0.051	0.011	0.070	0.043	0.005

Legend: BAS — Below Average Semen (group with less progressively motile sperm in a dose); AAS — Above Average Semen (group with more progressively motile sperm in a dose).

Table 2. Results of CASA analysed kinetic parameters.

Groups	All samples (N =11)	Group BAS (N = 6)	Group AAS (N = 5)	t-test; p =
VCL, μm/s	48.9 ± 2.80	45.2 ± 4.24	53.3 ± 2.67	0.16
VSL, μm/s	32.5 ± 1.74	28.7 ± 1.66	37.0 ± 1.73	0.007
VAP, μm/s	40.0 ± 2.28	36.1 ± 3.09	44.6 ± 2.08	0.049
LIN, %	67.0 ± 2.25	64.6 ± 2.72	70.0 ± 3.55	0.25
STR, %	81.7 ± 1.87	80.4 ± 2.50	83.2 ± 2.96	0.48
WOB, %	81.9 ± 1.25	80.3 ± 1.71	83.9 ± 1.55	0.15
ALH, μm	1.8 ± 0.09	1.8 ± 0.14	1.9 ± 0.11	0.76
BCF, Hz	7.4 ± 0.14	7.1 ± 0.15	7.7 ± 0.14	0.019
Circular tracks, %	15.9 ± 2.28	16.7 ± 3.60	15.0 ± 2.95	0.73

Results are expressed as mean ± SEM
Legend: VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; LIN = linearity of track; STR = straightness of track; WOB = wobble; ALH = amplitude of the lateral head displacement; BCF = beat cross frequency; BAS — Below Average Semen (group with less progressively motile sperm in a dose); AAS — Above Average Semen (group with more progressively motile sperm in a dose).

Cytomorphological analysis of supravitally stained sperm smear with eosin-nigrosine/in one step showed 80.24 ± 2.38 percent of total live sperm cells for all analysed batches, 15.36 ± 2.92% of cells with acrosome defects, 3.91 ± 0.85% of sperm cells with protoplasmic droplets and the 27.39 ± 3.84% of total pathological forms of spermatozoa. Most dominant sperm pathology type was simple bent principal piece. None of the semen quality parameters as determined by cytomorphology were found significantly different between groups (Table 3).

The results of acrosome and sperm membrane integrity test indicated satisfactory percentage of total live sperm (ΣL – 71.40 ± 7.82%) and live sperm with intact acrosome (LIA – 52.36 ± 3.29%). However, higher percentage of live sperm with damaged acrosome (LDA – 19.04 ± 3.86%) and high percentage of total damaged acrosome (ΣDA – 38.92 ± 3.02%) were also noted. Statistically significant difference was found between the groups in live sperm cells with damaged acrosome (LDA – 21.02 ± 1.38% and 16.66 ± 1.40%), as determined by the test of membrane and acrosome integrity by flow cytometry (Table 4).

Mean values for 11 SCSA analysed semen samples indicated relatively high degree of chromatin damage (8.49% ± 1.23%), (where the sperm in 3 samples had ≥ 10% of damaged chromatin on SCSA test). The test results of sperm chromatin structure (SCSA) indicated presence of the DNA damage in 9.84 ± 2.02% of sperm cells in group BAS and 6.88 ± 1.00% in group AAS, but no difference was observed ($p > 0.05$) (Table 5).

Membrane permeability test (Sybr-14/PI) indicated presence of 74.54 ± 3.16% sperm with intact membrane (live cells) and 25.46 ± 3.16% with damaged membrane (dead cells). No statistical significance between groups was indicated (Table 5).

Of the total of 59 inseminated sows, 44 were pregnant and farrowed (74.58%). In group BAS, 19 of 32 inseminated sows were pregnant (59.38%), with farrowing ranging from 0.0 to 67%. In group AAS, 25 of 27 sows farrowed (92.59%), with farrowing ranging from 83.33–100%. Statistical analysis indicated highly significant difference in farrowing percentage between the groups, as well as in number of born piglets per litter and in number of live born piglets per litter (Table 6).

Number of progressively motile sperm cells per dose and farrowing rate, but not litter size, was significantly correlated. Both farrowing rate and litter size were significantly correlated to percentage of live sperm with damaged acrosome, as determined by flow cytometry PNA-FITC/PI test, but neither was correlated to percentage of sperm with chromatin damage as determined by SCSA test (Table 7).

4. Discussion

Fertilizing capacity of semen for AI significantly depends on the quality of used semen [11]. Classical methods used for semen evaluation measure the sperm concentration, progressive motility, percentage of viable sperm cells and acrosome morphology. These assays are poor in predicting sperm fertility because only the samples with remarkably poor quality can be detected. The development of new sperm tests and combination of several tests measuring certain sperm functions are an attempt to solve this

Table 3. Quality of semen on the basis of cytomorphology test in all samples and in two studied groups with different count of progressive motile sperm.

Groups	Detection of specific subpopulations of spermatozoa (%)					
	Σ L	LIA	LDA	Σ DA	Σ PPD	Σ PAT
All samples N =11	80.24 ± 2.38	47.82 ± 2.96	4.21 ± 1.65	15.36 ± 2.92	3.91 ± 0.85	27.39 ± 3.84
Group BAS	81.61 ± 3.75	49.50 ± 2.59	3.06 ± 1.88	12.00 ± 3.11	4.33 ± 0.98	28.39 ± 5.52
Group AAS	78.60 ± 3	45.80 ± 6.01	5.60 ± 2.96	19.40 ± 4.99	3.40 ± 1.54	26.20 ± 5.86
t-test; p =	0.557	0.561	0.471	0.224	0.610	0.793

Results are expressed as mean ± SEM
Legend: Sperm subpopulations: Σ L — Total live; LIA — live sperm with intact acrosome; LDA — live sperm with damaged acrosome; åDA — total damaged acrosome; ΣPPD- Protoplasmic droplet; Σ PAT- Total sperm abnormalities; Groups: BAS — Below Average Semen (group with less progressively motile sperm in a dose); AAS — Above Average Semen (group with more progressively motile sperm in a dose).

Table 4. Results of sperm membrane and acrosome integrity test by flow cytometry.

Groups	Sperm membrane and acrosome integrity (PNA-FITC/PI)*					
	%LIA	%DIA	% LDA	%DDA	Σ L	Σ DA
All samples (N =11)	52.36 ± 3.29	8.71 ± 1.09	19.04 ± 1.16	19.89 ± 2.00	71.40 ± 2.36	38.92 ± 3.02
Group BAS (N = 6)	48.40 ± 4.64	8.60 ± 1.21	21.02 ± 1.38	21.98 ± 2.53	69.42 ± 3.4	43.00 ± 3.66
Group AAS (N = 5)	57.12 ± 4.08	8.84 ± 0.54	16.66 ± 1.40	17.38 ± 3.08	73.78 ± 3.26	34.02 ± 4.36
t-test; p =	0.201	0.920	0.05	0.273	0.385	0.146

Results are expressed as mean ± SEM
Legend: Sperm subpopulations: %LIA — live sperm with intact acrosome; %DIA- dead sperm with intact acrosome; %LDA — live sperm with damaged acrosome; %DDA- dead sperm with damaged acrosome; Σ%L- Total live; ΣDA- total damaged acrosome; Groups: BAS — Below Average Semen (group with less progressively motile sperm in a dose); AAS — Above Average Semen (group with more progressively motile sperm in a dose); *PNA-FITC/PI – Peanut Agglutinin Fluorescein Isothiocyanate / Propidium Iodide semen co-staining - Refers to Flow cytometry Acrosome Integrity Assay test.

problem [8, 12–14]. In the present study, a combination of CASA, flow cytometry and cytomorphological examination of supravital stained sperm was used to more accurately determine the semen quality.

In addition to tests stated above, the most reliable indicator of fertility for porcine semen is conception rate and number of (live) piglets born per litter. Fertility parameters and semen quality had sharp-cut correlation: a highly significant difference was found for farrowing rate between the groups (group AAS from 83% to 100% and group BAS from 0 to 68.75%), as well as for the number of live born piglets per litter (group BAS - 14.53, group AAS - 15.71 on average).

Motility is one of the most important traits/properties affecting the fertilizing capacity of sperm [15,16]. CASA is objective and precise tool for determining the number of total and progressive motility of spermatozoa. Sows in the group AAS were inseminated with semen of significantly better quality parameters in relation to percentage and number of motile and progressive motile sperm in a dose and in milliliter, compared to the sows in group BAS. CASA analyses of kinetic parameters also showed significantly higher values for VSL, VAP and BCF parameters in semen from group AAS, indicating more vigorous (higher BFC, alongside higher VCL and ALH) and progressive sperm cells (higher VSL and VAP). Boar semen analysed in this study originated from well-known and quality standardized laboratory. The study showed considerable motility and sperm concentration variances (coefficient of variance ≈ 20% on CASA analyses), that were consistent with farrowing rate. The average concentration of motile spermatozoa in a dose for artificial insemination was 1193.40×10^6, which was less than 2 billion motile spermatozoa which the manufacturer had stated in its quality declaration for

Table 5. Results of viability assay and sperm chromatin structure assay by flow cytometry.

Groups	Viability Assay *(%)*		Chromatin structure *(%)* SCSA test	
	Intact	Damaged	Intact	Damaged
All samples (N =11)	74.54 ± 3.16	25.46 ± 3.16	91.51 ± 1.23	8.49 ± 1.23
Group BAS (N = 6)	72.54 ± 5.38	27.46 ± 5.38	90.16 ± 2.02	9.84 ± 2.02
Group AAS (N = 5)	76.93 ± 2.95	23.07 ± 2.95	93.12 ± 1.00	6.88 ± 1.00
t-test; p =	0.520	0.522	0.231	0.252

Results are expressed as mean ± SEM
Legend: BAS — Below Average Semen (group with less progressively motile sperm in a dose); AAS — Above Average Semen (group with more progressively motile sperm in a dose); SCSA — Sperm Chromatin Structure Assay.

Table 6. Reproductive results in sows from two studied groups.

Groups	No. of AI sows	No. of farrowed sows	% farrowed	No. of live piglets	No. of dead piglets	Total No. of piglets*
Group BAS N = 6						
Mean/Total:	32+	19+	59.38	14.53	0.83	15.36
SEM				2.61	31.47	3.51
CV%				6.4	77.1	8.59
Group AAS N = 5						
Mean/Total:	27+	25+	92.59	15.71	0.95	16.66
SEM				0.55	0.21	0.76
CV%				7.89	56.63	10.26
Chi square; p =			0.003			
t-test; p =				0.001	0.998	0.009

Results are expressed as mean, SEM and CV% for number of piglets born per sow; Number of inseminated and farrowed sows was expressed as total+
*Total No of piglets (both alive and dead) was expressed per sow as mean value resulted from statistical analyses preformed.
Legend: Groups: BAS — Below Average Semen (group with less progressively motile sperm in a dose); AAS — Above Average Semen (group with more progressively motile sperm in a dose).

Table 7. Spearman's rank correlation coefficients (r) between sperm quality traits and fertility parameters

Fertility parameters	Number of progressively motile cells per dose	Live sperm with damaged acrosome (PNA-FITC/PI)	Sperm with chromatin damage (SCSA)
Farrowing rate	r = 0.79*	r = – 0.42*	r = – 0.05 ns
Live litter size	r = 0.065ns	r = – 0.72*	r = – 0.13 ns

*$p < 0.05$; ns - non-significant
Legend: PNA-FITC/PI — Peanut Agglutinin Fluorescein Isothiocyanate / Propidium Iodide semen co-staining — Refers to Flow cytometry Acrosome Integrity Assay test; SCSA — Sperm Chromatin Structure Assay.

produced semen for artificial insemination of sows, so the number was almost twice lower in value than producer certified-declared number. Considerable variation among boars in regard to the fertilizing capacity of semen during storage can exist too [17]. However, this fact can only be attributed to technical failure at the time of semen dilution.

Flow cytometry is recognized methodology within animal andrology and has moved from being a research tool to the routine test in the assessment of animal semen [6]. The introduction of flow cytometry in andrological tests created possibility of testing a large number of sperm cells in few seconds, excluded subjectivity and resulted in high precision. Flow cytometric sperm analysis, with high precision and accuracy and low costs, can be proposed for routine use in clinical laboratories [18].

The percentage of sperm having intact acrosome membrane is considered an important parameter of semen quality [19]. Detection of damage to the membrane and acrosome by flow cytometry provides a more precise value for the assessment of their structure compared to cytomorphological analysis [13]. Cytomorphological analysis is more of a routine but also a very subjective analysis in fact, as this study showed. In the present study, there were differences in proportion of sperm cells with altered morphology between studied groups in terms of cytomorphological analysis and flow cytometry, as shown in tables 2 and 3. Contrary to cytomorphology analysis which found semen from group BAS to be better with less damage to sperm cell acrosome and more live intact cells, PNA-FITC/PI test, however, showed live sperm cells with damaged acrosome to differ significantly between groups, with semen in group BAS having more damaged acrosome cells than semen in group AAS. As a result, field fertility of sows showed higher farrowing rate as well as bigger litter size in sows from group AAS.

Boar spermatozoa are especially sensitive to cold shock when temperatures drop below 15°C [20–25]. The plasma membrane integrity due to temperature fluctuations is manifested with acrosome damage, which may not be revealed by SYBR-14/PI staining (data for semen stored at 5°C). This reduced motility was consistent with decreased sperm mitochondrial transmembrane potential and oxidoreductive capability [26]. Remarkable differences in the individual resistance of boar semen to long term storage at 10 °C were observed [27], but less at 12°C. However, according to Althouse et al. [28] no differences were observed in the farrowing rate, total offspring born or number of live-born piglets when stored ejaculates were exposed to temperatures between 12 °C and 17°C. In the present study, 3 batches of porcine semen had a peak of transport temperature of 11°C, and, for the other one, temperature range was 11–15°C. This didn't result in clear negative temperature influence on farrowing rate, but no such influence was specifically tested in this study. Zheng at al. [29] investigated possibilities of different thawing solutions for frozen semen of boars to improve sperm motility and sow reproductive performance and concluded that Dulbecco's modified Eagle medium + Fatal bovine serum (D-F) freezing diluent can improve sperm motility of frozen boar semen after thawing, prolong sperm survival time, and increase sow reproductive performance. They also stated that it's necessary to further verify the effects of fatal bovine serum on sperm motility, survival time, metabolic mode, and fertilization ability at room temperature, low temperature, and ultralow temperature.

Chromatin instability in boar sperm is associated with plasma membrane changes, which hinder the binding of chromatin-unstable sperm to the oviductal epithelia, thereby reducing their number in the functional sperm reservoir of the female reproductive tract [30]. Chromosome damage greater than 2.1% had negative impact, reducing the number of piglets per litter [4]. Results of the present study indicated much higher average values than the value of 2.1% reported by Boe-Hansen et al. [4], but no significant difference was noted between groups, probably because small sample size cannot support evident numerical distinction between groups BAS and AAS (9.84% vs. 6.88%, respectively). However, sows from group AAS had higher farrowing rate as well as more piglets born per litter. Didion et al. [31] proposed that DNA fragmentation index higher than 6% places certain commercial boars into a statistical group that produces a reduced farrowing rates and average number of piglets born, but they also stated that this threshold may be revaluated do to the limited number of boars used in the study.

According to Tsakmakidis et al. [8] and Boe-Hansen et al. [4], significant relationship exists between the percentage of live morphologically normal spermatozoa with stable chromatin structure and the farrowing rate and litter size in sows. In the current study, data presented in Table 6 shows that sows from group AAS, which was found by PNA-FITC/PI and SCSA tests to have more total live, live intact, less total acrosome damaged sperm cells and less cells with damaged chromatin structure, had significantly higher farrowing rate as well as bigger litter size. Tested correlations showed significant relationships between live sperm with damaged acrosome percentage and both farrowing rate and litter size, as well as between number of progressive sperm cells per dose and farrowing rate. However, tested chromatin structure differences between groups were not found to be significantly correlated to fertility traits, which is different from the study of Tsakmakidis et al. [8] who found correlation to farrowing rate. Therefore, more research is needed to confirm subsequent connection and correlation intensity to farrowing rate and number of piglets per litter. As proposed by Martinez [30], to evaluate effectively the fertilization capacity of semen, based on chromatin instability, it would be advisable to use the Hannover

Gilt Model [32], which uses fertilization rate, the rate of normal embryos and accessory sperm counts as tools to evaluate semen quality. Also, further research could be done in terms of using oxytocin for artificial insemination in order to increase farrowing rate in sows. Duzinski et al. [33] found that addition of oxytocin to seminal doses (5 IU to 100 mL of seminal dose) improved the farrowing rate after artificial insemination during the summer season. Supplementing semen with oxytocin when motility is reduced could perhaps insure higher farrowing rates as oxytocin stimulates uterine contractions. This plays a specific role in the transport of sperm, since these contractions accelerate the arrival of spermatozoa at their destination.

5. Conclusion

As shown, prolonged transport negatively affected sperm quality in terms of motility and chromatin structure stability. The decrease in motility was more than expected. The average concentration of motile spermatozoa in a dose for artificial insemination for sows was two times less the value manufacturer stated in their quality declaration. These results can serve as feedback information that could/should be of interest and considered by semen producers/sellers as a minimal required parameters for ongoing trade.

Fertility of sows was affected by semen quality in terms of concentration and sperm motility parameters, as well as ratio of live sperm cells population with damaged acrosome, as determined by flow cytometry.

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