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Effects of Storage in Different Commercial Semen Extenders on Sperm Motility, Viability and Membrane Integrity of Korean Native Boar Spermatozoa

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ABSTRACT

The objective of this study was to compare the effect of semen extenders on the sperm motility, viability, acrosome integrity and functional integrity of plasma membrane (HOST: hypo-osmotic swelling test) during liquid preservation of Korean Native boar semen. In this experiment, semen was diluted in Androhep plus, Beltsville Thawing Solution (BTS), Modena^{TM}, Seminark and Vitasem LD. Sperm-rich fractions were collected from three Korean Native boars and sub-samples were diluted (30×10^6 spermatozoa/ml) in different semen extenders. Semen samples were stored at 17℃ for 96 hours. On everyday (0, 24, 48, 72, 96 h) after storage, the sperm characteristics relevant for fertility, such as sperm motility, viability, acrosome integrity and HOST positive were evaluated. The motility of spermatozoa stored in different extenders was no significantly different among other extenders ($P>0.05$). Also, no difference was observed among samples processed with different extenders in the percentage of sperm viability, acrosome integrity and HOST positive. All extenders maintained a high percentage (70%) of sperm motility, viability and acrosome integrity through 96 h of storage. The result of this study show that there was no significant differences among extenders in their capacity to preserve motility, viability and membrane integrity of spermatozoa from normal, fertile Korean Native boars for 96 h of liquid preservation at 17℃.

(Key words: semen extender, motility, viability, acrosome integrity, Korean native boar)

INTRODUCTION

Artificial insemination of swine is widely performed around the world today. In Korea, the swine industry use artificial insemination over 90%. When compared with natural mating, artificial insemination is a very useful tool to introduce superior genes into sow herds, with a minimal risk of disease (Maes *et al*., 2008). In practice, artificial insemination with fresh liquid semen is performed immediately after dilution or keeping for a day at 15～20℃, using a dose of 3 billion spermatozoa with a volume of 80～100 ml.

Among mammalian species, boar spermatozoa are extremely sensitive towards cold shock because of their peculiar plasma membrane composition (White, 1993). A different composition of the phospholipids in the membrane of boar spermatozoa compared to bull spermatozoa, a low cholesterol/phospholipid ratio and an asymmetrical distribution of cholesterol within the membrane render boar spermatozoa very susceptible to cold temperatures resulting in increased permeability and loss of controlled membrane processes (De Leeuw *et al*., 1990). Consequently, two factors are very important for storage of liquid boar semen: the temperature of collection and storage, and the composition of the storage medium (Johnson *et al*., 2000). The storage media for liquid boar semen aim to prolong sperm survival, to provide energy to the sperm cells, to buffer the pH of the suspension and to avoid the growth of bacteria (Vyt *et al*., 2004). Boar semen extenders can be divided into two major groups: those designed for short-term preservation (less than 1～3 days) and extenders for long-term semen preservation (over 4 days) (Gadea, 2003). The storage tolerance of spermatozoa depends on many factors such as semen extender, storage time, storage temperature, semen quality and dilution concentration (Haugan *et al*., 2007; Martin-Hidalgo *et al*., 2011; Paulenz *et al*., 2000; Waterhouse *et al*., 2004). Martin-Hidalgo *et al*. (2013) reported that both extender and breed influence motility characteristics of liquid-stored boar semen. Also, Sonderman

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and Luebbe (2008) reported that boar breeds differed in the sustainability of motility in extended semen stored for 5 days.

There are many boar semen extenders available in Korea. However, no study has been carried out to compare the efficacy among commercial semen extenders on the sperm characteristics during cold storage of Korean Native boar semen. Increasing concern on effective breeding program of scarce Korean Native pig raises questions in the field regarding the choice of semen extender. Consequently, the objective of this study was to examine the effect of semen extenders on the sperm motility, viability and membrane integrity during liquid preservation of Korean Native boar semen.

MATERIALS AND METHODS

1. Animals

Three mature Korean Native boars ranging from 1 to 2 years age were selected based on normal semen quality (e.g. initial motility of >80% and >85% morphologically normal spermatozoa) and proven fertility after artificial insemination with liquid-preserved semen. Boars were kept under uniform feeding and handling conditions in National Institute of Animal Science, Rural Development Administration, Korea.

2. Reagents and Media

All chemicals used in this study were purchased from Sigma-Aldrich Corporation (St. Louis, Mo, USA) unless otherwise stated. The five commercial semen extenders were Androhep plus (Minitube of America. INC., USA), BTS (Kruuse, Germany), Modena™(Swine Genetics International Ltd., USA), Seminark (Noah Biotech., Korea) and Vitasem LD (Magapor S.L., Spain).

3. Semen Processing and Experimental Design

Sperm-rich fractions were collected from three Korean Native boars using the gloved-hand manual collection method. Semen was filtered to remove the gel fraction of the ejaculates. At collection, sperm-rich fraction from a single boar was split into five aliquots, immediately diluted 1:1 with Androhep plus®, BTS, Modena™, Seminark and Vitasem LD pre-warmed at 37℃. Single AI doses (3×10^9 spermatozoa/100 ml) were then prepared with the same extender formerly used and stored in a semen storage unit at 17℃ for 96 h. Assessment of sperm characteristics was performed at 0, 24, 48, 72 and 96 h after storage.

4. Assessment of Sperm Characteristics

Sperm motility was assessed for %motile characteristics using computer-assisted sperm analysis (CASA; Medical Supply, Korea) on each day post collection. Five μl of semen sample was placed in a pre-warmed (38℃) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel).

The viability was measured by SYBR-14/Propidium iodide stain. Ten μl of diluted spermatozoa were mixed with 5 μl of the working solution of SYBR-14 and 10 μl of propidium iodide. After incubation at 37℃ for 15 min, a total of 200 spermatozoa were assessed under fluorescence microscope at magnification of × 400. The nuclei of spermatozoa with intact plasma membrane stained green with SYBR-14, while those with damaged membranes stained red with propidium iodide.

Acrosome integrity was evaluated using fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining. Ten μl of Ethidiumhomodimer-1 and incubated at 37℃ for 15 min. Five μl of the mixture was smeared on a slide glass and fixed with 95% ethanol for 30 sec. Fifty μl Fit C-PNA (dilute Fit C-PNA with PBS 1:10 v/v) was spread over the slide and incubated in a moist chamber at 4℃ for 30 min. After incubation, it was rinsed with cold PBS and air dried. The percentage of acrosome-intact spermatozoa was assessed under fluorescent microscope at magnification of × 1,000. At least 200 spermatozoa were counted for each sample.

The functional integrity of the sperm plasma membrane was assessed using a short hypo-osmotic swelling test (HOST) (Chanapiwat *et al*., 2009). Spermatozoa were incubated at 38℃ for 30 min, with 75 mOsm/kg a hypo-osmotic solution that consist of 0.368% (w/v) Na-citrate and 0.675% (w/v) fructose in distilled water. After this incubation, 200 μl of the semen hypo-osmotic solution was fixed in 1,000 μl of a hypo-osmotic solution plus 5% formaldehyde. At least 200 spermatozoa were counted under a phase contrast microscope at magnification of × 400. The coiled tail (HOST positive) spermatozoa found following incubation were functional intact plasma membrane.

5. Statistics

Data were analyzed by ANOVA using the General Linear Models procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). When F-test results were significant in ANOVA, individual data were further tested by Duncan's mul-

tiple-range test. Differences with values of $P<0.05$ were considered to be statistically significant. Data are presented as mean ± standard error of the mean (SEM).

RESULTS

This study was shown the effects of semen extenders and storage duration on sperm motility, viability, acrosome integrity and HOST during liquid preservation (for 96 h at 17℃) of Korean Native boar semen. All extenders maintained a high level (80%) of sperm motility through 96 h of storage. There was no significant difference ($P>0.05$) in sperm motility among extenders (Table 1). The viability of spermatozoa was gradually decreased by increasing the duration time of storage. However, there was no significant difference in sperm viability among extenders. The percentage of alive sperm was above 70% in all extenders through 96 h of semen storage (Table 2). Also, all extenders can maintain acrosome integrity of more than 70% up to 96 h after collection (Table 3). In case of acrosome integrity and HOST, there was no significant different with regardless to extenders type or length of storage. The percentage of HOST positive varied between 18 and 25% (Table 4).

DISCUSSION

In this study, we compared five different commercial semen extenders with regard to motility, viability, acrosome integrity and HOST of Korean Native boar spermatozoa during semen storage for 96 h at 17℃. Generally, spermatozoa are changes in motility, viability and alterations membrane integrity during long term storage in low temperature (17℃). These changes can be explained by the fact that sperm have a high content of unsaturated fatty acids in their membranes (Johnson *et al*., 1969; Park and Lynch, 1992) and they lack a significant cytoplasmic component containing antioxidants (de Lamirande and Gagnon, 1995).

For evaluating the boar semen quality, sperm motility is an important parameter because the movement of spermatozoa

Table 1. Effect of different commercial semen extenders on sperm motility (%) of Korean Native boar semen stored for up to 96 h at 17℃

Storage time (h)	Extender type				
	Androhep plus	BTS	Modena™	Seminark	Vitasem LD
0	80.1 ± 6.0	89.7 ± 5.6	88.3 ± 5.0	89.1 ± 5.1	85.3 ± 6.8
24	85.2 ± 6.9	92.7 ± 4.9	88.9 ± 6.5	91.6 ± 5.7	84.1 ± 4.9
48	83.0 ± 4.8	86.3 ± 6.5	90.3 ± 8.4	86.4 ± 5.8	85.0 ± 4.3
72	84.8 ± 5.8	92.0 ± 4.4	89.6 ± 5.5	86.0 ± 5.4	88.1 ± 4.7
96	80.5 ± 5.0	85.1 ± 7.5	87.7 ± 10.1	81.1 ± 3.6	83.6 ± 7.9

Value are expressed as mean ± SEM.

Table 2. Effect of different commercial semen extenders on sperm viability (%) of Korean Native boar semen stored for up to 96 h at 17℃

Storage time (h)	Extender type				
	Androhep plus	BTS	Modena™	Seminark	Vitasem LD
0	80.0 ± 4.7	79.2 ± 4.6	78.3 ± 4.1	79.6 ± 4.3	79.9 ± 4.8
24	81.1 ± 6.3	83.9 ± 5.2	78.9 ± 7.3	79.6 ± 5.8	82.3 ± 3.6
48	75.4 ± 12.4	72.0 ± 5.5	74.8 ± 7.1	79.5 ± 6.8	78.3 ± 5.2
72	73.9 ± 7.9	76.3 ± 6.6	71.7 ± 5.9	74.8 ± 5.6	75.8 ± 4.1
96	72.9 ± 5.0	73.5 ± 7.4	72.8 ± 5.7	75.8 ± 4.1	74.2 ± 3.5

Value are expressed as mean ± SEM.

Table 3. Effect of different commercial semen extenders on acrosome integrity (%) of spermatozoa of Korean Native boar semen stored for up to 96 h at 17℃

Storage time (h)	Extender type				
	Androhep plus	BTS	Modena™	Seminark	Vitasem LD
0	79.1 ± 4.1	79.5 ± 3.0	76.5 ± 5.0	77.8 ± 5.5	79.7 ± 5.9
24	78.7 ± 5.9	79.7 ± 8.3	78.0 ± 3.8	78.8 ± 6.5	74.1 ± 3.9
48	80.2 ± 6.4	76.3 ± 9.2	70.5 ± 6.0	74.2 ± 7.9	76.1 ± 5.6
72	79.7 ± 7.7	78.7 ± 6.4	74.8 ± 5.8	74.5 ± 5.7	78.1 ± 4.7
96	75.7 ± 6.8	73.1 ± 7.5	76.2 ± 8.0	73.9 ± 6.9	73.1 ± 7.5

Value are expressed as mean ± SEM.

Table 4. Effect of different commercial semen extenders on HOST (%) of spermatozoa of Korean Native boar semen stored for up to 96 h at 17℃

Storage time (h)	Extender type				
	Androhep plus	BTS	Modena™	Seminark	Vitasem LD
0	23.1 ± 9.5	19.9 ± 6.4	22.4 ± 8.9	20.4 ± 5.1	19.2 ± 10.8
24	21.8 ± 4.6	23.2 ± 6.9	21.3 ± 7.9	22.1 ± 8.7	20.4 ± 6.7
48	25.4 ± 7.8	24.5 ± 9.5	24.7 ± 8.6	18.2 ± 5.8	19.6 ± 7.3
72	24.9 ± 4.3	22.1 ± 6.7	18.6 ± 6.5	19.8 ± 5.4	22.5 ± 9.3
96	21.3 ± 5.8	19.4 ± 5.3	21.1 ± 5.4	23.8 ± 6.7	22.8 ± 8.9

Value are expressed as mean ± SEM.

indicates active metabolism, membrane integrity and fertilizing capacity (Vyt *et al*., 2004; Estienne *et al*., 2007). The minimum percentage of motile spermatozoa for artificial insemination dose has been established to be at least of 60% because, under this threshold, lower farrowing rates can be experienced (Flowers, 1997). The results of several showed that semen extender influences sperm quality parameters during storage (De Ambrogi *et al*., 2006; Estienne *et al*., 2007; Waterhouse *et al*., 2004). In this study, there was no significant difference in sperm motility among five different commercial semen extenders (Androhep plus, BTS, Modena™, Seminark and Vitasem LD). Also, the percentage of motile sperm was above 80% in all extenders for 96 h. Interestingly, even samples extended with short-term extender such as BTS was not different in sperm motility from other extenders. Consistent with our results, Laforest and Allard (1995) reported that the percentage of motile spermatozoa in semen extended in short-term and long-term extenders was not different significantly by extender or day of storage. Waberski *et al.* (2011) reported that ther were no significant changes in membrane integrity as assessed by flow cytometry between 1 and 3 days of storage.

On the other hand, the viability of spermatozoa was gradually decreased by increasing the duration time of storage. However, there was no significant difference in sperm viability among extenders. The percentage of alive and acrosome-intact sperm was above 70% in all five semen extenders through 96 h of storage. Also, there were not marked any differences in acrosome integrity and HOST among the extenders, regardless of the storage time. Vyt *et al*. (2004) reported that there were no major differences in the percentage of dead cells in semen extended with BTS or long-term extenders. Huo *et al*. (2002) reported that there were no major differences in spermatozoa preserved in BTS or Androhep plus, when boar semen was extended and stored for 120 h post-collection. In the present study, even samples extended with short-term extender such as BTS did not show any change in sperm motility and membrane integrity after 96 h storage.

In conclusion, the present study showed that there was no

significant difference among extenders in their capacity to preserve motility, viability, acrosome integrity and HOST of spermatozoa from Korean Native boars for 96 h of *in vitro* storage at 17℃. Therefore, it seems that even using a short-term extender can provide good preservation of semen characteristics such as motility and membrane integrity, at least for 96 h of storage. The result of this study shows that even inexpensive extenders were reasonably effective in preserving spermatozoa quality of Korean Native boar, when diluted semen is to be used within 3 to 4 days after collection.

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