Liquid Storage of Boar Semen Using Commercial Extenders

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Abstract

The research was focused on the modern evaluation of boar semen parameters, using computer assisted sperm analysis (CASA), before and after liquid storage at 15°C. Semen was collected from 15 sexually mature boars by manual stimulation. Macroscopical and microscopical evaluation of semen was performed, followed by a detailed CASA analysis of all ejaculates. Subsequently, semen was diluted using 4 different extenders (Semtest, Androstar, MIII and Cronos) and stored at 15°C for 24 hours. Next, evaluation of progressive motility, total motility and viability was performed, using the same CASA system. All experiments were performed in triplicates and results were statistically analyzed. The average progressive motility after 24 hours was as follows: 69.56 ± 6.38 for MIII, 65.92% ± 2.63 for Semtest, 67.07% ± 5.58 for Androstar Plus and 68.93% ± 3.40 for Cronos. The viability results after 24 hours were: 86.34% ± 1.38 for Semtest extender, 93.55% ± 3.38% for Androstar Plus, 89.19% ± 3.42 for MIII and 91.35% ± 2.37 for Cronos. The findings of this study suggest that the use of commercial extenders for short-term storage of swine semen is important in order to increase sperm longevity with minimal sperm function deterioration.

Keywords: boar semen, CASA, liquid storage

Introduction

Artificial insemination with freshly diluted or liquid stored boar semen led to the improvement of several important morphological features, but especially to increase in production. Several recent studies were made to show the influence of various extenders as well as bacterial contamination and supplementation with different antioxidants on the quality of liquid stored boar ejaculates at 15-17°C (Fang *et al.*, 2017; Schultze *et al.*, 2017; Pinart *et al.*, 2017).

Classical sperm analysis is based on the subjective evaluation of spermatoc parameters, such as motility, viability and morphology. Currently, computerized semen analysis is used in many andrology laboratories and offers much more accurate data (Budworth *et al.*, 1988; Cooper *et al.*, 1999; Verstegen *et al.*, 2002).

Compared to the established methods of analysis, the images obtained with these systems allow analysis of several spermatoc parameters, such as: concentration, motility, morphology, degree of seminal DNA fragmentation, and especially seminal morphometry. Automatic image measurement systems in general and the Sperm Class Analyzer (SCA), in particular, are based on the sequential analysis of captured images coming from a microscope. These images are subsequently digitized, the sperm cells are identified and for each spermatozoon the trajectory is established. Eventually, results are generated in the form of reports. These results reflect a series of measurements derived from the two-plane analysis of the spermatozoa head over time (Foote, 2002; Cremades *et al.*, 2005).

Figure 1. Semen collection in boars by manual stimulation

The result of the analysis depends on a number of factors such as the laboratory's climatic conditions, the semen volume, the storage conditions, the type of counting chamber, the status of the sperm population (fresh or frozen samples, concentration, extender used), as well as measurement systems (optics used, illumination type, instrument calibration) (Holt *et al.*, 2007).

CASA systems are capable to objectively determine the morphological parameters and classification of spermatozoa in morphological categories, such as: normal and abnormal, spermatozoa with primary, secondary abnormalities, and also allow differentiation of different sperm subpopulations depending on motility or head shape (Mortimer, 2000).

Therefore, the current research was focused on the modern evaluation of boar semen parameters, using computer assisted sperm analysis (CASA), before and after liquid storage at 15°C.

Materials and methods

Semen was collected from 15 sexually mature boars, belonging to four different breeds

(PIC337, Landrace, Pietrain and Great White) by manual stimulation (Figure 1). Macroscopical and microscopical evaluation of semen was performed followed by a detailed CASA (Sperm Class Analyzer® CASA System, Microoptic) analysis of all ejaculates.

Criteria for the inclusion of ejaculates in research were based on the following characteristics of the fresh material: white color, lactescent consistency, good sperm motility and viability.

Criteria for exclusion of ejaculates from the research referred to modified (abnormal) color, lack of motility and sperm viability.

Subsequently, each ejaculate was diluted using 4 different extenders (Semtest, Androstar, MIII and Cronos) and stored at 15°C for 24 hours. Dilution was carried out in two stages, namely, in the first instance, the initial dilution was carried out immediately after semen collection and sampling for the required examinations. Semen was introduced into double-walled graded flasks over which the same amount of diluent was added at 37 ° C (1:1 v/v). After 20-30 minutes, which is required for equilibration at laboratory

Table 1. Results of fresh semen analysis

Boar no.	Volume (ml)	Motility (%)	Viability (%)	Concentration
B1	230	85%	85%	0.645x10 ⁹
B2	219	95%	95%	0.578x10 ⁹
B3	198	75%	75%	0.721x10 ⁹
B4	267	75%	75%	0.429x10 ⁹
B5	200	90%	90%	0.639x10 ⁹
B6	365	85%	85%	0.768x10 ⁹
B7	120	90%	90%	0.659x10 ⁹
B8	416	75%	75%	0.468x10 ⁹
B9	180	75%	75%	0.340x10 ⁹
B10	182	75%	75%	0.623x10 ⁹
B11	280	90%	90%	0.835x10 ⁹
B12	250	85%	85%	0.779x10 ⁹
B13	220	85%	85%	0.543x10 ⁹
B14	340	90%	90%	0.876x10 ⁹
B15	352	90%	90%	0.359x10 ⁹

temperature, final dilution was carried out with the extender at laboratory temperature.

The final dilution was made taking into account the following formula:

$$GD = \frac{N \times a}{n}$$

where: GD=dilution degree; N=total number of spermatozoa from the ejaculate; a=motility; n=number of spermatozoa/insemination dose.

Each insemination dose was prepared at 90 ml with a concentration of 2x10⁶ spermatozoa/ml.

Next, evaluation of progressive motility, total motility and viability was performed, using the same CASA system. For the motility analysis, the Leja 20µl chambers and the heating plate were used. An aliquot of 3µl was analyzed from each sample. The analysis was carried out by phase contrast, using the 10x lens and the green filter. Each captured field had a capacity of 512 x 512 x 8, providing a digitized image of 264,144 pixels and 256 gray levels. The image resolution was 0.15 and 0.14 µm/pixel on the horizontal and vertical axis, respectively. A total of 500 spermatozoa were analyzed from each sample. After completion of the analysis process, the system software automatically performs the detection of cross-trajectories, the calculations for the information obtained from each analyzed image, as well as all the fields, in order to calculate the average and the final result of the sample.

Viability assessment was performed using the FluoVit (Microoptic) kit. Thus, 10 µl of Blue dye (FluoVit, Hoechst and Trihydrochloride trihydrate) solution, preheated to 37°C, was added to 10 µl of diluted sperm. The sample was incubated for 5 minutes at room temperature, after which 1 µl of Red dye (FluoVit, propidium iodide) was added. The samples thus prepared were evaluated using the Nikon Eclipse C1 microscope, the Vitality module with the DAPI filter and the 20x lens. A total of 100 spermatozoa/sample were evaluated.

The entire experimental protocol was performed 3 times, at the second and third repeats using only diluents and concentrations with satisfactory results in the first experiment. The results were statistically analyzed using the GraphPad Prism program, and the results were expressed as averages ± standard error (SE).

Results and discussions

The evaluation of fresh sperm was made in order to assess the quality of the ejaculates, which is essential for obtaining the best results after dilution and liquid storage. The evaluation of semen was focused on the determination of macro- and microscopic parameters specific to each individual boar, as follows (Table 1):

Based on these results, the ejaculates were diluted and transported to the Andrology Laboratory of the Faculty of Veterinary Medicine Cluj-Napoca. Samples were evaluated using the

Table 2. Results obtained for fresh semen using the Sperm Class Analyzer CASA system

Boar no.	Concentration M/ml	No. spermatozoa/ sample	Progressive motility %	Total motility %	Vitality %
B1	19.73	1874.35	40.95	78.23	79.02
B2	16.44	1561.8	83.64	96.36	98.44
B3	25.25	2474.5	59.41	86.27	89.38
B4	27.12	2712	57.65	89.55	90.78
B5	37.3	3730	74.96	90.05	93.33
B6	30.68	3066	82.85	93.45	94.12
B7	24.13	2413	70.72	87.45	96.67
B8	27.33	2733	75.5	90.17	93.33
B9	27.78	2876	82.53	95.05	96.63
B10	30.75	3075	81.99	93.1	94.43
B11	17.4	1740	78.01	83.65	86.36
B12	18.19	1890	76.16	85.94	90.37
B13	20.29	2029	82.28	89.18	87.18
B14	16.76	1676	76.69	83.03	85.19
B15	23.45	2345	52.45	69.54	73.58

Table 3. Overall analysis of average total motility after 24 h of storage

24 h	Total motility			
	Semtest	Androstar Plus	MIII	Cronos
PIC 337	85.86%	90.77%	83.2%	91.7%
Landrace	87.89%	91.34%	89.21%	89.34%
Pietrain	81.24%	93.32%	91.21%	90.56%
Great White	81.32%	89.32%	87.45%	87.32%
Total	84.07% ± 1.65	91.18% ± 1.65	87.76% ± 3.41	89.73% ± 1.87

CASA system and the results were statistically evaluated. In order to perform the statistical analysis, the samples were evaluated in triplicate.

The average results are shown in Table 2.

As shown above, total and progressive motility was superior for boars B2, B6, B9, B10, B13. Nevertheless, all of the ejaculates met the quality criteria for semen undergoing the storage process.

The most significant samples for each extender and breed were identified for re-evaluation after 24 hours. Total motility, progressive Type-A motility, progressive B-type motility, C-type non-progressive motility, immotile D-type cells as well as viability were considered. The results obtained for every breed and extender are presented in the tables below:

The average total motility after 24 hours was $88.19\% \pm 0.93$, while the best results were recorded for Androstar Plus where the average total motility was $91.18\% \pm 1.65$, followed by

the Cronos extender where average motility was $89.73\% \pm 1.87$. Average motility for samples diluted with MIII was $87.76\% \pm 3.41$, and for Semtest extender the average motility was $84.07\% \pm 1.65$. Beside total motility, other sperm parameters were evaluated in each breed, as follows:

Overall analyzes of the results indicate that semen collected from the PIC 337 breed had the best qualities, where the average progressive motility after 24 hours of storage was $70.99\% \pm 2.66$, compared to the rest of the breeds where progressive motility was $68.99\% \pm 1.84$ for Landrace, $68.25\% \pm 2.87$ for Pietrain and $63.24\% \pm 2.35$ for Great White. Nevertheless, the differences are not statistically significant.

Regarding the extenders used for semen dilution, the MIII extender showed best results in what progressive motility was concerned (the average progressive motility after 24 hours of liquid storage was 69.56 ± 6.38). The average

Table 4. Sperm parameters in PIC337 after 24h of storage

PIC 337				
	Semtest	Androstar Plus	MIII	Cronos
Progressive motility%	67.76	74.91	68.2	73.09
Non-progressive motility%	18.1	15.85	15	18.61
Non-motile%	14.14	9.23	16.8	8.3
Progressive motility type A%	18.97	20.03	19.6	11.8
Medium progressive type B%	48.79	54.88	48.6	61.21
Non progressive type C%	18.1	15.85	15	18.61
Non-motile type D%	14.14	9.23	16.8	8.3
Viability%	86.32	91.21	84.45	93.45

Table 5. Sperm parameters in Landrace after 24h of storage

Landrace				
	Semtest	Androstar Plus	MIII	Cronos
Progressive motility%	65.21	63.7	78.43	68.65
Non-progressive motility%	12	14.43	4.32	12.65
Non-motile%	10.6	12.64	6.46	8.04
Progressive motility type A%	20.21	24.16	23.21	25.3
Medium progressive type B%	45	39.54	55.22	43.35
Non progressive type C%	12	14.43	4.32	12.65
Non-motile type D%	10.6	12.64	6.46	8.04
Viability%	88.32	93.23	90.35	90.2

Table 6. Sperm parameters in Pietrain after 24h of storage

Pietrain				
	Semtest	Androstar Plus	MIII	Cronos
Progressive motility%	68.21	67.2	68.4	69.21
Non-progressive motility%	8.3	13.5	12.6	12.8
Non-motile%	4.73	12.62	10.21	8.55
Progressive motility type A%	24.3	21.09	25.21	21.3
Medium progressive type B%	43.91	46.11	43.19	47.91
Non progressive type C%	8.3	13.5	12.6	12.8
Non-motile type D%	4.73	12.62	10.21	8.55
Viability%	85.32	98.43	92.54	93.21

progressive motility for Semtest was 65.92% $\pm$ 2.63, while the Androstar Plus and Cronos averages were 67.07% $\pm$ 5.58 and 68.93% $\pm$ 3.40, respectively.

The overall analysis of sperm cell viability results after 24 hours can be summarized as follows:

For the Semtest extender, the average viability was 86.34% $\pm$ 1.38, 93.55% $\pm$ 3.38 for the Androstar Plus, 89.19% $\pm$ 3.42 for MIII and 91.35% $\pm$ 2.37 for Cronos. All four tested extenders have the ability to maintain the viability

of sperm cells within normal limits, but statistically significant better results were obtained with Androstar Plus.

The overall better results obtained for Androstar Plus are probably due to the fact that this is a long-term extender for boar semen which also contains a CSP (cold shock protector).

The Computer Assisted Sperm Analysis (CASA) system provides information on the motility of each sperm cell in particular by processing sperm electronic images, reconstructing the trajectory of each sperm cell, simultaneously and objectively

Table 7. Sperm parameters in Great White after 24h of storage

Great White				
	Semtest	Androstar Plus	MIII	Cronos
Progresive motility%	62.5	62.5	63.21	64.78
Non-progressive motility%	8.9	13.6	12.3	11.2
Non-motile%	9.92	13.22	11.94	11.34
Progresive motility type A%	29.2	26.3	28.1	21.5
Medium progresive type B%	33.3	36.2	35.11	43.28
Non progresive type C%	8.9	13.6	12.3	11.2
Non-motile type D%	9.92	13.22	11.94	11.34
Viability%	85.43	91.34	89.43	88.56

Table 8. Overall analysis of average progresive motility after 24 h of storage

	Semtest	Androstar Plus	MIII	Cronos
PIC 337	67.76%	74.91%	68.2%	73.09%
Landrace	65.21%	63.7%	78.43%	68.65%
Pietrain	68.21%	67.2%	68.4%	69.21%
Great White	62.5%	62.5%	63.21%	64.78%

Table 9. Overall analysis of average viability after 24h of storage

	Semtest	Androstar Plus	MIII	Cronos
PIC 337	86.32%	91.21%	84.45%	93.45%
Landrace	88.32%	93.23%	90.35%	90.2%
Pietrain	85.32%	98.43%	92.54%	93.21%
Great White	85.43%	91.34%	89.43%	88.56%

evaluating each sperm component so that minor changes in sperm motility can be detected (Estienne *et al.*, 2007, Gil *et al.*, 2009).

Regarding the working methods, we consider that the computerized analysis of semen was an extremely useful method, being quick, easy to execute and very precise, removing the subjectivity in the evaluation of the parameters. The major disadvantage is the cost of purchasing the equipment, but also of the consumables, but in the case of a specialized laboratory processing a large amount of semen daily, it is a perfectly justified investment.

In order to produce pigs for fattening and to meet the needs of pork consumers, a large number of inseminations are carried out and management practices aim at maximizing success rates. This provides the opportunity to analyze large sets of data from optimized and standardized field conditions to determine whether the fertilization capacity of semen can be predicted by CASA parameters (Flowers, 2002).

Thus, the study confirmed that the method is very objective, demonstrating that the CASA system is very valuable for the selection of boar ejaculates, and implicitly for the selection of boars.

In the case of extenders, the findings of this study suggest that their use for short-term storage of swine semen is important to increase sperm longevity with minimal sperm function deterioration.

According to the storage conditions used in this study, all four types of diluents possess the necessary qualities to preserve the metabolic activity and integrity of the swine sperm membrane during short-term storage (up to 7 days) of liquid semen.

Conclusion

The findings of this study suggest that the use of commercial extenders for short-term storage of swine semen is important in order to increase sperm longevity with minimal sperm function deterioration.

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References

1. Budworth PR, Amann RP, Chapman PL (1988). Relationships between computerized measurements of motion of motion of frozen-thawed spermatozoa and fertility. *Journal of Andrology*, 9: 41-54.
2. Cooper TG, Atkinson AD, Nieschlag E (1999). Experience with external quality control in spermatology. *Human Reproduction*, 14 : 765-769.
3. Cremades T, Roca J, Rodriguez-Martinez H, Abaigar T, Vazquez JM, Martinez EA (2005). Kinematic Changes During the Cryopreservation of Boar Spermatozoa. *Journal of Andrology*, 26: 610-618.
4. Estienne MJ, Harper AF, Day J (2007). Characteristics of sperm motility in boar semen diluted in different extenders and stored for seven days at 18°C. *Reproductive Biology*, 7: 221-231.
5. Fang Q, Wang J, Hao YY, Li H, Hu JX, Yang GS, Hu JH (2017). Effects of iodine methionine on boar sperm quality during liquid storage at 17°C. *Reprod Dom Anim*, 52: 1061-1066.
6. Flowers WL (2002). Increasing fertilization rate of boars: Influence of number and quality of spermatozoa inseminated. *J Anim Sci*, 80: E47-E53.
7. Foote RH (2002). The history of artificial insemination: Selected notes and notables. *J Anim Sci*, 80: 1-10.
8. Gil MC, García-Herreros M, Barón FJ, Aparicio IM, Santos AJ, García-Marín LJ (2009). Morphometry of porcine spermatozoa and its functional significance in relation with the motility parameters in fresh semen. *Theriogenology*, 71: 254-263.
9. Holt WV, O'Brien J, Abaigar T (2007). Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. *Reproduction, Fertility and Development*, 19: 709-718.
10. Mortimer TS (2000). CASA – Practical Aspects. *Andrology Lab Corner*, 515-524.
11. Pinart E, Domènech E, Bussalleu E, Yeste M, Bonet S (2017). A comparative study of the effects of *Escherichia coli* and *Clostridium perfringens* upon boar semen preserved in liquid storage. *Animal Reproduction Science*, 177: 65-78.
12. Schulze M, Ammon C, Schaefer J, Luther A-M, Jung M, Waberski D (2017). Impact of different dilution techniques on boar sperm quality and sperm distribution of the extended ejaculate. *Animal Reproduction Science*, 182: 138-145.
13. Verstegen J, Iguer-Ouada M, Onclin K (2002). Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology*, 57: 149-179.