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Increasing Extender Viscosity Improves the Quality of Cooled Boar Semen

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Abstract

The use of several types of gelling extenders for the storage of semen from several domestic species in the solid state has been shown to have beneficial effects on some semen quality parameters. The objective of this study was to evaluate the effect of a new high-viscosity semen extender, Zoosperm ND-5 3D® (Import-Vet, Centelles, Spain), on the the quality of boar spermatozoa at preserved at 17ºC for 7 days. Sodium alginate was used for the first time to increase the viscosity of the extender for the liquid storage of boar semen. The same extender, but without increased viscosity, was used as a control extender (Zoosperm ND-5®, Import-Vet, Centelles, Spain). Sixteen ejaculates from four Pietrain boars were evaluated for motility (by the CASA system), and for viability, acrosome status, plasma membrane fluidity, externalization of phosphatidylserine at the plasma membrane of the spermatozoa and mitochondrial membrane potential (by flow cytometry). In samples diluted with the Zoosperm ND-5 3D® viscous extender, the STR (straightness) parameter and the number of progressively motile spermatozoa were higher compared to those of the non-viscous extender ($p < 0.05$). In addition, the number of spermatozoa with damaged acrosomes, an unstable sperm plasma membrane and externalization of phosphatidylserine at the plasma membrane was lower in samples treated with the viscous extender ($p < 0.05$). In conclusion, an increase in extender viscosity improves quality of boar spermatozoa following long-term storage.

Keywords: Zoosperm ND-5 3D®, boar, extender, storage, viscosity

1. Introduction

The role that artificial insemination (IA) based on cooled semen technology has played in global boar production is widely acknowledged. Among other advantages, this technology allows for maximizing the genetic potential of high-quality breeding stock. Among the facts that have contributed to the advances in this technology are better knowledge of sow management and physiology, improvements in semen processing and design of better extenders.

Because seminal plasma alone does not allow for long-lasting semen preservation, the addition of an extender permits an increase in the ejaculate volume, extends the average life span of spermatozoa and maintains an adequate fertility level in the sow herd (Gadea, 2003). Because boar spermatozoa are highly susceptible to cold shock, they need to be stored at temperatures of 15-20ºC, which limits storage period.

During the storage of extended boar semen, a further decrease in the fertility potential as a result of the natural ageing process of spermatozoa cannot be prevented. In several studies, functional and structural changes of spermatozoa during storage have been measured. The data include changes in motility, viability, pH, DNA integrity, acrosome intactness, mitochondrial activity, bacterial contamination, tyrosine phosphorylation and more recently, screening for the sperm proteome, cytome and lipidome (De Ambrogi et al., 2006; Dubé, Beaulieu, Reyes-Moreno, Guillemette, & Bailey, 2004; Huo, Ma, & Yang, 2002; Martín-Hidalgo et al., 2013; Waberski, Henning, & Petrunkina, 2011).

Extenders are a factor influencing the cold shock tolerance of spermatozoa. Over the last 10 years, new extenders for boar semen, both for short-term (1-3 days) and long-term preservation (more than 4 days), have been designed (Riesenbeck, 2011). The use of long-term extenders is suitable in situations where work overload is expected at the AI center, when large sow herds are involved or when seminal doses are prepared from low demand or rarely used boars. They also allow for long-distance transportation of the semen. Artificial insemination with cooled semen is likely to remain the most widely used sperm preservation technology in swine rearing programs, given the excellent fertility results achieved until now (Roca et al., 2006).

An increase in extender viscosity has been shown to improve the quality of stored semen. However, research in boars about this subject is scarce. Corcini et al. (2011) observed that adding gelatin to the short-term Beltsville Thawing Solution (BTS) extender for boar semen produced a less marked fall in the percentage of motile and morphologically normal spermatozoa during the preservation period compared to the samples to which no gelatin was added, although no significant differences between extenders were observed in either parameter (Corcini et al., 2011). Coy, Gadea, Rath and Hunter (2009) showed that the re-suspension of boar spermatozoa in a viscous medium increased the stability of the sperm membrane, decreased reactive oxygen species production and increased the capacity of spermatozoa to penetrate oocytes *in vitro*. The use of gelling extenders, mostly by the use of gelatin, has proven to be effective in prolonging the preservation of rabbit (López-Gatius et al., 2005; Nagy, Sinkovics, & Kovacs, 2002; Rosato & Iaffaldano, 2011), ovine (Yániz et al., 2005) and caprine (Salvador, Yániz, Viudes-de-Castro, Gómez, & Silvestre, 2006) sperm in a solid state. However, it remains to be seen whether the reported benefits are also applicable to semen stored in a liquid form. Those benefits were attributed to possible effects on sperm sedimentation and the reduction of metabolic activity due to the reduced sperm motility. Alginate or alginic acid is an anionic polysaccharide distributed widely in the cell walls of brown algae. It has a large gelling capacity and is widely used for the microencapsulation of spermatozoa before storing. Microencapsulation in barium alginate membranes has been found to protect sperm cells during storage, preserving morpho-functional characteristics (Spinaci et al., 2013; Torre et al., 2000; Vigo et al., 2002). We could find no reference in the literature to the use of alginate in long-term viscous extenders for the long-term preservation of boar semen in liquid form. For this reason, Import-Vet, S. A. (Centelles, Spain) has designed Zoosperm ND-5 3D® (hereafter 3D), a viscous extender for long-term preservation. This study evaluates for the first time the effectiveness of this new viscous extender in terms of its capacity to preserve the sperm quality of boar semen aliquots after 7 days at 17ºC. In addition, the same extender but without increased viscosity was used as a control extender (Zoosperm ND-5®, Import-Vet, S. A., hereafter ND-5). On the basis of previous studies of viscous extenders for semen storage in the solid state, our hypothesis is that 3D will improve the sperm quality of the liquid stored semen.

2. Materials and Methods

2.1 Chemicals and Sources

ND-5® and ND-5 3D® were provided by Import-Vet, S. A. (Centelles, Spain). Live/dead spermatozoa viability kits including propidium iodine (PI) and SYBR-14, M540 and YoPro-1 probes were purchased from Molecular Probes (Leiden, The Netherlands). Annexin-V-FITC was obtained from Immnostep (Salamanca, Spain). FITC-PNA was purchased from Sigma-Aldrich® (St. Louis, MO, USA). The coulter isoton II diluent was obtained from Beckman Coulter Inc. (Brea, CA, USA) and the JC-1 (5,5′,6,6′–tetrachloro-1,1′,3,3′ tetraethylbenzymidazolyl carbocyanine iodine) probe was purchased from Life Technologies Ltd. (Grand Island, NY, USA).

2.2 Sample Preparation and Assessment of Sperm Quality Parameters

2.2.1 Sample Preparation

Sixteen ejaculates from four healthy, sexually mature (2-3 years old) and fertile Pietrain boars were analyzed. The animals belonged to an AI center in Segovia (Spain). The boars were subjected to regular semen collection three times every two weeks for commercial use. All the males were kept in individual pens in a controlled environment (15-20ºC) and all were fed the same diet. The ejaculate was collected using the "gloved hand" technique and was then gently homogenized and divided in two equal parts. After an initial assessment of motility (assessed subjectively by light microscopy at 10X), morpho-anomalies and concentration (both by light microscopy in a Bürker chamber after dilution in 10% formaldehyde-saline solution at 40X), one of the fractions was diluted to a 33 x 10^6 cells/mL final concentration with ND-5 extender and the other fraction, at the same concentration level, was diluted with 3D viscous extender. The composition of the extenders differed only in the hydrocolloid compound (sodium alginate) added to the 3D extender in order to increase viscosity to a 13 centipoises value at 17ºC. Viscosity measurement was performed by a specialized laboratory (Brookfield viscometer, CETAEX, Badajoz, Spain). The quantitative composition is unknown because of commercial interests, so only qualitative composition is done (glucose, sodium citrate, EDTA, sodium bicarbonate, potassium chloride, acetylcysteine,

MOPS and antibiotics). Both extenders were prepared following the manufacturer's instructions. Dilutions of both ejaculate fractions were carried out within 15 min after they were obtained. Only those ejaculates containing at least 70% of motile spermatozoa and less than 20% of morphologically abnormal spermatozoa were selected for the trial. The diluted semen was then bottled in 90 ml plastic bottles with clip-tops (Import-Vet, S. A., Centelles, Spain).After lowering the temperature of the semen doses to 20-22ºC over approximately 2 h at room temperature, 3 bottles of each ejaculate diluted in ND-5 or 3D were stored at 16-17ºC until their transport to the laboratory of the Veterinary Faculty of Cáceres. This journey, which took 12 h, occurred at night. The samples were carried in isothermal boxes by a courier with no special precautions. On arrival at the laboratory, the semen doses were stored in a refrigerated incubator (FOC 225 I, VELP Scientifica, Usmate, Italy) at 16-17ºC for 7 days (collection day = day 0). On days 1, 4 and 7, two aliquots were extracted from one bottle of each extender. A total of 192 semen samples were analyzed. The following sperm quality parameters were measured in each of the aliquots: motility, viability, acrosome status, plasma membrane fluidity, phosphatidylserine externalization at the plasma membrane of spermatozoa and mitochondrial membrane potential. The motility analysis was carried out by a CASA (Computer Assisted Sperm Analysis) system and the other parameters were measured by flow cytometry.

2.2.2 Assessment of Sperm Motility

Immediately after gentle mixing, 1mL of semen was taken from each bottle and examined for motility pattern using the CASA system (ISAS® program, Proiser R+D, Paterna, Valencia, Spain). Before the motility analysis, the seminal samples were incubated at 38ºC for 1 h (Mini Galaxy A, RS Biotech, United Kingdom). A total of 2 µl of sample was placed in a pre-warmed counting chamber (Leja®, Luzernestraat, The Netherlands). Sperm motility analysis was based on the examination of 25 consecutive digitalized images obtained from several fields using a 10X negative-phase contrast objective and a heated stage at 38ºC. At least 300 spermatozoa per sample were analyzed. Images were taken with a time lapse of 1s. The following sperm motility parameters were recorded: total motile spermatozoa (% TMS, spermatozoa with an average path velocity, VAP > 10µm/s), progressively motile spermatozoa (% PMS, spermatozoa with a straightness coefficient > 0.8, 80%), VCL (curvilinear velocity in µm/s), VSL (straight-line velocity in µm/s), VAP (average path velocity in µm/s), STR (straightness coefficient in %), WOB (wobble coefficient in %) and ALH (amplitude of lateral head displacement in µm). Spermatozoa with an average path velocity (VAP) < 10 µm/s were considered immotile. Spermatozoa deviating < 10% from a straight line were designated as linearly motile and spermatozoa with a radius < 25 µm were classified as circularly motile (Saravia et al., 2005).

2.2.3 Flow Cytometry Analyses

Flow cytometry analyses were performed using a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter Ltd.) The fluorophores were excited by a 200 mV argon ion laser operating at 488 nm. A total of 10,000 gated events based on the forward scatter and side scatter of the sperm population recorded in the linear mode were collected per sample with a running rate of approximately 500 events/s. The fluorescence data were collected in the logarithmic mode and analyzed using a FACStationTM and EXPOTM 32 ADC software (Beckman Coulter, Inc.).

2.2.3.1 Assessment of Sperm Viability

Fluorescent staining using the LIVE/DEAD Sperm Viability Kit was performed to assess porcine spermatozoa viability (Aparicio et al., 2007). Briefly, 5 µl of SYBR-14 (2 µM) and 10µl of propidium iodide (PI 5 µM) were added to 500 µL of diluted semen sample (33 x 10^6 cells/mL) in isotonic buffered diluent Coulter Isoton II and incubated for 20 min at room temperature in the dark. After incubation, the cells were analyzed and the percentage of viable spermatozoa was expressed as the percentage of SYBR 14 - positive and propidium iodide - negative spermatozoa.

2.2.3.2 Assessment of Acrosome Status of Spermatozoa

The acrosome integrity of the spermatozoa was assessed after staining the spermatozoa with fluorescein isothiocyanate - peanut agglutinin (FITC-PNA), as a marker for acrosomal status, and PI (Waterhouse et al., 2004). Aliquots of 100 µL of each semen sample (33 x 10^6cells/mL) were incubated at room temperature in the dark for 5 min with 5 µL (1 µg/mL) PNA-FITC and 5 µL (6µmol/L) PI. Just prior to flow cytometry, 400 µl of isotonic buffered diluents was added to each sample and remixed. The cells were analyzed and the percentage of spermatozoa with acrosomes that were damaged or reacted was expressed as the percentage of PNA - positive and PI - negative spermatozoa.

2.2.3.3 Assessment of Plasma Membrane Fluidity

The plasma membrane fluidity of the spermatozoa was assessed by staining the sperm with merocyanine 540 (M540) and plasma membrane permeability was assessed by staining with YoPro-1 (Harrison, Ashworth, & Miller,

1996). Aliquots of 100 µl of each semen sample (33 x 10^6 cells/mL) were diluted in 400 µl of isotonic buffered diluent containing 75 nmol/L YoPro-1. The samples were then mixed and incubated at 38ºC for 15 min. Just before flow cytometry, M540 was added to each sample to a final concentration of 2 µmol/L, incubated for 2 min and remixed. After incubation, the cells were analyzed and the results were expressed as the percentage of viable sperm with an unstable plasma membrane (YoPro-1-negative/M-540 - positive).

2.2.3.4 Evaluation of the Phosphatidylserine Externalization at the Plasma Membrane of Spermatozoa

The study of phosphatidylserine (PS) externalization in plasma membrane spermatozoa was performed using Annexin-V-FITC to specifically detect PS translocation from the inner to the outer leaflet of the sperm plasma membrane.

Aliquots of 300 µl of each semen sample (33 x 10^6 cells/mL) were diluted in 200 µl of the following buffer: 96 mmol/l NaCl, 4.7 mmol/l KCl, 0.4 mmol/l $MgSO_4$, 0.3 mmol/l NaH_2PO_4, 5.5 mmol/l glucose, 1 mmol/l sodium pyruvate, 21.6 mmol/l sodium lactate, 20 mmol/l HEPES (pH 7.45), and 2.5 mmol/l $CaCl_2$. Then, a 100µl aliquot was transferred to a 5 ml tube and stained with 5 µl AnnexinV-FITC and 4µl propidium iodide (PI) by incubation for 15 min in the dark at room temperature. Finally, 400 µl of isotonic buffered diluents was added to each sample and mixed before flow cytometry analysis. For statistical analysis, the results were expressed as the percentage of viable sperm with PS externalization (AnnexinV-FITC - positive/PI - negative).

2.2.3.5 Assessment of Mitochondrial Membrane Potential Status

Mitochondrial membrane potential was evaluated using the specific probe JC-1. JC-1 reversibly changes its fluorescence from green (monomeric status) to orange (multimeric status, formation of aggregates, J_{agg}) when the mitochondrial membrane potential is high (Amaral & Ramalho-Santos, 2010). Because J_{agg} formation depends on the chemical environment (Reers, Smith, & Chen, 1991), seminal samples (1.2 mL) were centrifuged at 1500 g for 1.5 min and the pellet was resuspended in 0.8 mL of ND-5 extender. From each sperm sample, 100 µL (30 × 10^6 cells/mL) was diluted in 400 µL of isotonic buffered diluent containing 0.15 mmol/L JC-1 and then mixed and incubated at 38ºC for 30 min. The samples were remixed before flow cytometry analysis. The percentage of orange stained cells was recorded; these cells were defined as the cells with a high mitochondrial membrane potential (hMMP).

2.3 Statistical Analysis

The mean and the standard error of the mean were calculated for descriptive analysis. Q-Q plots were used to check for departures from the normal distribution. The effects of extender (ND-5 and 3D) and storage time (1, 4 and 7 days) on seminal characteristics were assessed using a General Linear Model for Repeated Measures. A mixed-effects model (with boars and ejaculates within boars as random effects and extender and storage time as fixed effects) was applied to the experimental design.

All statistical analyses were performed using the libraries Linear and Nonlinear Mixed Effects Models from the statistical package R 3.0.1 (Pinheiro, Bates, Debroy, Sarker, & The R Development Core Team, 2013). Statistical significance was defined as $p < 0.05$, 0.001.

3. Results

Table 1 shows the pH, osmolarity and viscosity characteristics of both extenders.

Table 1. Characteristics of ND-5® and ND-5 3D® extenders

Extender	pH	Osmolarity (mOsm/L)	Viscosity at 16ºC (cP)	Viscosity at 38ºC (cP)
ND-5	6.9	295	10	8
ND-5 3D	6.9	295	13	11

cP: centipoise.

Table 2 shows the results of the motility analysis. The increase in viscosity did not modify the percentage of motile spermatozoa (TMS). For the rest of the parameters, the values were significantly different between the extenders for some or all preservation days.

Table 2. Effects of extender on motility characteristics on specific days after collection

Parameter	Extender	Day 1	Day 4	Day 7	p value (time)	Interaction (extender x time)
VCL (μm/s)	ND-5	71.19±1.90	74.05±1.89	78.88±1.89	<0.001	n.s.
	ND-5 3D	55.60±1.90	62.11±1.89	65.13±1.93		
	p value (extender)	<0.001	<0.001	<0.001		
VSL (μm/s)	ND-5	49.94±2.17	49.27±2.16	48.07±2.16	n.s.	n.s.
	ND-5 3D	37.00±2.17	38.41±2.16	37.50±2.18	n.s.	
	p value (extender)	<0.001	<0.001	<0.001		
VAP (μm/s)	ND-5	61.82±2.00	63.04±1.99	65.57±1.99	<0.001	n.s.
	ND-5 3D	45.01±2.00	48.19±1.99	48.37±2.02		
	p value (extender)	<0.001	<0.001	<0.001		
STR	ND-5	0.79±0.01	0.77±0.01	0.72±0.01	<0.001	0.052
	ND-5 3D	0.81±0.01	0.79±0.01	0.77±0.01	<0.001	
	p value (extender)	0.018	0.017	<0.001		
WOB	ND-5	0.86±0.01	0.84±0.01	0.82±0.01	<0.001	0.017
	ND-5 3D	0.82±0.01	0.78±0.01	0.75±0.01	<0.001	
	p value (extender)	<0.001	<0.001	<0.001		
ALH (μm)	ND-5	2.14±0.04	2.29±0.04	2.55±0.04	<0.001	n.s.
	ND-5 3D	1.99±0.04	2.26±0.04	2.40±0.04		
	p value (extender)	0.005	n.s.	0.008		
BCF (μm)	ND-5	8.09±0.11	7.86±0.11	7.76±0.11	0.019	<0.001
	ND-5 3D	8.21±0.11	8.92±0.11	9.33±0.11	<0.001	
	p value (extender)	n.s.	<0.001	<0.001		
TMS (%)	ND-5	87.3±0.02	85.8±0.02	82.0±0.02	<0.001	n.s.
	ND-5 3D	87.8±0.02	86.7±0.02	82.5±0.02		
	p value (extender)	n.s.	n.s.	n.s.		
PMS (%)	ND-5	56.1±0.03	48.7±0.03	40.3±0.03	<0.001	n.s.
	ND-5 3D	59.1±0.03	53.1±0.03	47.8±0.03		
	p value (extender)	n.s.	0.012	0.001		

TMS indicates percentage of total motile sperm; PMS, percentage of progressively motile sperm.
Values are means ± standard error of the mean (SEM).
n. s.: not significant ($p > 0.05$).

It is worth noting that both the STR, which indicates the straightness of the sperm trajectory, and the percentage of spermatozoa with progressive motility were higher in 3D. The differences between extenders were not the same throughout the preservation period; the difference was higher on day 7 in relation to days 1 and 4. On day 7, STR

increased 6.9% on average in 3D in relation to the ND-5 extender, whereas on days 1 and 4, a 2.5% and 2.6% increase was observed, respectively. Regarding the percentage of spermatozoa with progressive motility, a 9% increase (day 4) and an 18.6% increase (day 7) was obtained with the 3D extender.

It can also be seen that values for the velocity parameters VCL, VSL and VAP were higher in the ND-5 non-viscous extender; however, for these variables, the behavior of each extender through the storage period was no different, with no effect of storage time in the case of VSL.

The amplitude of lateral head displacement (ALH) was lower in the 3D extender, whereas flagellar beat cross frequency (BCF) was higher, given the higher viscosity of the medium. In addition, for BCF, the behavior throughout the storage time was different for each extender: there was a decrease over time in the ND-5 extender and an increase in the 3D extender.

Table 3 shows the results for the sperm quality variables in both extenders as measured by flow cytometry.

Table 3. Effects of extender on flow cytometry characteristics on specific days after collection

Parameter	Extender	Day 1	Day 4	Day 7	p value (time)	Interaction (extender x time)
VS	ND-5	92.59±0.46	93.32±0.45	93.34±0.45	n.s.	n.s.
	ND-5 3D	93.75±0.46	93.27±0.45	93.79±0.45		
	p value (extender)	n.s.	n.s.	n.s.		
DAS	ND-5	6.93±0.96	7.60±0.95	7.73±0.95	n.s.	n.s.
	ND-5 3D	3.24±0.96	3.40±0.95	3.47±0.95		
	p value (extender)	<0.001	<0.001	<0.001		
VUM	ND-5	6.80±0.43	9.11±0.42	8.67±0.42	0.001	0.028
	ND-5 3D	5.86±0.43	6.24±0.42	6.74±0.44	n.s.	
	p value (extender)	n.s.	<0.001	<0.001		
EPS	ND-5	8.93±0.64	10.45±0.78	11.27±0.87	n.s.	n.s.
	ND-5 3D	5.77±0.62	6.04±0.78	7,74±1.00		
	p value (extender)	<0.001	<0.001	<0.001		
hMMP	ND-5	76.95±3,94	73.36±5,52	79.93±3,39	n.s.	n.s.
	ND-5 3D	81.87±3,67	79.77±3,21	80.54±2,61		
	p value (extender)	<0.001	n.s.	n.s.		

VS indicates viable spermatozoa with an intact plasma membrane (SYBR-14+/PI -); DAS, viable spermatozoa with a damaged acrosome (PNA+/PI-); VUM, viable spermatozoa with an unstable plasma membrane; EPS, viable spermatozoa with phosphatidylserine externalization in plasma membrane; hMMP, spermatozoa with high mitochondrial membrane potential; MF, mean fluorescence intensity (JC-1).

Values are means ± standard error of the mean (SEM).

n. s.: not significant ($p > 0.05$).

There were no differences between the extenders in the percentage of viable spermatozoa with an intact plasma membrane (VS). The percentage of spermatozoa with a high mitochondrial membrane potential (hMMP) was only different on day 1 ($p < 0.001$), with higher values for the 3D extender. Indeed, for the live spermatozoa population, a 4 point average reduction in the percentage of spermatozoa with damaged acrosomes (DAS) (reduction of 54.5%) ($p < 0.001$) and in the percentage of spermatozoa with PS externalization at the plasma membrane (EPS)

(reduction of 36.2%) was obtained with the 3D extender, a decrease that remained constant throughout the 7 preservation days. The use of this extender also resulted in a significant decrease ($p < 0.001$) in the percentage of spermatozoa with an unstable plasma membrane (VUM) on days 4 and 7 (M540 - negative/YoPro-1- negative spermatozoa).

4. Discussion

The sperm velocity parameters were different for the two extenders. The most likely reason for this is their different densities. It has been shown that the viscosity of the medium surrounding the spermatozoon influences its movement (Hirai et al., 1997; Hunter, Coy, Gadea, & Rath, 2011; Kirkman-Brown & Smith 2011; Smith, Gaffney, Gadelha, Kapur, & Kirkman-Brown, 2009). Thus, in the case of a bull spermatozoon, the flagellar beat frequency decreases almost exactly with the square root of the viscosity (Rikmenspoel, 1984). While the hyperactive movement of a mouse spermatozoon is not linear in a low viscosity medium, it becomes more linear in a highly viscous or viscoelastic solution (Suárez & Dai, 1992). Our results are a further contribution to the study of the significant differences in the motility of spermatozoa in media of differing viscosity, and the greater consideration that this fact is being given in the measurement of *in vitro* motility, with a view to their subsequently being correlated to *in vivo* motility. Our study shows that an increase in the viscosity of the extender produced a reduction in the VCL, VSL and VAP velocity parameters. These results are similar to those reported by Suárez and Dai (1992) for mouse spermatozoa.

The number of motile spermatozoa, in particular those with progressive motility, is one of the most frequent measures used to estimate the quality of an ejaculate, given its importance for sperm migration through the female genital tract and penetration of the oocyte membrane. Thus, it is considered to be one of the factors determining *in vitro* fertilization rates (Simon & Lewis, 2011; Turner, 2006). In this study, the 3D viscous extender produced an increase both in the straightness of the trajectory of the spermatozoa and in the percentage with progressive motility. In addition, the difference between the extenders was greater at the end of the preservation period. Coy et al. (2009) used a plant extract to increase the viscosity of a medium for swine spermatozoa and observed an increase in the STR index and the percentage of progressively motile spermatozoa, although they also reported lower VCL, VSL and VAP values in comparison to the control medium. Corcini et al. (2011) found an increase in the percentage of motile spermatozoa after adding gelatin to the BTS extender for short term (three days) preserved swine semen. In our study, this parameter was not different between the extenders. The literature suggests that changes to motility parameters are dependent on the agent used to increase the viscosity (because it determines whether liquefaction will occur at the temperature at which motility is measured), as well as the degree of viscosity (Corcini et al., 2011; Coy et al., 2009; Salvador et al., 2006; Yániz et al., 2005). Different viscous media, therefore, influence the motility characteristics of the spermatozoa.

Various authors have indicated the importance of *in vitro* evaluation of sperm motility in viscous media. During transit through the sow's genital tract, the spermatozoon is surrounded by fluids that are not only of different chemical composition but also of different viscosity. The oviduct secretes a viscous glycoprotein (Hunter, 2002; Hunter, 2005; Jansen, 1978; Jansen, 1980). In experimental *in vitro* conditions, however, the level of viscosity is very low, close to that of water, which is very much lower than that of the oviduct. According to Coy et al. (2009), a certain degree of viscosity is desirable in order for the spermatozoon to interact with the oocyte in *vitro* (culture medium) or *in vivo* (oviduct) once it is no longer in contact with seminal plasma components. For this reason, they suggest that the viscosity of the medium is a parameter that must be taken into account when *in vitro* or *in vivo* experiments are being designed. Smith et al. (2009) also noted the need to measure sperm motility in media with viscosity similar to that found in uterine physiological conditions. In their study, they note how viscosity significantly affects sperm characteristics, such as planarity, torsion, wave form, trajectory and sperm progression and beat cross frequency, modifications that were observed in our study (BCF, ALH and WOB parameters). Those authors conclude that observations carried out in low viscosity liquids, which is the case in the majority of *in vitro* experiments, can provide little information with regard to motility *in vivo*. Furthermore, the fact that a viscous medium reduces the rolling rate allows the full waveform to be captured in a precise way for a period of one or more beats, which is convenient for bi-dimensional techniques for measuring flagellar movement. In the view of these researchers, given the important advances which are being made in the digital capture and processing of high-speed images, the use of a viscous medium has the advantage of allowing a more detailed analysis of the development and propagation of the flagellar wave and so establishes a better correlation between cellular progression in liquids with viscosities similar to physiological ones. These are matters that will surely be taken into consideration in the development of the new generation of CASA systems.

Various studies have attempted to relate the CASA parameters with *in vivo* fertility results. The results are contradictory (Broekhuijse, Sostaric, Feitsma, & Gadella, 2012; Budworth, Amann, & Chapman, 1988; Didion,

2008; Liu, Clake, & Baker, 1991). Because spermatozoa exposed to a viscous medium have a greater capacity to move, unite with and penetrate the oocyte under *in vitro* conditions (Coy et al., 2009), and their movements are more similar to those that occur in the oviduct, it would be interesting if future studies were to correlate the motility in viscous solutions with fertility results.

The 3D viscous extender produced a reduction in the number of spermatozoa with damaged acrosomes and an unstable plasma membrane. These are standard parameters for measuring semen quality. This favorable effect on semen quality was observed by Nagy et al. (2002) in rabbit spermatozoa suspended in an extender with gelatin. Coy et al. (2009) also found greater viability and less generation of reactive oxygen species in swine spermatozoa extended in Androhep® (Minitube, Germany) to which a powdered plant extract was added to increase its viscosity, compared with the viscosity achieved using Androhep® on its own. Unlike other studies (Coy et al., 2009; Nagy et al., 2002; Yániz et al., 2005), we did not find an increase in sperm viability with the viscous extender.

The liquid preservation of boar semen is associated with apoptotic-like changes in the sperm, such as a decrease in the mitochondrial membrane potential and externalization of phosphatidylserine from the inner to the outer leaflet of the sperm membrane (Trzcinska, Bryla, & Smorag, 2011). A decrease in the mitochondrial membrane potential of spermatozoa has frequently been associated with an increase in the number of abnormalities in the semen (Espinoza, Schulz, Sánchez, & Villegas, 2009) and a reduction in fertility (Gallon, C. Marchetti, Jouy, &P. Marchetti, 2006; Grunewald, Said, Paasch, Glander, & Agarwal, 2008). The percentage of spermatozoa with high mitochondrial potential did not differ significantly between extenders except on day 1, when the value for the viscous extender was higher, although there was a tendency towards greater values in the samples diluted in the viscous extender through the storage period.

The assessment of PS externalization is included in the evaluation of cooling - induced damage to spermatozoa. The number of spermatozoa with these early apoptotic changes has been correlated with poor fertility in breeding bulls (Anzar, He, Buhr, Kroestsch, & Pauls, 2002) and with male infertility in humans (P. Marchetti & C. Marchetti, 2007). In this study, the 3D viscous extender was associated with a reduction in the number of spermatozoa with PS externalization at the plasma membrane of spermatozoa. We cannot compare our results with other authors because this parameter is not reported in other studies.

The mechanism by which the 3D extender improves sperm quality remains unclear. López-Gatius et al. (2005) suggest that a viscous medium, because it limits the movement of the spermatozoa during storage, also probably causes a reduction in the metabolic demands made on them. According to Nagy et al. (2002), even when buffers are added to the extenders to minimize pH fluctuations, the sedimentation which inevitably occurs during the preservation probably leads to a lowering of the pH in the region of the sedimented cells as a result of the accumulation of toxic metabolites. Because the gelling extender (solid storage) avoids sedimentation, there is a more homogenous distribution of the spermatozoa, which in turn allows the buffer to act more efficiently. The spermatozoa also benefit from a more homogenous distribution of the various components of the suspension. However, in this study, the slight increase in 3D extender viscosity was not enough to maintain the spermatozoa in suspension through the storage period, and sedimentation was inevitable. The proposed justification is, in this case, not applicable. On the other hand, sperm microencapsulation in barium alginate membranes protects sperm cells during storage. This procedure has been shown to preserve morpho-functional characteristics such as motility, *in situ* enzymatic activity and acrosome integrity (Spinaci et al., 2013; Torre et al., 2000; Vigo et al., 2002). An explanation is that the “dilution shock” (Watson, 1995), with its damaging effects, that spermatozoa suffer when they are stored under refrigeration in extender is avoided. In our study, the fact that sedimentation and dilution were not avoided during storage suggests that some protective effect of the viscous medium is present. More research needs to be done to confirm this statement, including field fertility trials.

5. Conclusion

The use of the Zoosperm ND-5 3D® viscous extender for the long-term storage of swine semen improves sperm quality in terms of a lower percentage of spermatozoa with unstable plasma membranes, externalization of PS and damaged acrosomes. These results introduce a new possibility in the design of new boar extenders for different storage conditions.

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