The effects of three extenders on refrigerated boar semen

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Abstract
This study aims to evaluate the quality of boar sperm that was refrigerated for 14 days at 17 ºC with three extenders. For this study, samples from four boars were collected twice a week using the gloved-hand technique. After collection, only ejaculates showing total motility of 75% or greater were submitted to the refrigeration process. Ejaculates were diluted in Androhep, MR-A® or Reading. Each portion was kept for two hours at 22 ºC, then sperm motility was assessed through contrast microscopy. Sperm mitochondrial activity, viability and acrosome integrity were measured by the flow cytometric technique. The remaining diluted semen was maintained at 17 ºC for 336 h, and the same analysis being repeated every 48 h. Semen diluted in Androhep revealed no significant quality deterioration in percentage of live spermatozoa during refrigeration. However, after 144 h, viability decreased significantly for MR-A® and Reading (63.3% ± 7.0 and 66.4% ± 6.2, respectively), and after 336 h, this decrease was accentuated (56.3% ± 3.9 and 18.4% ± 6.2, respectively, for MR-A® and Reading). On average, for all three extenders, acrosome integrity values did not differ statistically up to 144 h, ranging from 48.3 ± 2.3 for MR-A® to 62.4 ± 3.2 for Androhep. Then values decreased towards the end of the experiment, with Androhep always presented the higher values, while Reading resulted in the lowest values (46.3 ± 3.2 and 5.6 ± 1.4, respectively). No significant changes in mitochondrial membrane potential were observed during the refrigeration period. Results of this study indicate that Androhep achieves the best results for the various parameters studied over time.

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Introduction
Storage of extended semen at low temperatures prolongs sperm viability by reducing energy consumption and cell metabolic product formation. Refrigeration at 15 - 20 ºC for a limited time is still the preferred method of storage for boar semen that is to be used for artificial insemination (AI) (Johnson *et al.*, 2000). Pursel *et al.* (1973) have shown that extended porcine semen stored at 15 ºC yields acceptable fertilization rates, while exposure to temperatures below 15 ºC results in cold shock and cell death. Studies that address the effects of cold shock on boar sperm cells have used temperatures of 5 ºC or colder because these temperatures are known to induce the desired cold shock effects, which lead to irreversible sperm damage (Althouse *et al.*, 1998). The exact temperature at which perturbation of the boar sperm cell occurs has not been established. If this critical temperature was determined, it would be beneficial to the pig industry in establishing guidelines for handling, shipping and storing extended semen. The proposed critical temperature (<15 ºC) appears to have originated from previous research as the optimum storage temperature of undiluted fresh boar semen. For this purpose, several extenders have been developed to preserve boar semen for up to four days. Moreover, extenders protect sperm against seminal plasma, which can be toxic, providing protection for sperm by reducing its metabolic rate in low or chilling temperatures during storage. Dilution also allows multiple inseminations from a single ejaculate. Extenders can act as energy sources for sperm metabolism (Mapeka *et al.*, 2012), providing pH buffering from sperm cell waste and from antibiotics. They prevent growth of micro-organisms that may cause diseases (Knox, 2011).

Although short-term extenders are being used widely with good results, the possibility of using refrigerated semen beyond five days is much more encouraging as it would greatly improve production efficiency and economic profit for the AI industry in maintaining the farrow rate and litter size.

Nevertheless, it is well known that the fertility of semen is gradually lost if refrigerated for long periods. Not only is sperm viability lost, but motility and cell metabolism are drastically reduced, which can diminish

the fertilizing rate, as only spermatozoa with intact plasma membranes, acrosomes and sufficient cell metabolism are able to fertilize an oocyte *in vivo* (Yanagimachi, 1994). Several studies have shown the influence of various extenders on boar sperm survival (Vyt *et al.*, 2004), especially quality (Huo *et al.*, 2002; Waterhouse *et al.*, 2004; Ambrogi *et al.*, 2006), for short periods. The aims of the present study were to investigate the influence of three commercial extenders, namely Androhep, MR-A® and Reading, during the holding time, on boar semen quality stored at 17 ºC for up to 14 days. Sperm quality was evaluated through examining viability, mitochondrial membrane potential and acrosome integrity using flow cytometric methodology.

Materials and Methods

In the present study, the effects were studied of three long-term extenders on the viability, mitochondrial potential and acrosome integrity of boar sperm that was refrigerated at 17 ºC for 14 days (336 h). Evaluation was performed eight times at different time intervals (0, 24, 96, 144, 192, 240, 288 and 336 h). Unless otherwise noted, all reagents were purchased from Sigma Chemical (St. Louis, Mo). Use and care of the boars in this study were approved by the Ethics Committee of the authors' institution, and the experimental procedures were sanctioned by this committee. Care was taken to minimize the number of animals.

Sperm-rich ejaculate fractions of four healthy and sexually mature animals (Serviços de Desenvolvimento Agrário, Terceira, Portugal) were collected using the gloved-hand method and evaluated for conventional semen characteristics. Eight ejaculates containing more than 75% motile sperm (evaluated by contrast-phase microscopy) was selected. After evaluation, semen was placed in flasks, set in isolated containers at 37 ºC, transported to the laboratory, and processed 10 minutes after collection. On arrival, sperm concentration was evaluated using the Neubauer chamber. The semen was split into three portions and each one was diluted to a final concentration of 40×10^6 sperm/mL in three long-term extenders, MR-A® (Kubus, S.A., Madrid, Spain), Androhep and Reading. Androhep and Reading were prepared according to Gadea (2003).

For each extender, the sperm was conserved for two hours at 22 ºC, and then at 17 ºC. Aliquots of 5 mL were withdrawn, and parameters of sperm cells activity, namely viability, acrosomal status and mitochondrial membrane potential, were assessed at 0, 24, 96, 144, 192, 240, 288 and 336 h, corresponding to T0; T1; T2 T3; T4; T5; T6 and T7, respectively. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif) was used after excitation at 469 nm and emission of green fluorescence at 541 nm through a 15-mW argon ion laser, at a rate of 500 - 1000 cells/s, using FACSflow as sheath fluid. Data were then analysed using CellQuest software (Becton Dickinson). Mean fluorescence intensity of the analysed sperm cells (n = 10 000) was determined after gating the cell population by forward and side light scatter signals.

For viability tests, sperm was assessed using SYBR-14 (Molecular Probes, Eugene, Oreg, USA) and propidium iodide (PI). A staining solution was prepared by adding *5* µL of a 2 mM solution of PI (in water) and 2 µL of a 100 mM solution of SYBR-14 (in anhydrous DMSO) to 2 mL of phosphate buffered saline (PBS). Just prior to measurement, 495 µL of a staining solution was added to 5 µL of semen and incubated for 15 minutes at room temperature in the dark, then analysed by flow cytometry.

Evaluation of acrosome integrity was performed by staining sperm with PI and pisum sativum agglutinin conjugated to fluorescein isothiocinate (FITC-PSA), as described by Franco *et al.* (2013). The semen was diluted in PBS to 1x106 sperm/mL in a final volume of 2 mL. Then, 296 µL of this dilution was withdrawn and placed in a cytometric tube, preheated to 37 ºC, stained with 6 µL of PI (0.5 g/mL) and 20 µL of FITC-PSA (100 mg/mL), then incubated for 10 minutes at 37 ºC. Immediately before analysing, 300 µL of PBS was added and samples measured by flow cytometry. Sperm was then allocated to one of four groups based on their FITC-PSA and PI staining patterns: i) FICT-PSA negative and PI negative, acrosome intact live (AIL); ii) FICT-PSA negative and PI positive, acrosome intact dead (AID); iii) FICT-PSA positive and PI negative, acrosome reacted/damaged live (ARL); and iv) FICT-PSA positive and PI positive, acrosome damaged dead (ARD).

To identify mitochondria with low and high membrane potential, lipophilic cationic compound JC-1 (5,50,6,60-tetrachloro-1,10,3,30 tetraethylbenzimidazolyl carbocyanine iodine) was used according to Franco *et al.* (2013). After diluting the semen in PBS ($1x10^6$ sperm/mL in a volume of 2.0 mL) 288 µL was withdrawn and placed in a cytometry tube preheated to 37 ºC. Samples were stained with 12 µL of JC-1 (153 mM, T-3168; Molecular Probes Inc., Eugene, Oreg) and incubated for 10 min at 37 ºC. Then sperm was diluted ($0.5x10^6$ sperm/mL) by adding 300 µL of PBS and analysed by flow cytometry.

For statistical analysis, results obtained for each boar were compared among extenders and times (T0, T1, T2, T3, T4, T5, T6 and T7) before being analysed by one-way ANOVA and expressed as means ± SEM. Data expressed as percentages were normalized through arc sine transformation. Then the data were submitted to homogeneity testing, followed by variance analysis (one-way ANOVA) with post hoc least

significant difference test by IBM SPSS version 20 Statistics Program software (SPSS Inc. Chicago, Il). For all analyses, a *P* value of <0.05 was considered statistically different.

Results and Discussion

In the present study, the effects of three long-term extenders on boar sperm viability, mitochondrial membrane potential and acrosome integrity were challenged against refrigeration at 17 ºC for 336 h. Evaluation was performed eight times at various time intervals (0, 24, 96, 144, 192, 240, 288 and 336 h).

Changes in the percentage of total motile spermatozoa throughout the 14 days of refrigeration in each extender are presented in Table 1. At T0, T1 and T2 no statistical differences were observed among extenders. For the remainder of the study, the sperm motility for the three extenders differed statistically (*P* <0.05), and the highest values were observed for Androhep.

Table 1 Percentage of sperm motility (mean ± SE) of boar semen with three extenders at eight incubation times

Storage Period	Semen Extenders		
	MR-A®	**Androhep**	**Reading**
T0	79.3[a,A] ± 2.26	90.2[ab,A] ± 0.87	86.3[a,A] ±1.05
T1	77.5[a,AB] ± 2.26	82.3[a,AB] ± 2.01	78.8[a,AB] ± 2.15
T2	74.8[a,BC] ± 1.85	75.3[a,BC] ± 1.86	71.2[a,B] ± 1.42
T3	70.7[a,C] ± 0.80	71.3[a,BCD] ±1.26	62.8[b,C] ± 1.11
T4	65.2[a,D] ± 0.79	67.7[a,CD] ± 1.09	54.7[b,C] ± 0.56
T5	60.5[a,DE] ± 1.12	66.3[b,CD] ± 1.26	40.2[c,D] ± 1.01
T6	55.3[a,E] ± 1.26	64.2[b,D] ± 1.42	36.2[c,D] ± 1.08
T7	42.8[a,F] ± 1.49	50.3[b,E] ± 1.99	17.3[c,E] ± 0.88

T0 = 0 h, T1 = 24 h, T2 = 96 h, etc.
[a,b,c] Different superscripts within the same row indicate significant differences, *P* <0.05.
[A,B,C,D] Different superscripts within the same column indicate significant differences, *P* <0.05.

Table 2 Percentage live sperm (mean ± SE) of boar semen with three extenders at eight incubation times determined by SYBr-14/PI staining

Storage Period	Semen Extenders		
	MR-A®	**Androhep**	**Reading**
T0	78.5[a,AB] ± 1.93	79.7[a,AB] ± 2.91	79.7[a,AB] ± 2.67
T1	83.0[a,A] ± 2.46	86.4[a,A] ± 2.11	84.6[a,A] ± 2.19
T2	84.3[a,A] ± 3.85	87.3[a,A] ± 2.72	85.6[a,A] ± 3.39
T3	63.3[a,A] ± 7.03	73.6[a,AB] ± 4.65	66.4[a,B] ± 6.17
T4	49.7[a,BC] ± 7.44	71.8[b,B] ± 3.62	37.3[a,C] ± 7.94
T5	47.3[a,C] ± 5.60	72.8[b,B] ± 2.74	37.3[a,C] ± 7.30
T6	50.9[a,C] ± 6.23	73.3[b,B] ± 1.74	28.9[c,CD] ± 7.42
T7	56.3[a,C] ± 3.91	73.6[b,B] ± 3.34	18.4[c,D] ± 6.21

T0 = 0 h, T1 = 24 h, T2 = 96 h, etc.
[a,b,c] Different superscripts within the same row indicate significant differences, *P* <0.05.
[A,B,C,D] Different superscripts within the same column indicate significant differences, *P* <0.05.

Results of sperm viability are shown in Table 2. From T0 to T3, no statistical differences were observed among extenders (*P* >0.05). However, from T4 to T7, sperm viability diluted with Androhep was

statistically higher (*P* <0.05) when compared with the other two extenders. For mitochondrial membrane potential, the results obtained with Androhep were higher than those obtained from the other two extenders (Table 3).

Table 3 Percentage of sperm with high mitochondrial membrane potential (hMMP) (mean ± SE) of boar semen with three extenders at eight incubation times determined by JC-1 staining

Storage Period	Semen Extenders		
	MR-A®	Androhep	Reading
T0	52.5[ab,A] ± 4.27	59.9[a,A] ± 3.06	44.5[b,A] ± 2.73
T1	53.4[a,BC] ± 5.07	54.6[b,AB] ± 4.98	41.3[ab,AB] ± 2.13
T2	48.1[a,A] ± 4.03	49.8[a,ABC] ± 3.74	34.6[a,ABC] ± 6.16
T3	48.5[a,AB] ± 2.58	46.0[a,CDE] ± 4.17	33.6[a,ABC] ± 4.57
T4	42.4[a,BC] ± 4.79	38.7[a,BCD] ± 6.30	33.0[a,ABCD] ± 5.98
T5	31.3[a,C] ± 5.02	34.5[a,DEF] ± 3.15	29.4[a,BCD] ± 4.89
T6	30.8[a,C] ± 4.18	34.3[a,EF] ± 2.91	26.3[a,CD] ± 4.06
T7	21.6[a,C] ± 3.03	32.7[a,F] ± 2.58	17.0[a,D] ± 4.58

T0 = 0 h, T1 = 24 h, T2 = 96 h, etc.
[a,b,c] Different superscripts within the same row indicate significant differences, *P* <0.05.
[A,B,C,D] Different superscripts within the same column indicate significant differences, *P* <0.05.

For viable sperm with Intact acrosome (AIL) (Table 4), despite values obtained for Androhep extender from T0 to T7 were always higher than those observed for the other two extenders, only from T4 were values significantly higher (*P* <0.05).

Table 4 Percentage of sperm acrosome integrity (mean ± SE), acrosome intact and live cells after eight incubation times, in boar semen with three extenders

Storage Period	Semen Extenders		
	MR-A®	Androhep	Reading
T0	57.2[a,A] ± 0.87	62.4[b,A] ± 2.42	60.0[ab,A] ± 1.59
T1	55.3[a,AB] ± 2.18	63.2[b,A] ± 2.06	61.0[ab,A] ± 1.69
T2	57.4[a,A] ± 3.44	63.8[a,A] ± 3.35	59.4[a,A] ± 3.21
T3	48.3[a,ABC] ± 2.34	62.4[b,A] ± 3.23	55.1[ab,A] ± 5.80
T4	46.5[a,BC] ± 4.65	56.9[a,A] ± 3.31	33.2[b,B] ± 5.36
T5	45.4[a,C] ± 2.62	59.7[b,A] ± 1.85	28.3[c,B] ± 3.92
T6	29.8[a,D] ± 3.84	47.7[b,B] ± 5.34	15.6[c,C] ± 4.61
T7	27.9[a,D] ± 3.69	46.3[b,B] ± 3.24	5.6[c,C] ± 1.36

T0 = 0 h, T1 = 24 h, T2 = 96 h, etc.
[a,b,c] Different superscripts within the same column indicate significant differences, *P* <0.05.
[A,B,C,D] Different superscripts within the same row indicate significant differences, *P* <0.05.

Numerous semen extenders are available for use by commercial pig producers. In the current study, the longevity of three extenders, MR-A®, Androhep and Reading, was evaluated regarding sperm viability, acrosome integrity and mitochondrial activity for 14 days refrigerated at 17 ºC. Although these extenders are used widely (Johnson *et al.*, 2000), they revealed differences in maintaining sperm motility, viability, mitochondrial activity and acrosome intactness for an extended period (Kaeoket *et al.*, 2011). Our study

demonstrated that semen quality gradually reduced during the 336 h of storage, a result that is in agreement with other investigations (Perez *et al.*, 1991; Waberski *et al.*, 1994). In our results, a significant decrease in sperm motility was observed after 144 h for all extenders, the highest values being recorded with Androhep and the lowest with Reading. The most likely reason is their different densities as, according to Anaya *et al.* (2014), the viscosity of the medium surrounding the spermatozoa influences its movement. Nevertheless, as the quantitative composition of these extenders is unknown for commercial reasons, only qualitative composition is done. Results for mitochondrial activity were similar among extenders, Androhep revealed higher values along the storage time. Weitze (1991) found that bovine serum albumin (BSA) in the Androhep extender could selectively bind to plasma membrane, surrounding the mid and principal piece of boar spermatozoa, neutralizing the metabolic by-products from spermatozoa and bacteria, and may have antiperoxidative activity (Alvarez & Storey, 1983). In the present study, over 70% of the sperm cells in the Androhep extender remained alive on the last day of storage. However, it has previously been suggested that semen extended with Androhep should be used for AI within three days for optimal fecundity in gilts (Kuster & Althouse, 1999).

In general, for all evaluations, lower values for all parameters were obtained when sperm was extended with Reading, perhaps because it is the only tris-based extender. In our conditions, based on research (Revell & Glossop, 1989), trehalose and potassium chloride were added to Reading, which stabilized the lipid bilayer and protected the sperm membrane from cold temperatures. This extender also contains ethylenediaminetetraacetic acid (EDTA), a chelator for divalent ions, this can limit Ca^{2+} movement across the plasma membrane during dilution and cooling (Watson, 1990: Johnson *et al.*, 2000), contributing to sperm fertility. MR-A® is known to protect the structure of the sperm's membrane after the dilution process, reducing oxidation and apoptosis, and having high effectiveness and durability during preservation. For the mitochondrial potential, although no statistical differences were observed, Androhep was the only extender to keep sperm qualities during the storage period. It is possible that differences became evident only after longer storage.

Acrosome reaction is related to sperm fertility and is essential in the process of fertilization. However, its early occurrence during long-term semen storage decreases the viability and fertility of the stored sperm (Lee & Park, 2015). The results from this trial indicated that the acrosome is more susceptible to damage during storage than the organelles, being the structural basis of motility. This presumption is in accordance with experiments performed by Buhr (1990), who stated that the decrease of membrane fluidity during storage is greater for head plasma membranes than for sperm body membranes. This is not surprising as storage of diluted semen to some extent may cause sperm capacitation, possibly followed by acrosome reaction (Vishwanath & Shannon, 1997). Therefore, the decrease in acrosome integrity might be because of acrosome reaction in addition to membrane damage (Larsson, 1985).

Despite an expected variability among ejaculates, the extender factor did not seem to affect sperm viability in the first 72 h in the present trial. Sperm viability surprisingly increased with storage time in samples extended with MR-A®, Androhep and Reading. This increase has no immediate biological behaviour, and perhaps can be attributed to an underestimation of the viability at T0. A recent study reported an unexpected increase in sperm viability after 72 h, but did not provide a proper explanation for the phenomenon (Boe-Hansen *et al.*, 2005). All extenders are long term, so that preservation of the viability up to 96 h (and more) is expected. Because a decrease in viability was detectable only after 144 h, this adds value to this affirmation. In our study no differences in the percentage of dead spermatozoa between the extenders used were observed up to 96 h. After this period Androhep was the only extender that kept higher values of viability, in compliance with results published by Lipenský *et al.* (2013).

Conclusion

Boar sperm motility, viability and acrosome integrity do not seem to be significantly affected by the kind of extender that is used up to 96 h of storage at 17 ºC. This is an interesting result, because in the AI industry semen is often preserved for short periods and rarely for more than 5 days (120 h). Our results indicate that Androhep is the most desirable choice for preserving seminal quality for long-term storage.

Acknowledgments

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