Scientific Annals of Polish Society of Animal Production - Vol. 11 (2015), No 3, 47-53

The use of new antibiotics for preservation of boar semen in liquid form*

Magdalena Bryła, Monika Trzcińska

National Research Institute of Animal Production,
Department of Biotechnology of Animal Reproduction,
ul. Krakowska 1, 32-083 Balice k. Krakowa

The aim of this study was to prepare a modification of the diluent used for liquid storage of boar semen, containing the antibiotics polymyxin B, florfenicol or daptomycin. The study was conducted in order to obtain a diluent enabling semen preserved in liquid form for six days to retain its high biological value. Semen from six boars (30 ejaculates) was diluted in a control extender (Biosolwens Plus) and extenders R1, R2 and R3 containing polymyxin B, florfenicol and daptomycin, respectively. The quality of the stored semen was evaluated on the basis of sperm motility, using a CASA system (TM: total motility; PM: progressive motility) and on the percentage of viable sperm (YO-PRO-1$^-$/PI$^-$), sperm with apoptotic-like changes (YO-PRO-1$^+$/PI$^-$), sperm with mitochondrial activity (JC-1$^+$) and sperm with DNA fragmentation (TUNEL$^+$). Our research showed that supplementation with 200 µg florfenicol ensures the highest quality of boar semen during 6-day storage.

KEY WORDS: boar / semen / antibiotics / YO-PRO-1 / JC-1 / TUNEL

Artificial insemination is an increasingly widespread method in pig breeding, as it enables intensification of production and maximum exploitation of valuable breeding boars. Mainly semen preserved in liquid form is using for insemination, and depending on the extender used, the degree of dilution, and storage conditions, it can remain suitable for several days. Problems persist, however, involving a rapid decline in the fertilization value of semen with the passage of time after dilution and a reduction in semen quality in boars maximally exploited and selected for favourable characteristics. A number of changes take place in the ejaculate during storage, including a reduction in sperm motility, changes in cell membrane permeability, a decrease in mitochondrial transmembrane potential, and an increase in sperm DNA fragmentation [3, 10, 11]. Our previous research has shown an increase with storage time in the percentage of sperm

***The study was carried out as part of the statutory activity of the National Research Institute of Animal Production in Balice, subtask 02-5.07.1**

with low mitochondrial transmembrane potential and in the population of apoptotic sperm with disturbed cell membrane integrity [10, 11]. A reduction in the fertilization capacity of semen during storage may also be linked to bacterial infections. Bacterial contamination is routinely observed during semen collection and storage. Maroto Martin et al. [8] reported the presence of bacterial strains in 62.5% of fresh ejaculates and 79% of insemination doses. A high level of bacterial contamination in semen is associated with an increase in agglutination, acrosome damage, decreased sperm motility, and shortened semen storage time [2]. For this reason, in insemination practice extenders are commonly supplemented with antibiotics. However, while antibiotics inhibit the growth of pathogenic bacteria, they are not without impact on semen quality [9]. It has been demonstrated that the presence of antibiotics may negatively affect the motility and viability of sperm cells and the structure of cell membranes [1]. Therefore attempts are made to find antibiotics which eliminate bacteria without significantly reducing the biological value of semen.

At the same time, research on improving techniques for liquid preservation of boar semen aims at precise diagnosis of semen quality parameters, which will enable assessment of the extenders used. Currently available methods of semen quality analysis are increasingly based on characteristic morphological and biochemical changes indicating apoptotic changes in the sperm cell.

The aim of the study was to modify the composition of an extender using new antibiotics to enable preservation of boar semen for six days without reducing its biological value.

Material and methods

Antibiotics which have not previously been used in preservation of boar semen were tested: polymyxin B, florfenicol and daptomycin. At the same time, in addition to basic semen quality parameters, new evaluation methods were used to determine the effect of these antibiotics on the biological value of preserved semen. These methods are based on analysis of apoptotic changes using the fluorochrome YO-PRO-1, measurement of mitochondrial transmembrane potential ($\Delta\Psi$) using JC-1 dye, and evaluation of the degree of DNA fragmentation by the TUNEL method.

The study was conducted on semen collected from 5 boars (6 ejaculates from each boar) used for insemination at the Boar Exploitation Station in Klecza Dolna. The boars were housed in standard conditions and fed complete mixed rations, with permanent access to water. Semen was collected twice a week from October to November from crossbreeds (Polish Landrace and Polish Large White) with a body weight of 254.3 ±4.2 kg, at the age of 17.9 ±0.8 months. Semen was collected by the manual method. Following preliminary evaluation under a microscope, only ejaculates in which 80% of sperm had normal morphology and progressive motility exceeding 80% were used for further analysis.

The semen was diluted in Biosolwens Plus (Biochefa, Poland) as a control extender and in extenders R1, R2 and R3, in which 200 µg of gentamicin was replaced with polymyxin B (200 µg), florfenicol (200 µg), and daptomycin (200 µg), respectively. The extenders were prepared in sterile conditions in a laminar flow cabinet. The semen was diluted to obtain a standard insemination dose of $2.5x10^9$ sperm cells in 80 ml of extender, and then the semen was stored at 15ºC in a chill cabinet for six days. Sperm motility and apoptotic changes were evaluated immediately after dilution (day 0) and on the 3rd and 6th day of storage. Sperm motility was assessed using the CASA system (Computer Assisted Semen Analysis), determining total motility (TM, %) and progressive motility (PM, %). Apoptotic changes were evaluated on the basis of changes in cell membrane permeability using the fluorochrome YO-PRO-1 (Vybrant Apoptosis Assay Kit≠4, Molecular Probes, USA). For this purpose samples containing $1x10^6$ sperm cells were washed in buffered saline solution (PBS) and centrifuged for 10 minutes. Following removal of the supernatant the sperm were placed in PBS solution containing 2 µl YO-PRO-1 and 1 µl propidium iodide (PI). Incubation was carried out in the dark, at room temperature, for 20-30 minutes. Mitochondrial transmembrane potential (ΔΨm) was measured using JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide) (Molecular Probes, USA). The semen ($1x10^6$ sperm cells) was suspended in PBS solution without calcium and magnesium ions and then centrifuged for 10 min. Following removal of the supernatant the sperm were again placed in PBS solution, containing 1 µl JC-1. The samples were incubated for 10 min in a water bath at 37°C. An In Situ Death Detection Kit (Roche Diagnostics, Germany) was used to determine the degree of sperm DNA fragmentation. Semen ($1x10^6$ sperm cells) was suspended in PBS solution, centrifuged for 10 minutes, and following removal of the supernatant, fixed in 200 µl 1% paraformaldehyde for 10 minutes at room temperature and then permeabilized for 15 minutes with 0.1% Triton X-100. The samples were washed three times and then incubated in 50 µl of reaction mixture for 60 minutes. To remove unincorporated nucleotides the samples were washed twice in PBS solution and transferred to VECTASHIELD solution with DAPI (Vector Laboratories, Burlingame, CA).

Individual subpopulations of cells (200 cells per sample) were observed under a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan) using appropriate filters. Fluorescence assessment of sperm quality was based on the percentage of sperm exhibiting apoptotic changes ($YO\text{-}PRO\text{-}1^+/PI^-$), the percentage of live sperm ($YO\text{-}PRO\text{-}1^-/PI^-$), the percentage of sperm with high mitochondrial transmembrane potential ($JC\text{-}1^+$), and the percentage of sperm exhibiting DNA fragmentation ($TUNEL^+$).

The results were analysed statistically by one-way analysis of variance – ANOVA (according to the formula $y_{ij} = \mu + a_i + e_{ij}$, where y – variable tested, μ – mean value, a_i – day of semen evaluation, type of extender used, e_{ij} – random error) using Statistica 10 software (StatSoft, Tulsa, USA). Significance of differences between means in groups was estimated by Duncan's Multiple Range Test. Differences between parameters at $P<0.01$ were considered statistically significant.

Results and discussion

The results obtained pertaining to the quality of semen stored for 6 days in the extenders tested are presented in the table.

The study showed that storage of semen for three days does not cause differences in the percentage of motile sperm, either in total motility (TM) or progressive motility (PM), between the extenders tested. The results differ from those reported by Kommisrud et al. [7] and De Ambrogi et al. [4], who after just 48 hours and after 102 hours of storage observed a significant decrease in sperm motility. In the present study, it was not until day 6 that the percentages of sperm with total motility in the control (51.2 ±5.4) and extender R1 (55.5 ±4.7) were statistically significant different ($P<0.01$) from the percentages observed for extenders R2 (64.9 ±3.7) and R3 (62.9 ±4.6). Comparison of the percentages of sperm with progressive motility (PM) between extenders showed the highest value, significantly statistically different from the others, for the extender containing daptomycin (61.5 ±4.6).

A study by Althouse and Lu [2] showed that the presence of bacteria in an extender induces apoptotic changes in semen, which reduces its biological value. Therefore assessment of these changes seems to be a crucial criterion in evaluating the suitability of extenders for long periods of semen storage. In the present study, apoptotic changes were assessed using the fluorochrome YO-PRO-1/PI, which detects characteristic micropores in the outer cell membrane of spermatozoa. The results indicate that statistically significant differences between extenders are not observed until the sixth day of storage, and induction of apoptotic changes is determined by the antibiotic used. Wysocki et al. [12] also found a link between the type of extender used and the degree of apoptotic changes during storage of boar semen at temperatures above 0. In our study the lowest percentage of sperm cells exhibiting apoptotic changes ($YO\text{-}PRO\text{-}1^{+}/PI^{-}$) and the highest percentage of living cells ($YO\text{-}PRO\text{-}1^{-}/PI^{-}$) were observed in the semen preserved in the extender containing florfenicol (R2). The highest level of apoptotic changes in sperm (16.4 ±3.2) was observed in the extender supplemented with 200 µg of daptomycin.

Changes in mitochondrial transmembrane potential were measured using the dye JC-1, which accumulates in living cells in the form of aggregates visible under a fluorescence microscope as orange-red fluorescence. The percentages of cells with high mitochondrial transmembrane potential ($JC\text{-}1^{+}$) in the extenders are shown in the table. On the sixth day of storage the highest percentage of sperm with high $\Delta\Psi m$ was noted in the extenders supplemented with florfenicol (63.4 ±5.6) and daptomycin (62.9 ±4.8) ($P<0.01$). The data indicate that as storage time increased, the percentage of sperm with high mitochondrial transmembrane potential decreased. Such a correlation was observed in an earlier study [10], and is also confirmed by results obtained by Dziekońska et al. [5].

The study found no increase in DNA fragmentation in the semen stored for a period of 6 days. This result differs from that reported by Boe-Hansen et al. [3], who observed an in-

Table
Results of assessment of sperm quality during liquid storage

Sperm parameters	Day of storage	Extenders			
		control	extender 1	extender 2	extender 3
Total motility (%)	0	91.3 ±5.3	95.4 ±7.1	91.8 ±6.5	94.3 ±5.2
	3	84.2 ±4.9	84.4 ±6.2	87.9 ±6.4	86.5 ±6.1
	6	51.2 ±5.4^{A}	55.5 ±4.7^{A}	64.9 ±3.7^{B}	62.9 ±4.6^{B}
Progressive motility (%)	0	88.6 ±3.3	91.3 ±6.4	89.3 ±7.1	90.1 ±7.4
	3	74.5 ±5.6	76.7 ±5.4	75.4 ±9.5	77.0 ±4.3
	6	53.9 ±5.1^{A}	54.3 ±5.9^{A}	55.9 ±3.7^{A}	61.5 ±4.6^{B}
Viable spermatozoa (YO-PRO-1^{-}/PI^{-}) (%)	0	89.4 ±6.1	90.3 ±7.2	91.3 ±8.1	91.6 ±4.5
	3	74.2 ±3.7	76.4 ±6.1	75.4±7.2	76.2 ±7.3
	6	45.3 ±6.8^{A}	43.4 ±8.6^{A}	57.2 ±6.3^{B}	49.7 ±4.3^{A}
Viable spermatozoa with apoptotic-like changes (YO-PRO-1^{+}/PI^{-}) (%)	0	4.5 ±1.1	4.0 ±0.9	3.8 ±0.5	4.0 ±1.3
	3	6.4 ±2.5	7.3 ±0.6	5.9 ±2.4	8.4 ±2.0
	6	11.3 ±3.6^{A}	11.9 ±4.1^{A}	6.1 ±1.8^{B}	16.4 ±3.2^{C}
Mitochondria with high ΔΨm (JC-1^{+}) (%)	0	92.5 ±6.7	94.4 ±5.3	94.8 ±3.3	93.9 ±2.7
	3	79.1 ±5.3	83.4 ±6.9	84.0 ±5.1	84.9 ±4.0
	6	52.5 ±3.2^{A}	53.6 ±6.1^{A}	63.4 ±5.6^{B}	62.9 ±4.8^{B}
TUNEL^{+} (%)	0	1.9 ±0.1	1.8 ±0.1	1.9 ±0.3	2.0 ±0.2
	3	2.1 ±0.4	1.9 ±0.7	2.2 ±0.1	2.1 ±0.4
	6	2.2 ±0.2	2.2 ±0.2	2.2 ±0.3	2.4 ±0.3

A, B, C – values expressed as mean ±SD with different superscript letters in rows differ significantly ($P<0.01$)

crease in DNA fragmentation after 72 h of storage at 18°C. A study by Fraser and Strzeżek [6] showed changes in DNA integrity during semen storage. These discrepancies may be due to different methods of assessing the degree of DNA fragmentation, different storage temperatures, or different extenders.

To sum up, storage of semen for a period of three days in extenders supplemented with gentamicin, polymyxin B, florfenicol or daptomycin does not reduce its quality in terms of any of the parameters tested. Statistically significant differences between extenders were not noted until the 6th day of semen storage. The extender supplemented with daptomycin ensured the highest percentage of sperm with progressive motility (PM). At the same time, the highest level of apoptotic changes (YO-PRO-1^{+}/PI^{-}) was noted in this extender. During 6-day semen storage the highest percentage of sperm with total motility (TM), of living sperm (YO-PRO-1^{-}/PI^{-}) and of sperm with high mitochondrial transmembrane potential (JC^{-}), together with the lowest percentage of sperm exhibiting apoptotic changes (YO--PRO-1^{+}/PI^{-}), was observed in the extender supplemented with florfenicol. The results of the study indicate that semen stored for six days in the extender with florfenicol retains its full biological value.

REFERENCES

1. ALAVI-SHOUSHTARI S.,M., AHMADI M., SHAHVARPOUR S., KOLAHIAN S., 2007 – Effects of tiamulin, neomycin, tetracycline, fluorophenicol, penicillin G, Linco-Spectin, erythromycin and oxytetracycline on controlling bacterial contaminations of the river buffalo (*Buballus bubalis*) semen. ***Pakistan Journal of Biological Science*** 10, 3200-3204.
2. ALTHOUSE G.C., LU K.G., 2005 – Bacteriospermia in extended porcine semen. ***Theriogenology*** 63, 573-584.
3. BOE-HANSEN G.B., ERSBØLL A.K., GREVE T., CHRISTENSEN P., 2005 – Increasing storage time of extended boar semen reduces sperm DNA integrity. ***Theriogenology*** 63, 2006-2019.
4. DE AMBROGI M., BALLESTER J., SARAVIA F., CABALLERO I., JOHANNISSON A., WALLGREN M., ANDERSSON M., RODRIGUEZ-MARTINEZ H., 2006 – Effect of storage in short-and long-term commercial semen extenders on the motility, plasma membrane and chromatin integrity of boar spermatozoa. ***International Journal of Andrology*** 29, 543-552
5. DZIEKOŃSKA A., FRASER L., MAJEWSKA A., LECEWICZ M., ZASIADCZYK Ł., KORDAN W., 2013 – Effect of commercial long-term extenders on metabolic activity and membrane integrity of boar spermatozoa stored at 17°C. ***Polish Journal of Veterinary Science*** 16, 517-525.
6. FRASER L., STRZEŻEK I., 2004 – The use of comet assay to assess DNA integrity of boar spermatozoa following liquid preservation at 5 degrees C and 16 degrees C. ***Folia Histochemica et Cytobiologica*** 42, 49-55.
7. KOMMISRUD E., PAULENZ H., SEHESTED E., GREVLE I.S., 2002 – Influence of boar and semen parameters on motility and acrosome integrity in liquid boar semen stored for five days. ***Acta Veterinaria Scandinavica*** 43, 49-55.
8. MAROTO MARTÍN L.O., CRUZ MUÑOZ E., DE CUPERE F., VAN DRIESSCHE E., ECHEMENDIA-BLANCO D., MACHADO RODRÍGUEZ J.M., BEECKMANS S., 2010 – Bacterial contamination of boar semen affects the litter size. ***Animal Reproduction Science*** 120, 95-104.

9. OKAZAKI T., MIHARA T., FUJITA Y., YOSHIDA S., TESHIMA H., SHIMADA M., 2010 – Polymyxin B neutralizes bacteria-released endotoxin and improves the quality of boar sperm during liquid storage and cryopreservation. ***Theriogenology*** 74, 1691-1700.
10. TRZCIŃSKA M., BRYŁA M., SMORĄG Z., 2008 – Effect of liquid storage on membrane integrity and mitochondrial activity: a new diagnostic method of evaluating boar sperm quality. ***Journal of Animal and Feed Sciences*** 17, 372-380.
11. TRZCIŃSKA M., BRYŁA M., SMORĄG Z., 2011 – Apoptotic-like changes in the spermatozoa of fresh and stored boar semen and the quality of embryos produced *in vivo*. ***Animal Reproduction Science*** 124, 90-97.
12. WYSOCKI P., ŁYJAK A., KORDAN W., 2013 – Analysis of proapoptotic changes in boar spermatozoa stored at 16°C in different short-term semen extenders. ***Bulletin of Veterinary Institute in Pulawy*** 57, 425-428.