Animal (2009), **3:2**, pp 269–274 © The Animal Consortium 2008
doi:10.1017/S1751731108003443

animal

Evaluation of swine fertilisation medium (SFM) efficiency in preserving spermatozoa quality during long-term storage in comparison to four commercial swine extenders

P. Fantinati, A. Zannoni, C. Bernardini, M. Forni, A. Tattini, E. Seren and M. L. Bacci†

Department of Veterinary Morphophysiology and Animal Production (DIMORFIPA), University of Bologna, Via Tolara di Sopra 50, Ozzano Emilia 40064 (BO), Italy

(Received 9 April 2008; Accepted 16 September 2008; First published online 28 October 2008)

In pig production, artificial insemination is widely carried out and the use of fresh diluted semen is predominant. For this reason, there are increasing interests in developing new extenders and in establishing the optimal storage conditions for diluted spermatozoa. In the last few decades, we utilised a homemade diluent (swine fertilisation medium (SFM)) for spermatozoa manipulation and biotechnological application as the production of transgenic pigs utilising the sperm-mediated gene transfer technique. The purpose of the present study is therefore to analyse the ability of SFM, in comparison to four commercial extenders, in preserving the quality of diluted boar semen stored at 16.5°C till 15 days. We utilised some of the main predictive tests as objectively measured motility, acrosome and sperm membrane integrity, high mitochondrial membrane potential and pH. Based on our in vitro *study, SFM could be declared as a good long-term extender, able to preserve spermatozoa quality as well as Androhep Enduraguard for up to 6 to 9 days and more.*

Keywords: boar, spermatozoa quality, extender, semen storage

Introduction

Artificial insemination (AI) is used routinely because it is simple, economical and successful (Vishwanath, 2003); it remains the main vehicle, together with embryo transfer, for the rapid dispersal of valuable genes and reduces the sexual transmission of diseases (Thacker *et al.*, 1984). The use of fresh diluted semen, preserved at 15 to 20°C, is predominant (99% AI carried out worldwide/year) while AI with frozen semen, due to the low sperm survival after thawing, accounts for only 1% (Gerrits *et al.*, 2005). For this reason, there has been increased interest in developing new extenders and in establishing the optimal storage conditions for diluted spermatozoa. In the last few decades, we utilised a homemade diluent (swine fertilisation medium (SFM)) for swine spermatozoa manipulation and biotechnological applications as the production of transgenic pigs utilising the sperm-mediated gene transfer technique (Lavitrano *et al.*, 2002; Lavitrano *et al.*, 2003), with good fertility results as well as in AI (Webster *et al.*, 2005) or surgical or laparoscopic inseminations (Fantinati *et al.*, 2005; Manzini *et al.*, 2006). Nevertheless, this SFM has never been evaluated methodically in comparison to other well-defined commercial diluents.

Even if extenders, in association with low temperature, can prolong the spermatozoa lifespan, the physiological senescence of sperm cells still cannot be completely avoided. Such a phenomenon can be composed of non-regulated capacitation-like modifications (Bailey *et al.*, 2000), structural and functional changes (Johnson *et al.*, 2000), loss of DNA integrity (Fraser and Strzezek, 2004) and membrane fatty acid peroxidation (Cerolini *et al.*, 2000); these modifications can be postponed by using various extenders (Huo *et al.*, 2002). Long-term extenders have some advantages; they allow better organisation at semen collection centres, long-distance transport and the possibility of conducting tests on the semen before use (Gadea, 2003); however the storage of semen for long time before insemination reduces the subsequent viability of spermatozoa in the female genital tract, requiring an accurate timing of AI (Johnson *et al.*, 2000).

The purpose of the present study was therefore to analyse the ability of SFM, in comparison to four commercial extenders, which have been extensively tested also in field studies, in preserving the quality and the longevity of diluted boar semen stored at 16.5°C for 15 days, by evaluating

† E-mail: marialaura.bacci@unibo.it

parameters representing some of the main predictive tests for boar semen, which are relevant for their fertilising ability.

Material and methods

Extenders and semen collection and processing

All extenders, Androhep® Enduraguard™ (AeG, long term), Beltsville Thaw Solution (BTS, short term), Merk III® (M III, short term), Androstar® (AN, economic long term, BSA free), were from Minitube (Tiefenbach, Germany) except for SFM (11.25 g glucose, 10 g sodium citrate-2H_2O, 4.7 g ethylene diamine tetra-acetic acid (EDTA)-2H_2O, 3.25 g citric acid-H_2O, 6.5 g Trizma, 6 g BSA, 1 g ampicillin sodium salt per liter, adjusted to pH 6.8). Extenders were prepared by dissolving powder in pyrogen-free water at least 12 h before use and stored at 4°C till use.

Sperm-rich fractions were collected using the gloved-hand technique from six boars (two Large White and four Duroc, 1 to 2 years of age), trained to mount an artificial sow and of proven fertility, during routine AI operations from December to February (six ejaculates/boar were used). At collection, sperm-rich fraction from a single boar was split into five aliquots, immediately diluted 1 : 1 with AeG, BTS, MIII, AN and SFM pre-warmed at 37°C. The samples were then brought to the laboratory within 30 min in an isothermal box and spermatozoa morphology and concentration were assessed, after spermatozoa immobilisation in 1.5 M NaCl solution, under a phase-contrast microscope at 400× using a Thoma counting chamber. Single AI doses (3×10^9 sperm/100 ml) were then prepared with the same extender formerly used and stored in a water bath at 16.5°C for 15 days, being gently shaken by inversion every 12 h. Spermatozoa, stored in the five different extenders, were sampled and analysed at 12 h and at 1, 3, 6, 9, 12 and 15 days of storage. The 0 time point analyses refer to the SFM-diluted ejaculates for all experiments.

Semen quality assessment: motility

A SpermVision™ CASA system (Minitube, Tiefenbach, Germany) was used to evaluate the overall motility (OM) and progressive motility (PM) of spermatozoa incubated in different extenders. The complete Sperm Vision package consisted of a microscope (Olympus CX31) equipped with a negative high phase-contrast ×20 objective, an integrated heating system, a digital black and white camera, a PC including a special graphics card and Sperm Vision software for the digitalization and analysis of the images. Sperm aliquots were incubated for 5 min at 37°C before analysis. A pre-warmed (37°C) Counting Chamber 'Standard Count' (20 μm depth; Leja Products Nieuw-Vennep, The Netherlands) was filled with 2 μl of semen sample and the track sampling time was 20 s. The instrument setting used was the following: number of frames acquired: 30; frame rate: 60 Hz; and minimum size–maximum size: 25 to 120 μm^2. For individual cell motility, we used the standard Sperm Vision boar setting provided by Minitube. The OM of the sample was calculated as the sum of the percentages of the local motile and progressive spermatozoa as compared with the total number spermatozoa identified. PM is the percentage of progressive spermatozoa as compared with the sum of the percentages of the local motile and progressive spermatozoa. All samples were evaluated by the same operator and at least 400 spermatozoa for each sample were tracked.

Semen quality assessment: staining

For SYBR-14/PI/JC-1 staining, the solutions were prepared as previously described (Garner *et al.*, 1997; Huo *et al.*, 2002). In total, 50 μl of diluted boar semen was incubated with 10 μl of the staining solution at 37°C for 30 min under light-proof conditions. A 10 μl drop was then examined at 400× magnification with a fluorescence microscope (Eclipse E600, Nikon, Japan). Sperm heads with intact membranes displayed a bright green fluorescence (viable spermatozoa), while cells with damaged membranes showed a red fluorescence. Spermatozoa stained with JC-1 displayed either a green fluorescence (low/medium mitochondrial membrane potential (MMP)) or a red–orange fluorescence in the mid-piece (high MMP or active mitochondria) (Garner *et al.*, 1997). At least 250 spermatozoa were scored for each treatment and time point. Coomassie blue staining was performed as previously described (Turba *et al.*, 2007). Spermatozoa with absent or damaged acrosomes showed negligible staining in the apical region. A minimum of 100 spermatozoa were counted using a phase-contrast microscope (Diaplan, Leitz, Germany) at ×600 magnification.

pH analyses

For pH analyses, a 5 ml aliquot was taken from each dose after gentle mixing. The pH was measured at 37°C by means of a Micro pH 2002 and a Phoenix electrode K0203 (Crison Instrument A.G., Baar, Switzerland) calibrated before every measure with pH 4.0 and pH 7.0 standard solutions. The 0 time point pH was measured at 37°C in raw extenders after at least 12 h of calibration.

Statistical analyses

The study used the split ejaculate technique; means for the six collections for each boar, for each storage time point and for each diluent were subjected to the statistical analysis. All data were tested for normal distribution prior to analysis. The percentages of viable spermatozoa, high MMP, spermatozoa with damaged acrosomes, OM, PM and pH were analysed using two-way ANOVA for repeated measure during time. *Post-hoc* comparisons among groups were made based on the Duncan test. Data are presented as mean ± s.e.

Correlations between the different parameters were calculated by means of the Pearson correlation coefficient with $P < 0.05$ as the significance level. Statistical analyses were performed using SPSS 8.0 (SPSS Inc., Chicago, IL, USA).

Results

The correlation coefficients among different parameters and their statistical significance are shown in Table 1.

Table 1 *Correlation coefficients of various quality parameters in boar semen extended with five different diluents during long-term storage at 16.5°C*

Parameters	Membrane integrity	High MMP	Damaged acrosome	pH	Overall motility	Progressive motility
Membrane integrity		0.866**	−0.402**	−0.277**	0.681**	0.700**
High MMP	0.866**		−0.362**	−0.389**	0.626**	0.593**
Damaged acrosome	−0.402**	−0.362**		ns	−0.482**	−0.510**
pH	−0.277**	−0.389**	ns		−0.221**	−0.175**
Overall motility	0.681**	0.626**	−0.482**	−0.221**		0.889**
Progressive motility	0.700**	0.593**	−0.510**	−0.175**	0.889**	

**$P < 0.01$.
MMP = mitochondrial membrane potential.

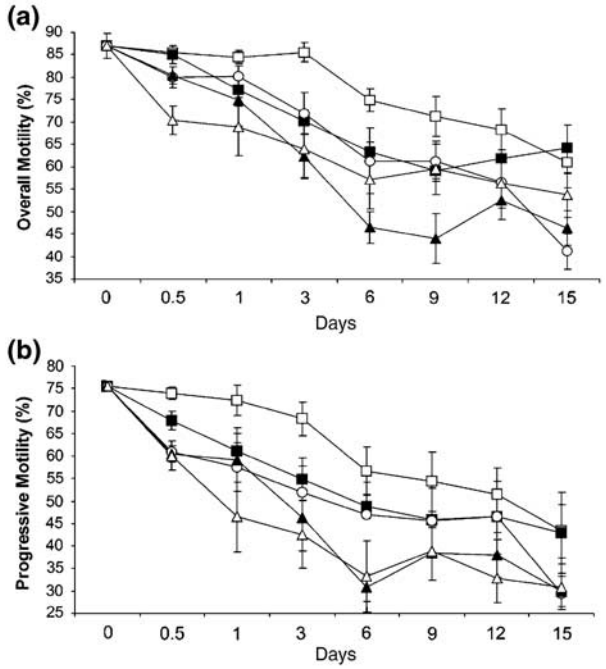

Figure 1 Overall (1a) and progressive (1b) motile spermatozoa, evaluated by SpermVision™ CASA (Minitube, Tiefenbach, Germany), during long-term storage (15 days) at 16.5°C (mean ± s.e.) in five different extenders. Diluent × time interaction for OM was $P = 0.001307$ and for PM was $P = 0.370744$. SFM □; AeG ■; BTS ○; AN ▲; M III △.

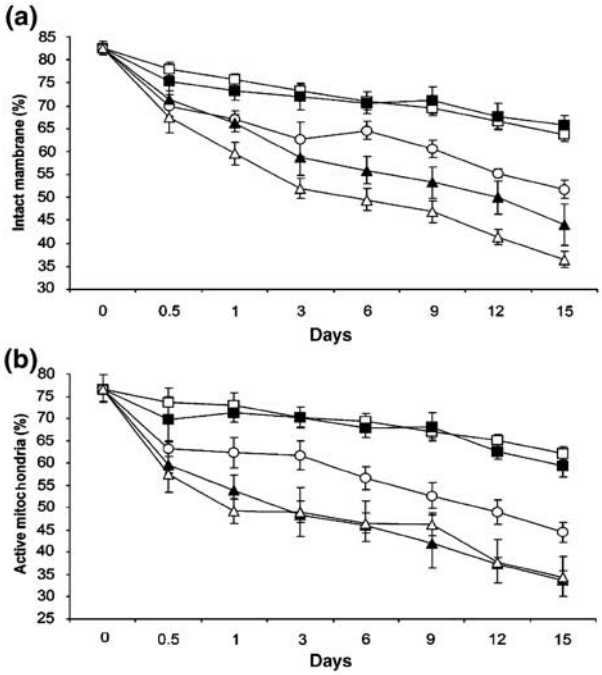

Figure 2 Spermatozoa with intact membranes (viable), evaluated by SYBR-14/PI staining (a) and spermatozoa with active mitochondria (high MMP), evaluated by JC-1 staining (b) during long-term storage (15 days) at 16.5°C (mean ± s.e.) in five different extenders. Diluent × time interaction for viability was $P < 0.0000001$ and for high MMP was $P = 0.000247$. SFM □; AeG ■; BTS ○; AN ▲; M III △.

Data on sperm overall motility in different extenders are shown in Figure 1a (Table 2 is published online as the supplementary data). The mean percentage of OM at time 0 was 87 ± 2.81 and this value decreased progressively over time for all extenders ($P < 0.0000001$). On day 1, none of the extenders significantly dropped below 70%. Motility of SFM- and AeG-diluted spermatozoa did not significantly drop below the threshold of 60% until day 15, even if the SFM samples showed significantly higher values than the AeG ones on days 3, 6 and 9. BTS and MIII performed well since the OM did not fall below the 60% threshold until day 12 of storage. For this parameter, AN showed the worst behaviour since the threshold was passed on day 6 of storage.

Sperm PM in different extenders is reported in Figure 1b (Table 3 is published online as supplementary data). Sperm PM decreased progressively over time for all extenders ($P < 0.0000001$). At time 0, PM was 75.61 ± 4.07%. The drop for this parameter was faster in MIII than in the other extenders; in fact, it decreased from 60.1 ± 3.24% at 12 h to 46.5 ± 7.70% on day 1, but on day 3 the value was no worse than that obtained for BTS and AN. The PM of AeG-, AN- and BTS-diluted spermatozoa significantly dropped below 60% on day 3 while spermatozoa diluted in SFM passed the threshold only on day 12. On day 15, no significant differences were observed among the five extenders, with values ranging from 29.4% to 43.3%.

Data on spermatozoa with intact membrane are shown in Figure 2a (Table 4 is published online as supplementary data alongside the electronic version of the article). At time 0, fresh semen showed a high percentage of membrane-intact spermatozoa (82.5 ± 1.44) and sperm viability remained over 70% for all extenders at least for 12 h after

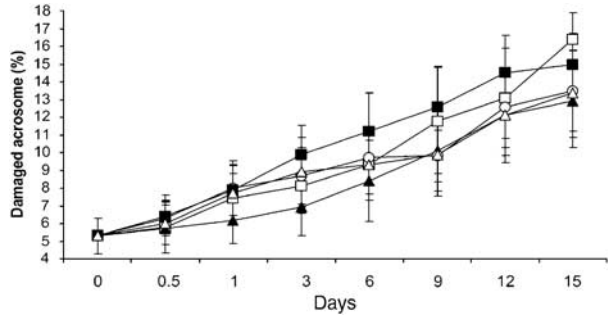

Figure 3 Spermatozoa with damaged acrosomes, evaluated by Coomassie blue stain, during long-term storage (15 days) at 16.5°C (mean ± s.e.) in five different extenders. Diluent × time interaction for spermatozoa with damaged acorsomes was $P = 0.976928$. SFM □; AeG ■; BTS ○; AN ▲; M III △.

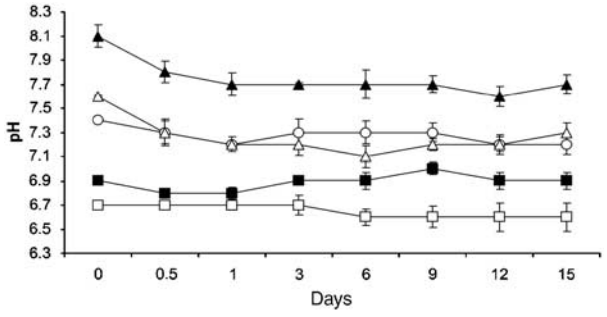

Figure 4 pH of AI doses prepared with the five extenders during long-term storage (15 days) at 16.5°C (mean ± s.e.). Time 0 points refer to the pH of the raw extenders. Diluent × time interaction for pH was $P = 0.370744$. SFM □; AeG ■; BTS ○; AN ▲; M III △.

dilution. Nevertheless, viability decreased over time for all extenders ($P < 0.0000001$). AeG- and SFM-diluted sperm still showed high viabilities at day 15 (65.75 ± 2.06% and 63.8 ± 1.46%, respectively) while MIII and AN showed viabilities lower than those of AeG and SFM (day 15) already at days 1 and 3, respectively, reaching a very low level on day 15 (36.5 ± 1.71%; 44.0 ± 4.59%). BTS-diluted spermatozoa had an intermediate viability as, only after day 9, the mean percentage of sperm with intact membranes falls below the AeG and SFM (day 15) threshold.

Results on spermatozoa with a high MMP are shown in Figure 2b (Table 5 is published online as supplementary data). At point 0, 76.67% of spermatozoa presented active mitochondria; then mean values decreased over time for all extenders ($P < 0.0000001$). SFM- and AeG-diluted spermatozoa behaved in the same manner until the last time point, reaching a value of approximately 60% only on day 15. Interestingly, after a small and initial decrease, AeG and SFM reached a plateau between 12 h and 9 days. On the contrary, MIII- and AN-diluted spermatozoa showed progressive and substantial decreases until the end (34.3 ± 1.50%; 33.8 ± 5.22%). Spermatozoa diluted in BTS had an intermediate behaviour, with good efficiency until day 6.

Data on damaged acrosomes are shown in Figure 3 (Table 6 is published online as supplementary data). At time 0, the mean percentage of spermatozoa with damaged acrosomes was 5.3 ± 3.67; this value increased over time for each extender with similar kinetics ($P < 0.0000001$), showing no statistical differences among diluents ($P = 0.951$). On day 15, the percentages of spermatozoa with damaged acrosomes in the five extenders ranged from 12.9% to 16.4%.

pH data are shown in Figure 4 (Table 7 is published online as supplementary data). After at least 12 h of equilibration, the pH values of the raw extenders showed significant differences. While SFM and AeG had a pH of approximately 6.8, BTS and MIII had a more neutral value. On the contrary, the initial pH value of AN was basic. After spermatozoa dilution, the pH of the doses showed two different behaviours. In two of them (SFM and AeG), the pH was maintained during the entire storage period. In the others, there were significant decreases on the first day and then the pH remained constant until the end. This effect was mainly evident for AN.

Discussion

The present study compared the efficiencies of different extenders in preserving boar semen during long-term storage in the liquid phase at 16.5°C. In particular, we compared SFM, a homemade diluent, to four commercial ones in order to establish its characteristics and preservation period.

Sperm motility, which is one of the most important characteristics for evaluating the fertility potential of spermatozoa ejaculates, has been objectively evaluated. Computer-assisted sperm analysis (CASA) is a reliable tool for overcoming subjective evaluations (Verstegen *et al.*, 2002), and a significant correlation between CASA and on-farm insemination outcomes has been reported (Holt *et al.*, 1997). The minimum percentage of motile spermatozoa for AI dose has been established to be at least of 60% because, under this threshold, lower farrowing rates can be experienced (Flowers, 1997). Among other parameters relevant for fertilisation, we evaluated spermatozoa membrane and acrosome conditions during storage. The SYBR-14/PI and the Coomassie blue stains are easy and reliable assays for determining cellular membrane integrity and acrosome damage, respectively (Huo *et al.*, 2002). The JC-1 stain is very helpful (Huo *et al.*, 2002; Turba *et al.*, 2007) in studying the metabolic activity of the mitochondria, which is highly correlated with spermatozoa motility (Garner *et al.*, 1997; Turba *et al.*, 2007). The pH of extenders/diluted semen samples has also been shown to correlate with semen quality (Vyt *et al.*, 2004).

Extenders are classified as long-term extenders when the claimed preservation period is longer than 4 days, so BTS (3 days) and MIII (3 to 4 days) are classified as short-term diluent, AN (claimed preservation period of 5 days) is classified as economic long-term diluent and AeG (claimed preservation period of 10 days) as long-term diluent. SFM, with results always comparable to those of AeG if not better, could therefore be declared as a long-term diluent.

Between short-term diluents, BTS has shown better results than MIII (during the first 3 days) but also better than AN (during the first 6 days).

The percentage of spermatozoa with damaged acrosome was influenced only by the time of storage, with no statistical differences among diluents. However, we did not obtain values significantly above the 15% threshold till the end of the experiment for any diluents. Also, PM was influenced only by the time of storage.

On the contrary OM, sperm viability and high MMP were strongly influenced by the diluent used and by the time.

In MIII and AN, sperm cells showed good OM only for the first 3 days while, with BTS, it remained satisfactory until day 9. AeG and SFM possessed greater ability in preserving spermatozoa motility, which reached the 60% threshold at the end of the trial. These two diluents contain BSA, which has been shown to have a potent effect on the stimulation (Harrison *et al.*, 1982; Waberski *et al.*, 1989) and preservation of motility.

For viability, we experienced the best results with AeG and SFM, which were able to preserve membrane integrity more efficiently and for more days as compared with the other extenders. AeG and SFM can provide better conditions with a more controlled pH and osmotic pressure. It has been found that, in boar, BSA can selectively bind to the plasma membrane surrounding the mid and principal piece of spermatozoa (Weitze, 1991). Moreover, BSA can neutralise the metabolic products from spermatozoa and bacteria, and may have an anti-peroxidative activity (Bamba and Sone, 1981; Alvarez and Storey, 1983). For these reasons, BSA and EDTA are added to semen extenders (as well in SFM) in an effort to prevent or retard unwanted alterations in the structure and the function of the plasma membrane (Levis, 2000).

The percentages of spermatozoa with active mitochondria decreased in all extenders over time, but were also strongly influenced by the type of extender. Furthermore, a high correlation between viability and MMP was present as reported previously (Garner *et al.*, 1997). The functionality of mitochondria is of primary importance for fertilisation as it is responsible for motility (Gallon *et al.*, 2006). This fact is also confirmed by the significantly high correlations of MMP and both OM and PM. Again, AeG and SFM showed the best results as they were able to preserve high MMP till the end of the trial and also significantly better than BTS, MIII and AN on the first 6 days.

The present study showed that the five extenders tested differ in their pH. In all extenders, the pH tended to slightly decrease or to remain constant after being mixed with semen, depending on their buffering capacity as has been noted by other authors (Paulenz *et al.*, 2000). As a matter of fact, pH showed a significant negative correlation with both OM and PM, MMP and sperm viability. Moreover, it must be noted that the extenders with the best performance in preserving spermatozoa quality are the ones with the lowest mean values of pH. These findings emphasise the importance of the buffering capacity of extenders and can contribute to explain the better results obtained with SFM and AeG. Johnson *et al.* (2000) reported that spermatozoa metabolism tended to decrease with an increased tendency of acidification (pH under 7.2). These authors stated that intracellular acidosis enables spermatozoa to survive longer due to an impaired glycolitic metabolism and a consequent reduction in their motility. Also, in bovine spermatozoa, intracellular pH is directly related to motility and the decrease of the intracellular pH with weak acids reversibly depresses motility (Jones and Bavister, 2000). Therefore, by decreasing motility, a lower pH increases spermatozoa life expectancy through a decrease in energy consumption. Before *in vitro* motility analyses, the semen samples are heated and re-oxygenated and this manipulation is sufficient to resume motility. This hypothesis has also been confirmed by the observation that, in the boar, the pH in the *cauda epididymis* is approximately 6.5 (Rodriguez-Martinez *et al.*, 1990) while, after ejaculation and mixing with accessory sex gland secretions, the pH increases from 7.2 to 7.5 (Johnson *et al.*, 2000). This increase is able to render spermatozoa fully motile for fertilisation processes.

SFM lacks sodium bicarbonate, a compound known to be a capacitating agent, and its buffering activity is substituted by Trizma. Again SFM is especially rich in BSA and EDTA, chelating compounds very effective in sequestering divalent metal ions, especially Ca^{2+}, preventing the initiation of capacitation (Johnson *et al.*, 2000). These are the main differential characteristics between SFM and others diluents tested.

From a practical point of view, the choice of an extender depends on its use. When diluted semen is to be used within 2 to 3 days after collection, the most rational decision is to use a short-term diluent, which is less expensive and can be quite effective in preserving spermatozoa quality. However, as shown by our trials, some extenders can demonstrate better performance also in the short term. When the use of semen doses is postponed (more than 3 to 4 days), in our opinion, it is necessary to use a long-term extender, which is more expensive but which can offer about a greater guarantee regarding semen longevity as confirmed by field trials (Johnson *et al.*, 1988; Waberski *et al.*, 1994). SFM could be a good choice because, as a homemade diluent, it is not so expensive but requires accuracy in preparation.

Acknowledgements

This research was supported by RFO (Ricerca Fondamentale Orientata) Bologna University. We thank Sanifarm SRL for donating the extenders and the use of the SpermVision™ CASA system (Minitube, Tiefenbach, Germany), and Dr Daniel Remondini for the statistical analyses.

References

Alvarez JG and Storey BT 1983. Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. Biology of Reproduction 29, 548–555.

Bailey JL, Bilodeau JF and Cornier N 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. Journal of Andrology 21, 1–7.

Bamba K and Sone M 1981. Factors affecting the quality of boar semen stored by means of dialysis. Journal of Reproduction and Fertility 62, 193–197.

Cerolini S, Maldjian A, Surai P and Noble R 2000. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Animal Reproduction Science 58, 99–111.

Fantinati P, Zannoni A, Bernardini C, Webster N, Lavitrano M, Forni M, Seren E and Bacci ML 2005. Laparoscopic insemination technique with low numbers of spermatozoa in superovulated prepuberal gilts for biotechnological application. Theriogenology 63, 806–817.

Flowers WL 1997. Management of boars for efficient semen production. Journal of Reproduction and Fertility 52 (suppl.), 67–78.

Fraser L and Strzezek J 2004. The use of comet assay to assess DNA integrity of boar spermatozoa following liquid preservation at 5°C and 16°C. Folia Histochemica et Cytobiologica 42, 49–55.

Gadea J 2003. Semen extenders used in the artificial insemination of swine. A review. Spanish Journal of Agricultural Research 1, 17–27.

Gallon F, Marchetti C, Jouy N and Marchetti P 2006. The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. Fertility and Sterility 86, 1526–1530.

Garner DL, Thomas CA, Joerg HW, Dejarnette JM and Marshall CE 1997. Fluorometric assessment of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biology of Reproduction 57, 1401–1406.

Gerrits RJ, Lunney JK, Johnson LA, Pursel VG, Kraeling RR, Rohrer GA and Dobrinsky JR 2005. Perspectives for artificial insemination and genomics to improve global swine populations. Theriogenology 63, 283–299.

Harrison RA, Dott HM and Foster GC 1982. Bovine serum albumin, sperm motility, and the dilution effect. Journal of Experimental Zoology 222, 81–88.

Holt C, Holt WV, Moore HDM, Reed HCB and Curnokz RM 1997. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. Journal of Andrology 18, 312–323.

Huo LJ, Ma XH and Yang ZM 2002. Assessment of sperm viability, mitochondrial activity, capacitation and acrosome intactness in extended boar semen during long-term storage. Theriogenology 58, 1349–1360.

Johnson LA, Aalbers JG and Groothe HJG 1988. Artificial insemination of swine: fecundity of boar semen stored in Beltsville Thawing Solution (BTS), modified Modena (MM), or MR-A and inseminated on one, three and four days after collection. Reproduction in Domestic Animals 23, 49–55.

Johnson LA, Weitze KF, Fiser P and Maxwell WMC 2000. Storage of boar semen. Animal Reproduction Science 62, 143–172.

Jones JM and Bavister BD 2000. Acidification of intracellular pH in bovine spermatozoa suppresses motility and extend viable life. Journal of Andrology 21, 616–624.

Lavitrano M, Bacci ML, Forni M, Lazzereschi D, Di Stefano C, Fioretti D, Giancotti P, Marfé G, Pucci L, Renzi L, Wang H, Stoppacciaro A, Stassi G, Sargiacomo M, Sinibaldi P, Turchi V, Giovannoni R, Della Casa G, Seren E and Rossi G 2002. Efficient production by sperm-mediated gene transfer of human decay accelerating factor (hDAF) transgenic pigs for xenotransplantation. Proceedings of the National Academy of Sciences of the United States of America 99, 14230–14235.

Lavitrano M, Forni M, Bacci ML, Di Stefano C, Varzi V, Wang H and Seren E 2003. Sperm mediated gene transfer in pig: selection of donor boars and optimization of DNA uptake. Molecular Reproduction and Development 64, 284–291.

Levis DG 2000. Liquid boar semen production: current extender technology and where do we go from here! In Boar Semen Preservation IV (ed. LA Johnson and HD Guthrie), Proceedings of the IV International Conference on Boar Semen Preservation, pp. 121–128. Allen Press Inc., Lawrence, KS, USA.

Manzini S, Vargiolu A, Stehle IM, Bacci ML, Cerrito MG, Giovannoni R, Zannoni A, Bianco MR, Forni M, Donini P, Papa M, Lipps HJ and Lavitrano M 2006. Genetically modified pigs produced with a nonviral episomal vector. Proceedings of the National Academy of Sciences of the United States of America 103, 17672–17677.

Paulenz H, Kommisrud E and Hofmo PO 2000. Effect of long-term storage at different temperatures on the quality of liquid boar semen. Reproduction in Domestic Animals 35, 83–87.

Rodriguez-Martinez H, Ekstedt E and Einarsson S 1990. Acidification of epididymal fluid in the boar. International Journal of Andrology 13, 238–243.

Thacker BJ, Larsen RE, Joo HS and Leman AD 1984. Swine disease transmissible with artificial insemination. Journal of the American Veterinary Medical Association 185, 511–516.

Turba ME, Fantinati P, Bernardini C, Gentilini F, Bacci ML and Forni M 2007. Relationships between innovative and traditional parameters to investigate semen quality in pigs. Animal Reproduction Science 99, 72–81.

Verstegen J, Iguer-Ouada M and Onclin K 2002. Computer assisted semen analyzer in andrology research and veterinary practice. Theriogenology 57, 149–179.

Vishwanath R 2003. Artificial insemination: the state of the art. Theriogenology 59, 571–584.

Vyt P, Maes D, Dejonckheere E, Castryck F and Van Soom A 2004. Comparative study on five different commercial extenders for boar semen. Reproduction in Domestic Animals 39, 8–12.

Waberski D, Weitze KF, Rath D and Sallman HP 1989. Effect of bovine serum albumin and zwitterionic buffers on stored liquid boar semen. Zuchthygiene 24, 128–133.

Waberski D, Meding S, Dirksen G, Weitze KF, Leiding C and Hahn R 1994. Fertility of long-term-stored boar semen: influence of extender (Androhep and Kiev), storage time and plasma droplets in the semen. Animal Reproduction Science 36, 145–151.

Webster NL, Forni M, Bacci ML, Giovannoni R, Razzini R, Fantinati P, Zannoni A, Fusetti L, Dalprà L, Bianco MR, Papa M, Seren E, Sandrin MS, Mc Kenzie IF and Lavitrano M 2005. Multi-transgenic pigs expressing three fluorescent proteins produced with high efficiency by sperm mediated gene transfer. Molecular Reproduction and Development 72, 68–76.

Weitze KF 1991. Long-term storage of extended boar semen. In Proceedings of the 2nd International Conference on Boar Semen Preservation (ed. LA Johnson and D Rath), pp. 231–253. Paul Parey Scientific Publishers, Berlin, Germany.