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The effect of dilution temperature by two extenders with different specifications on boar semen quality

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ABSTRACT

This study investigated the quality characteristics and the field fertility of boar semen after dilution with OptimIA, a common extender at 23 (n = 20, group A23) or at 30 °C (n = 20, group A30), and after dilution with OptimIA commercial extender at 23 °C (n = 20, group 23A) or with Androhep Plus, a membrane protective extender at 23 °C (n = 20, group 23B). Each sample of extended semen was stored (16-18 °C) and used for a double artificial insemination at 48 h and 72 h after its collection at the farm (n = 30 per group). The semen was assessed in the laboratory (kinetic parameters, morphology and DNA fragmentation) at collection (0 h) and at insemination hours (48 and 72 h). Most of the semen laboratory parameters deteriorated from 48 h to 72 h, regardless of dilution temperature or the use of the protective extender. However, in the special protective extender the percentages of rapid movement spermatozoa, VCL (curvilinear velocity), VAP (average path velocity) and WOB (wobble) did not differ between 48 h and 72 h. A lower farrowing rate was observed in the common extender group at 23 °C, and a lower number of live born piglets in the protective extender group compared to the other two groups. In conclusion, one step dilution of boar semen at 23 °C compared to dilution at 30 °C did not dramatically affect its *in vitro* quality characteristics after 72 h of storage, although field fertility was negatively influenced. Some of these negative effects can be compensated for by the use of a membrane protective extender.

Key words: boar semen, extender, field fertility, semen quality, temperature effect

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Introduction

The widespread application of artificial insemination in the swine industry demonstrates the demand for using good quality extended semen in practice. The dilution procedure and the temperature management of boar ejaculates are crucial processes. Boar spermatozoa are extremely sensitive to temperature variations because the sperm plasma membrane consists of a high concentration of polyunsaturated fatty acids (DE LEEUW et al., 1990; ROBERTSON et al., 1990; PARKS and LYNCH, 1992). This characteristic makes boar spermatozoa susceptible to oxidative damage (PIPAN et al., 2014). Temperatures below 12 °C are critical for boar sperm cells (ALTHOUSE et al., 1998). Boar semen is commonly stored at 16-18 °C and even though the critical temperature is not reached, lipid-phase separation happens when the semen is rapidly cooled. This causes increased membrane permeability (DROBNIS et al., 1993).

On pig farms, boar semen is usually extended according to two different protocols. The first protocol includes dilution of semen with an extender in one step at 32-33 °C. The second protocol includes a first step of semen dilution with an extender at 32-33 °C in a volume ratio of 1:1, followed by a second step of final dilution with an extender at 32-33 °C or at room temperature, about 23 °C (WABERSKI, 2009). Although older studies lent support to the fact that boar semen dilution at 32-33 °C protects spermatozoa during storage at 17 °C, PETRUNKINA et al. (2005) reported that a dilution temperature close to 33 °C does not decrease the spermatozoa's metabolism and leads to faster quality degradation during storage. Moreover, when the two step dilution protocol is used, stimulation during the first step should be avoided, because it can increase the spermatozoa's susceptibility during the final step of dilution (WABERSKI, 2009).

It has been perceived that simplification of the method of semen dilution, without the degradation of the extended semen's quality, would be beneficial for the productivity of a pig farm. One step boar semen dilution at room temperature is faster, easier and requires less material, but it can be stressful for the spermatozoa. However, there are not sufficient data available regarding the effect of this method on the basic quality semen characteristics or on farm fertility parameters. Moreover, the market provides boar semen protective extenders, which minimize membrane damage. Spermatozoa membrane protective extenders usually include chemicals, replacing the non-animal origin components, lending more support to the regulation of pH and osmotic pressure. They also prevent the morpho-functional changes and ageing of spermatozoa that occur *in vitro* during storage, and *in vivo* after insemination at the utero-tubal junction (RODRIGUEZ-MARTINEZ et al., 2005). Therefore, the first aim of this study was to compare boar semen laboratory characteristics after the one step dilution procedure with a common extender at 23 °C and at 30 °C. Furthermore, in this study we evaluated whether a cell membrane protective extender would improve the characteristics of semen compared to the use of

a common extender at 23 °C. Lastly, we compared fertility parameters after artificial insemination with semen processed by these three methods.

Materials and methods

Chemicals, extenders and equipment. In this study the following reagents were used: acridine orange (Acridine orange, A6014, Sigma Aldrich®, Seelze, Germany) and spermblue (SpermBlue, 08029, Mcroptic SL®, Automatic Diagnostic Systems, Barcelona, Spain). The boar semen extenders were OptimIA, as a common medium-term commercial extender (Magapor®, Spain), and Androhep Plus, as a membrane protective extender (Minitube, Germany). For the purposes of this study we used an Automatic Sperm Class Analyser® (Microptic SL, Automatic Diagnostic Systems, Barcelona, Spain), a Zeiss microscope (Axio, Scope A1, Germany), a Fluorescence microscope (Olympus BX 41, Japan) and a Photometer (SDM1, Minitube, Germany).

Experiment 1: In vitro study

Animals, sperm collection-dilution. Semen samples were collected from 7 mature cross-bred boars that were active in artificial insemination procedures on a 700 sow capacity farm. A total of 40 ejaculates (5-6 per boar) were obtained during a period of eight months. Summer was excluded to avoid the negative effect of heat stress on semen quality. Two hours before semen collection, the selected extenders OptimIA (A) and Androhep Plus (B) were prepared according to the instructions of the supplier company. The "sperm rich fraction" was collected by the gloved-hand technique, while the gel fraction was separated with a gauze filter. Raw semen was transported to the farm's laboratory in an isothermal glass vessel (37 °C). Initially, motility and morphology were microscopically assessed. Motility was subjectively evaluated in a light microscope with a heated stage (37 °C) at magnification ×100. A slide with a semen drop (20 μL) from each ejaculate was prepared, covered by a coverslip, and ten different optical areas were observed. Morphology was estimated using an eosin - nigrosin stained slide (SHIPLEY, 1999). In total, 200 spermatozoa were evaluated and the percentage of abnormal spermatozoa was determined. Semen density was also calculated with the use of a photometer (SDM1, Minitube, Germany). Ejaculates with >75% motile and <25% morphologically abnormal spermatozoa were accepted and further processed.

Each of the first 20 collected ejaculates was divided into 2 equal aliquots in terms of volume, and diluted in one step with either preheated extender A at 30 °C (n = 20, group A30) or extender A at room temperature (23 °C) (n = 20, group A23) to a concentration of 30×10^6 spermatozoa/mL.

Each of the last 20 collected ejaculates was divided into 2 equal aliquots in terms of volume. One of them was diluted with extender A (n = 20, Group 23A), while the second

was diluted with extender B (n = 20, Group 23B) in one step at 23 °C, to a concentration of $30X10^6$ spermatozoa/mL.

Laboratory semen evaluation. Diluted semen samples were stored in the laboratory for 72 h at 16-18 °C. An alarm sensor was used to ensure the appropriate temperature during storage. In order to evaluate semen quality at 48 and 72 h of storage (collection = 0 h), 1.5 mL of extended semen from each one of the samples was heated in Eppendorf vials in a water bath (37 °C).

Motility was estimated by Computer Assisted Semen Analysis (CASA).

The assessed parameters were:

Immotile spermatozoa %

Progressive spermatozoa %

Rapid, medium and slow spermatozoa % (10<slow<25<medium<45<rapid μm/sec)

Curvilinear velocity (VCL) (μm/sec), straight line velocity (VSL) (μm/sec), average path velocity (VAP) (μm/sec), amplitude of lateral head displacement (ALH) (μm), beat/cross-frequency (BCF) (Hz), straightness (STR) (VSL/VAPx100), linearity (LIN) (VSL/VCLx100), wobble (WOB) (VAP/VCLx100)

Hyperactivation (sperm subpopulation of increased VCL >97 μm/sec, ALH >3.5 μm and decreased LIN <0.32)

CASA was configured as follows: 10 fields and >500 spermatozoa, 25 frames/sec, region of particle control 10-18 μm, progressive movement of >45% of the parameter STR, circumferential movement <50% LIN, depth of field 10 μm and temperature of the microscope plate at 37 °C. In order to assess the motility of each sample, pictures of at least 4 fields were taken (×100), in order to record the movements of 500 spermatozoa or more. A number of objects incorrectly identified as spermatozoa were manually removed and final analysis was made for each sample. Analysis was performed by Sperm Class Analyzer software (SCA v.3, Microptic S.L., Spain). Each sample measurement was double replicated for reliability.

Morphology was estimated by the SpermBlue® staining method, according to the manufacturer's instructions. The assessment was done by microscopic observation (magnification ×400). Spermatozoa were classified into two categories: a) normal and b) with morphological abnormalities (head, neck, tail, protoplasmic droplets). Finally, 200 spermatozoa were scored and the % ratio per sample was calculated.

DNA integrity was estimated by the Acridine Orange Test (AOT) (TSAKMAKIDIS et al., 2011). The final assessment took place in a dark room, using an optical fluorescence microscope (Olympus BX 41, Japan) equipped with a digital camera and image analyzer computer software (U-TV 0.35 × C-2, Imaging Software System GmbH for Windows,

Olympus, Japan) at ×1000 magnification. Ten different fields were counted and estimated, and 200 spermatozoa were scored. The results were expressed in a % ratio.

Experiment 2: Field study

The evaluated ejaculates were used for conventional artificial insemination at the same time as they were laboratory assessed. During the experimental period and depending on the routine of the pig farm, cross-bred sows aged 20 to 36 (mean ± SEM, 28.4 ± 0.5) months and parity 2 to 4 (mean ± SEM, 2.9 ± 0.09) were checked once daily (08.00 am) for the onset of estrus by boar exposure. Those who exhibited estrus were inseminated twice with the stored boar semen (dose of 3×10^9 spermatozoa/80 mL). At the time of estrus detection, insemination was performed with semen that had been stored for 48 h. The second homospermic insemination followed 24 h later with semen of the same ejaculate that had been stored for 72 h. During the insemination, the sows received boar exposure to minimize semen loss and back-flow (WILLENBURG et al., 2003). A total of 90 sows were inseminated. Thirty of them were inseminated with semen diluted by extender A at 30 °C (group A30), 30 sows with semen diluted by extender A at 23 °C, representing groups A23 and 23A, and 30 sows with semen diluted by extender B at 23 °C (group 23B). All sows were monitored for estrus return. The farrowing rate and the number of live born piglets were documented.

Statistical analysis. The statistical analysis was performed using the Statistical Analysis Systems version 9.3 (SAS Institute Inc., 1996, Cary, N.C., U.S.A.). Normality of the data was tested using the Shapiro-Wilk Test (Proc Univariate). Only the parameters hyperactivated spermatozoa, abnormal morphology and DNA integrity did not follow a normal distribution and were normalized by square root transformation. A repeated measure ANOVA, using a general linear model within subjects effect across time was conducted using Proc Glm (SAS, USA). Student's *t*-test was used to evaluate differences within groups and between specific time points, using Proc Univariate (SAS, USA). Farrowing rates were compared between groups using the chi-square (Proc Freq). Data were presented as mean ± SEM. A statistically significant difference was defined as $P<0.05$.

Results

There were no significant differences in any lab parameters between two groups within the same time point (Table 1-4). Storage time had a significant negative effect on most, but not all, sperm parameters, regardless of dilution time or extender (Table 1-4). Specifically, after semen dilution with extender A at 23 or 30 °C, immotile spermatozoa increased and rapid moving spermatozoa decreased at 72 h compared to 48 h. However, rapid spermatozoa remained constant when extender B was used. Furthermore, VCL,

VAP and WOB decreased significantly during the storage period in extender A, both at 23 and 30°C (Table 1), but not in extender B (Table 3). Abnormal spermatozoa and DNA fragmentation were significantly increased, while hyperactivated spermatozoa remained constant in all groups between 48 h and 72 h (Table 2 and 4).

Table 1. The effect of dilution temperature with OptimIA extender at 23 (A23) and 30 °C (A30) on the kinetic parameters of boar spermatozoa (%) after 48 and 72 h of liquid storage (n = 20 ejaculates from 7 boars). Values are expressed in mean ± SEM.

	48 h		72 h	
Variable	A23	A30	A23	A30
Immotile	36.1 ± 4.0^{a,1}	33.5 ± 3.9^{a,1}	55.9 ± 4.5^{b,1}	50.3 ± 4.9^{b,1}
Progressive	28.3 ± 2.2^{a,1}	30.0 ± 2.7^{a,1}	16.7 ± 2.6^{b,1}	19.8 ± 2.8^{b,1}
Rapid	23.6 ± 3.2^{a,1}	26.5 ± 4.2^{a,1}	12.0 ± 2.6^{b,1}	13.1 ± 2.4^{b,1}
Medium	18.8 ± 1.6^{a,1}	18.8 ± 1.9^{a,1}	12.0 ± 1.7^{a,1}	14.8 ± 2.0^{a,1}
Slow	21.5 ± 1.1^{a,1}	21.2 ± 1.7^{a,1}	20.1 ± 1.6^{a,1}	21.8 ± 1.8^{a,1}
VCL	41.1 ± 2.2^{a,1}	42.5 ± 3.0^{a,1}	33.0 ± 2.5^{b,1}	31.9 ± 2.4^{b,1}
VSL	17.4 ± 1.0^{a,1}	16.7 ± 0.8^{a,1}	11.9 ± 1.0^{b,1}	12.5 ± 1.2^{b,1}
VAP	29.4 ± 1.6^{a,1}	28.6 ± 1.9^{a,1}	20.0 ± 1.8^{b,1}	20.6 ± 1.9^{b,1}
LIN	44.4 ± 3.2^{a,1}	41.9 ± 2.9^{a,1}	36.8 ± 2.8^{a,1}	38.6 ± 3.1^{a,1}
STR	60.7 ± 3.1^{a,1}	60.3 ± 2.6^{a,1}	59.6 ± 2.3^{a,1}	59.9 ± 2.4^{a,1}
WOB	71.9 ± 2.2^{a,1}	68.5 ± 2.3^{a,1}	60.3 ± 3.0^{b,1}	63.0 ± 3.3^{a,1}
ALH	2.0 ± 0.1^{a,1}	2.1 ± 0.1^{a,1}	2.0 ± 0.1^{a,1}	1.8 ± 0.1^{a,1}
BCF	6.9 ± 0.1^{a,1}	7.1 ± 0.1^{a,1}	6.5 ± 0.4^{a,1}	6.1 ± 0.4^{a,1}

VCL: curvilinear velocity (μm/sec); VSL: straight line velocity (μm/sec); VAP: average path velocity (μm/sec); LIN: linearity (VSL/VCL); STR: straightness (VSL/VAP); WOB: wobble (VAP/VCL); ALH: amplitude of lateral head displacement (μm); BCF: beat cross frequency (Hz). ^{a,b} Values within dilution temperature between time with different superscripts differ significantly (P<0.05). ^{1,2} Values within time between dilution temperature with different superscripts differ significantly (P<0.05).

Table 2. The effect of dilution temperature with OptimIA extender at 23 (A23) and 30°C (A30) on the characteristics of boar spermatozoa (%) after 48 and 72 h of liquid storage (n = 20 ejaculates from 7 boars). Values are expressed in mean. ± SEM.

	48 h		72 h	
Variable	A23	A30	A23	A30
Hyperactivated	0.8 ± 0.2^{a,1}	1.1 ± 0.3^{a,1}	0.8 ± 0.2^{a,1}	0.7 ± 0.2^{a,1}
Abnormal	17.0 ± 2.1^{a,1}	17.9 ± 2.4^{a,1}	26.5 ± 3.0^{b,1}	26.5 ± 3.5^{b,1}
DNA	1.3 ± 0.2^{a,1}	1.0 ± 0.2^{a,1}	2.0 ± 0.2^{b,1}	1.6 ± 0.1^{b,1}

^{a,b} Values within dilution temperature between time with different superscripts differ significantly (P<0.05). ^{1,2} Values within time between dilution temperature with different superscripts differ significantly (P<0.05).

Table 3. The effect of dilution with extenders OptimIA and Androhep Plus at 23°C (23A and 23B, respectively) on the kinetic parameters of boar spermatozoa (%) after 48 and 72 h of liquid storage (n = 20 ejaculates from 7 boars). Values are expressed in mean. ± SEM.

	48 h		72 h	
Variable	23A	23B	23A	23B
Immotile	37.3 ± 3.9^{a,1}	38.4 ± 4.1^{a,1}	57.6 ± 4.4^{b,1}	53.9 ± 4.8^{b,1}
Progressive	28.1 ± 2.3^{a,1}	28.8 ± 3.2^{a,1}	15.1 ± 2.3^{b,1}	16.9 ± 2.6^{b,1}
Rapid	22.8 ± 3.1^{a,1}	18.5 ± 3.4^{a,1}	10.3 ± 2.1^{b,1}	11.8 ± 2.3^{a,1}
Medium	18.5 ± 1.6^{a,1}	18.8 ± 1.7^{a,1}	11.2 ± 1.7^{b,1}	12.1 ± 1.8^{b,1}
Slow	21.4 ± 1.1^{a,1}	24.3 ± 1.3^{a,1}	20.9 ± 1.7^{a,1}	22.2 ± 2.5^{a,1}
VCL	41.0 ± 2.1^{a,1}	36.4 ± 2.4^{a,1}	32.2 ± 2.4^{b,1}	31.0 ± 2.7^{a,1}
VSL	17.5 ± 1.1^{a,1}	16.0 ± 1.2^{a,1}	11.5 ± 1.0^{a,1}	12.8 ± 1.3^{a,1}
VAP	29.2 ± 1.6^{a,1}	24.6 ± 1.7^{a,1}	19.2 ± 1.6^{b,1}	20.0 ± 2.0^{a,1}
LIN	44.4 ± 3.5^{a,1}	45.4 ± 3.4^{a,1}	36.8 ± 3.1^{a,1}	42.0 ± 3.0^{a,1}
STR	60.7 ± 3.2^{a,1}	65.3 ± 2.6^{a,1}	59.8 ± 2.5^{a,1}	64.5 ± 2.0^{a,1}
WOB	71.9 ± 2.5^{a,1}	68.1 ± 2.9^{a,1}	59.9 ± 3.2^{b,1}	64.6 ± 2.9^{a,1}
ALH	2.0 ± 0.1 ^{a,1}	2.0 ± 0.1^{a,1}	2.0 ± 0.1^{a,1}	1.9 ± 0.2^{a,1}
BCF	6.9 ± 0.1^{a,1}	7.0 ± 0.2^{a,1}	6.6 ± 0.5^{a,1}	6.4 ± 0.5^{a,1}

VCL: curvilinear velocity (μm/sec); VSL: straight line velocity (μm/sec); VAP: average path velocity (μm/sec); LIN: linearity (VSL/VCL); STR: straightness (VSL/VAP); WOB: wobble (VAP/VCL); ALH: amplitude of lateral head displacement (μm); BCF: beat cross frequency (Hz). ^{a,b} Values within extender between time with different superscripts differ significantly (P<0.05). ^{1,2} Values within time between extenders with different superscripts differ significantly (P<0.05).

Table 4. The effect of dilution with extenders OptimIA and Androhep Plus at 23 °C (A23, 23B, respectively) on the characteristics of boar spermatozoa (%) after 48 and 72 h of liquid storage (n = 20 ejaculates from 7 boars). Values are expressed in mean ± SEM.

	48 h		72 h	
Variable	23A	23B	23A	23B
Hyperactivated	0.7 ± 0.1^{a,1}	0.9 ± 0.2^{a,1}	0.7 ± 0.2^{a,1}	0.6 ± 0.2^{a,1}
Abnormal	16.1 ± 2.2^{a,1}	17.5 ± 2.7^{a,1}	25.2 ± 3.1^{b,1}	26.2 ± 3.6^{b,1}
DNA	1.3 ± 0.2^{a,1}	1.0 ± 0.2^{a,1}	1.9 ± 0.2^{b,1}	1.9 ± 0.3^{b,1}

^{a,b} Values within extender between time with different superscripts differ significantly (P<0.05). ^{1,2} Values within time between extenders with different superscripts differ significantly (P<0.05).

Fertility records are listed in Table 5. The farrowing rate was significantly lower in group A23, and the number of live born piglets was significantly lower in group 23B compared to the remaining groups.

Table 5. Farrowing rate and the number of live born piglets obtained after insemination with lab evaluated diluted boar semen in Androhep Plus and OptimIA extender at 23 °C (23B, A23/23A, respectively), and OptimIA extender at 30 °C (A30). Values of live born piglets are expressed in mean ± SEM.

Groups	N	Farrowing rate %	Number of live born piglets
23B	30	76.6[a]	9.96 ± 0.6[a]
A23/23A	30	46.6[b]	12.2 ± 0.4[b]
A30	30	83.3[a]	13.3 ± 0.5[b]

[a,b] Values within a column with different superscripts differ significantly (P<0.05)

Discussion

The present study evaluates the quality characteristics of boar semen under laboratory conditions, and the field fertility after one step dilution at 30 or at 23 °C, as well as the efficiency of a special sperm membrane protective extender after dilution at 23 °C. The *in vitro* sperm quality was evaluated by motility kinetics, morphology and sperm chromatin integrity determination, while the *in vivo* fertility was assessed by recording the farrowing rate and the number of live born piglets. Sperm motility affects fertilization directly, because it requires the integrity of the sperm membrane and active metabolism of the cell. In our study, different temperatures of dilution had no effect on boar semen kinetics, which impaired within 24 h (from 48 to 72 h) of storage. However, the use of a special extender seemed to delay the deterioration of rapid moving spermatozoa, VCL, VAP and WOB, which was evident after storage in the other groups. Androhep plus (B) assisted boar spermatozoa to maintain a higher level of movement, which is necessary to reach the oviduct and to interact and fertilize the oocyte. In humans, VCL and VAP have been shown to be important markers of the *in vitro* fertilizing ability of sperm (DE GEYTER et al., 1998). However, there are few data concerning the relationship between parameters evaluated with CASA and swine field fertility. HOLT et al. (1997) found a positive relationship between VAP and pig field fertility and VYT et al. (2008) between motility and the number of piglets born. In our study, the use of Androhep plus resulted in a higher farrowing rate, but also in a lower number of live born piglets compared to the common extender. More spermatozoa being capable of fertilization after 72 h in the Androhep plus group could partly explain the improvement in the farrowing rate. However, for the first insemination, semen of comparable quality (at 48 h of storage) was used and could have influenced the fertility results. In addition, it is well known that the *in vivo* fertilization process is more complicated and it is generally accepted that lab evaluated sperm parameters can indicate, but not totally predict field fertility results (RODRÍGUEZ-MARTÍNEZ, 2003).

Furthermore, previous studies reported that morphological abnormalities negatively affect the farrowing rate and the litter size (ALM et al., 2006; TSAKMAKIDIS et al., 2010). In the present study, all tested groups showed a significant increase of morphological abnormalities from 48 to 72 h of storage, independently of dilution temperature or extender. Similarly, chromatin integrity, which is related to low conception rate and litter size (BOE-HANSEN et al., 2005; EVENSON et al., 1994), was significantly higher after 72 h compared to 48 h of semen storage in all groups. The quality of sperm DNA is important in maintaining the reproductive potential of the male (WRIGHT et al., 2014). According to BOE-HANSEN et al. (2005), increased damage to chromatin integrity is presumed during semen storage. It is detected as early as 72 hours after collection and, for some boars, after only 24 hours. In contrast, DE AMBROGI et al. (2006) did not find any difference in the integrity of sperm chromatin associated with various diluents or preservation time, after sperm storage for 96 hours at 17 °C. Although temperature is one of the factors implicated in spermatozoa DNA damage (SAILER et al., 1997), we could not detect any difference after dilution at 23 or 30 °C.

In general, our study demonstrated the similar effects of the two dilution temperatures on the *in vitro* evaluated characteristics of semen, indicating that boar semen dilution in one step under room temperature does not dramatically affect the *in vitro* quality. This is in agreement with the results of a previous study (LOPEZ RODRIGUEZ et al., 2012), which concluded that dilution temperatures of 23 and 30 °C in the second step of a two-step dilution protocol, do not affect *in vitro* boar semen quality.

However, the results of the lab evaluated parameters were not exactly reflected in the field study. A significantly lower farrowing rate was observed in the OptimIA at 23 °C group compared to the remaining groups. Moreover, the protective effect of Androhep plus at 23 °C was expressed in the farrowing rate, but not in the number of live born piglets, that was significantly lower compared to the other tested groups. Parity, nutrition and differences among breeds are related with differences in the maternal ability to farrow a higher number of piglets (CAMPOS et al., 2012). Additionally, litter size depends on the time of insemination relative to ovulation, i.e. the fertilization rate is optimized when the artificial insemination is performed over a period of 24 h before and a few hours after ovulation (NISSEN et al., 1997). In our study, the sows were of the same sire and breed, of similar parity and were also housed and fed similarly, according to a complete commercial dietary. In addition, a traditional protocol of double artificial insemination during estrus was performed, with at least one insemination realized within an optimal time window for fertilization. Many factors that affect sperm transport and survival in the female reproductive tract and that have not been researched in this study, such as oxidative stress and phagocytosis, could have influenced our results.

In conclusion, one step dilution at 23 °C did not affect the *in vitro* quality characteristics of boar semen stored up to 72 h, compared to a dilution at 30 °C, however, field fertility was negatively influenced. A special type of extender can be beneficial for one step dilution under room temperature, but semen extension with a preheated extender at a temperature of around 30 °C is still superior for swine productivity.

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Conflict of interest

The authors declare that there is no conflict of interest.

References

ALM, K., O. A. T. PELTONIEMI, E. KOSKINEN, M. ANDERSSON (2006): Porcine field fertility with two different insemination doses and the effect of sperm morphology. Reprod. Dom. Anim. 41, 210-213.

ALTHOUSE, G. C., M. E. WILSON, C. KUSTER, M. PARSLEY (1998): Characterization of lower temperature storage limitations of fresh-extended porcine semen. Theriogenology 50, 535-543.

BOE-HANSEN, G. B., A. K. ERSBØLL, T. GREVE, P. CHRISTENSEN (2005): Increasing storage time of extended boar semen reduces sperm DNA integrity. Theriogenology 63, 2006-2019.

CAMPOS, P. H. R. F., B. A. N. SILVA, J. L. DONZELE, R. F. M. OLIVEIRA, E. F. KNOL (2012): Effects of sow nutrition during gestation on within-litter birth weight variation: a review. Animal 6, 797-806.

DE AMBROGI, M., J. BALLESTER, F. SARAVIA, I. CABALLERO, A. JOHANNISSON, M. WALLGREN, M. ANDERSSON, H. RODRIGUEZ-MARTINEZ (2006): Effect of storage in short- and long-term commercial semen extenders on the motility, plasma membrane and chromatin integrity of boar spermatozoa. Int. J. Androl. 29, 543-552.

DE GEYTER, C., M. DE GEYTER, B. KOPPERS, E. NIESCHLAG (1998): Diagnostic accuracy of computer-assisted sperm motion analysis. Hum. Reprod. 13, 2512-2520.

DE LEEUW, F., B. COLENBRANDER, A. VERKLEIJ (1990): The role membrane damage plays in cold shock and freezing injury. Reprod. Domest. Anim. 1, 95-104.

DROBNIS, E. Z., L. M. CROWE, T. BERGER, T. J. ANCHORDOGUY, J. W. OVERSTREET, J. H. CROWE (1993): Cold shock damage is due to lipid phase transitions in cell membranes: A demonstration using sperm as a model. J. Exp. Zool. 265, 432-437.

EVENSON, D. P., L. THOMPSON, L. JOST (1994): Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. Theriogenology 41, 637-651.

HOLT, C., W. V. HOLT, H. D. MOORE, H. C. REED, R. M. CURNOCK (1997): Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. J. Androl. 18, 312-323.

LOPEZ RODRIGUEZ, A., T. RIJSSELAERE, P. VYT, A. VAN SOOM, D. MAES, (2012): Effect of dilution temperature on boar semen quality. Reprod. Dom. Anim. 47, e63-e66.

NISSEN, A. K., N. M. SOEDE, P. HYTTEL, M. SCHMIDT, L. D'HOORE (1997): The influence of time of insemination relative to time of ovulation on farrowing frequency and litter size in sows, as investigated by ultrasonography. Theriogenology 47, 1571-1582.

PARKS, J. E., D. V. LYNCH (1992): Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology 29, 255-266.

PETRUNKINA, A. M., G. VOLKER, K. F. WEITZE, M. BEYERBACH, E. TOPFER-PETERSEN, D. WABERSKI (2005): Detection of cooling-induced membrane changes in the response of boar sperm to capacitating conditions. Theriogenology 63, 2278-2299.

PIPAN, M. Z., J. MRKUN, M. KOSEC, A. N. SVETE, P. ZRIMŠEK (2014): The effect of dialysis on the lipid peroxidation and antioxidant status of boar seminal plasma during liquid preservation. Vet. arhiv. 84, 129-141.

ROBERTSON, L., J. L. BAILEY, M. M. BUHR (1990): Effects of cold shock and phospholipase A2 on intact boar spermatozoa and sperm head plasma membranes. Mol. Reprod. Dev. 26, 143-149.

RODRÍGUEZ-MARTÍNEZ, H. (2003): Laboratory semen assessment and prediction of fertility: still utopia? Reprod. Dom. Anim. 38, 312-318.

RODRÍGUEZ-MARTÍNEZ, H., F. SARAVIA, M. WALLGREN, P. TIENTHAI, A. JOHANNISSON, J. M. VÁZQUEZ, E. MARTINEZ, J. ROCA, L. SANZ, J. J. CALVETE (2005): Boar spermatozoa in the oviduct. Theriogenology 63, 514-535.

SAILER, B. L., L. J. SARKAR, J. A. BJORDAHL, L. K. JOST, D. P. EVENSON (1997): Effects of heat stress on mouse testicular cells and sperm chromatin structure. J. Androl. 18, 294-301.

SHIPLEY, C. F. (1999): Breeding soundness examination of the boar. Swine Health Prod. 7, 117-120.

TSAKMAKIDIS, I. A., T. A. A. KHALIFA, A. G. LYMBEROPOULOS, I. A. MICHOS, C. M. BOSCOS (2011): Evaluation of common *in vitro* used chemicals' effect on motility and chromatin stability of boar spermatozoa. J. Hell. Vet. Med. Soc. 62, 118-124.

TSAKMAKIDIS, I. A, A. G. LYMBEROPOULOS, T. KHALIFA (2010): Relationship between sperm quality traits and field-fertility of porcine semen. J. Vet. Sci. 11, 151-154.

VYT, P., D. MAES, C. QUINTEN, T. RIJSSELAERE, W. DELEY, M. AARTS, A. DE KRUIF, A. VAN SOOM (2008): Detailed motility evaluation of boar semen and its predictive value for reproductive performance in sows. Vlaams Diergen. Tijds. 77, 291-298.

WABERSKI, D. (2009): Critical steps from semen collection to insemination. Proceedings of the Annual Meeting of EU-AI-Vets, 9-12 September. Ghent, Belgium. pp. 66-69.

WILLENBURG, K. L., G. M. MILLER, S. L. RODRIGUEZ-ZAS, R. V. KNOX (2003): Effect of boar exposure at time of insemination on factors influencing fertility in gilts. J. Anim. Sci. 81, 9-15.

WRIGHT, C., S. MILNE, H. LEESON (2014): Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. Reprod. Biomed. Online 28, 684-703.

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ALEXIOU, V., A. G. BASIOURA, G. TSOUSIS, E. D. TZIKA, C. M. BOSCOS, G. VATZIAS, I. A. TSAKMAKIDIS: Učinak temperature razrjeđivanja dvaju različitih razrjeđivača na kvalitetu sperme nerasta. Vet. arhiv 87, 197-208, 2017.

SAŽETAK

Istražena je kvaliteta i plodnost nerastove sperme u terenskim uvjetima nakon razrjeđivanja s dva različita razrjeđivača: OptimaIA – standardni razrjeđivač na 23 °C (oznaka skupine A23, n = 20) ili na 30 °C (oznaka skupine A30, n = 20) i OptimaIA - komercijalni razrjeđivač na 23 °C (oznaka skupine 23A, n = 20) ili sa zaštitnikom membrane Androhep Plus na 23 °C (oznaka skupine 23B, n = 20). Svaki uzorak razrijeđene sperme bio je pohranjen na 16-18 °C i korišten za dvokratno umjetno osjemenjivanje nakon 48 h i 72 h od prikupljanja na farmi (n = 30 po skupini). Sperma je u laboratoriju ocijenjena (pokazatelji pokretljivosti, morfologija i DNA fragmentacija) prilikom prikupljanja (0 h) i prilikom osjemenjivanja (48 h i 72 h). Većina pokazatelja je, bez obzira na temperature razrjeđivanja ili uporabu zaštitnog razrjeđivača, pokazala pogoršanje u laboratorijskim uvjetima od 48 h na 72 h. Ipak, kod posebnog zaštitnog razrjeđivača, razlike između 48 h i 72 h nisu utvrđene za postotak brzo pokretljivih spermija, valovitost gibanja, prosječnu brzinu i oscilirajuće pokretanje (treperenje). U usporedbi s ostalim dvjema skupinama, niža stopa oprasivosti opažena je kod primjene standardnog razrjeđivača na 23 °C, a niži broj živorođenih odojaka opažen je nakon primjene zaštitnog razrjeđivača. Zaključno, iako postoji negativni utjecaj na plodnost u terenskim uvjetima, nakon 72 sata pohranjivanja, jedan korak razrjeđivanja nerastove sperme na 23 °C, u usporedbi s 30 °C, ne utječe dramatično na njezinu kvalitetu *in vitro*. Neki od negativnih utjecaja mogu se nadomjestiti uporabom razrjeđivača koji štite membranu spermija.

Ključne riječi: sperma, nerast, razrjeđivač, plodnost, terenski uvjeti, kvaliteta, učinak temperature