Effects of Extender and Storage Time on Motility and Ultrastructure of Cooled-Preserved Boar Spermatozoa

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ABSTRACT

Artificial insemination (AI) using fresh diluted semen is used worldwide in the porcine industry. Viability of spermatozoa in diluted semen depends on several factors, such as the interaction with the type of extender and storage duration. The aim of this study was to evaluate the effects of extenders and storage time on the motility and ultrastructure of cooled-preserved boar spermatozoa. Semen samples were collected, diluted in BTS, Merck III or Androhep and stored at 15°C for 0, 1, 3, 5 and 7 d. The samples were warmed (37°C) and spermatozoa were evaluated for motility using light microscopy and ultrastructure using scanning (SEM) and transmission (TEM) electron microscopy. The results showed that motility did not differ significantly ($P > 0.05$) among extenders at days 0 to 3, while Androhep showed a significantly ($P < 0.05$) higher percentage of motility than BTS and Merck III from days 5 to 7. At day 7 of semen storage, plasma and acrosomal membrane damage were observed, as revealed by SEM and TEM. These findings suggest that the type of semen extender and cooled-preserved time affected motility and caused structural damage to boar spermatozoa, especially in the plasma and acrosomal membrane.

Keywords: boar spermatozoa, extender, SEM, TEM, ultrastructure

INTRODUCTION

It is well known that the survival of sperm cells is much greater following liquid rather than frozen storage of semen. As the temperature declines, there is an inevitable reduction in the proportion of spermatozoa that maintain normal membrane integrity, ultrastructure and biochemical components. Utilization of preserved semen for AI in pigs has increased approximately threefold in the past 15 y. More than 99% of the estimated 19 million inseminations per year conducted worldwide are made with semen that has been extended in the liquid state and used on the same day, or stored at 15 to 20°C for 1 to 5 d (Johnson *et al.*, 2000). Virtually all AI with liquid-stored semen is used for market hog production. Frozen boar semen has been available commercially since 1975, both in pellet form and in straws. However, less than 1% of all inseminations are made using frozen–thawed semen. The requirements of a liquid storage medium have been reviewed by Watson (1990). The important factors are the pH, ionic strength, type of ions and osmotic pressure of the medium. Anti-microbial substances are also commonly

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included in diluents. In freshly ejaculated boar semen, the pH varies between 7.2 and 7.5. Below this pH, the motility and metabolism of spermatozoa are reduced. The ionic strength of the diluent does not seem to be of primary importance in diluents for boar semen, where the osmolality is maintained by non-ionic components, such as glucose. This may explain the importance of surface-bound proteins, which are more readily solubilized in high ionic strength media (Watson, 1995). The ions in media for fresh semen are introduced as sodium bicarbonate and/or sodium citrate and, in certain diluents, potassium chloride. The former compounds are used as buffers, the latter in low concentration (minimum 4 mM K^+) to maintain the Na^+–K^+ pump of the cells in preventing intracellular K^+-exhaustion and a loss of motility (Alvarez and Storey, 1982). On the other hand, bicarbonate is known to cause changes in membrane lipid architecture within a few minutes of exposure, which initiates final membrane destabilization as an important step towards capacitation (Harrison, 1996). A number of extenders have been developed which decrease the metabolic activity of spermatozoa. Different modifications of glucose–sodium citrate media were mainly used. Ethylenediamine-tetra-acetic acid (EDTA), as a chelating substance, captures divalent metal ions, especially Ca^{2+} and is believed to limit their movement across the plasma membrane (Watson, 1990), preventing the initiation of capacitation and the acrosome reaction. At the present time, one of the most widely used extenders is Beltsville Thawing Solution (BTS). BTS is defined as a short-term semen-extender (stored for up to 3 d). Merck III and Androhep extenders are used for up to 4 and 5 d of semen storage in routine AI and have therefore been called medium and long-term extenders, respectively.

From a practical standpoint, a decrease in fertilizing ability during storage cannot be prevented, even when so-called "long-term"

media are used. The reduction of motility, which occurs during storage has long been the main parameter used to judge the decrease in fertilizing ability. The aging-related functional changes within different compartments of the spermatozoa, such as the mitochondria, flagellum, plasma membrane and acrosome, are poorly understood. Therefore, a link between sperm motility and morphological changes will provide a clearer picture of the semen-storage effect. The integrity of the sperm membranes, the acrosome in particular, is essential to achieve successful fertilization. SEM enables the detailed examination of the cell surface (Grondahl *et al.*, 1994) and TEM is a good complement to verify the type and extent of membrane damage.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless stated otherwise.

Semen collection and evaluation

Five boars housed at the Lopburi College of Agriculture and Technology, Lopburi province, Thailand were used in this study. Semen samples were collected by glove-hand technique and filtered through double layers of sterile cotton gauze to remove the gel particles and then transferred into an insulated vacuum bottle. The semen samples of the sperm-rich fractions were immediately assessed for volume, evaluated for sperm concentration using a haemocytometer and assessed visually for percentage of progressive motility using a phase contrast microscope. Ejaculates containing spermatozoa with greater than 80% progressive motility were included in this study.

Semen dilution and preservation at 15°C

This study was performed as a blinded

split sample trial. Each ejaculate was split into three aliquots and initial dilutions were made within 15 min after semen collection using the semen-extenders, BTS, Androhep (Johnson *et al.*, 2000) and Merck III (Maria *et al.*, 2006). BTS contains glucose (37.0 g/L), sodium citrate (6.0 g/L), sodium bicarbonate (1.25 g/L), EDTA (1.25 g/L), potassium chloride (0.75 g/L) and gentamycin (300 mg/mL). Androhep contains glucose (26.0 g/L), sodium citrate (8.0 g/L), sodium bicarbonate (1.2 g/L), EDTA (2.4 g/L), HEPES (9.0 g/L), bovine serum albumin (BSA, 2.5 g/L) and gentamycin (300 μg/mL). Merck III contains glucose (89.2%), sodium citrate (3.1%), sodium bicarbonate (4.2%), EDTA (3.1%) and gentamycin (300 μg/mL). After determination of the sperm concentration using a haemocytometer, the three sperm aliquots, which each had a concentration greater than 50×10^6 cell/mL, were diluted further in the same semen extender as earlier within 1 h, to a final concentration of approximately 30×10^6 cells/mL. Semen diluted in each of the three semen-extenders was split into five aliquots and stored in closed polypropylene plastic tubes at 15°C. Sperm motility and ultrastructural investigation were evaluated at days 0 (6 h after dilution) 1, 3, 5 and 7 of storage (15°C). Sample amounts of 5 μL of diluted semen were placed on warm glass slides (37°C) and progressive motility was assessed visually at room temperature under a phase-contrast microscope.

Sperm preparation for scanning electron microscopy (SEM)

Fresh and cooled-preserved semen samples were deposited onto a coverglass. Samples were fixed in 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer, washed three times in 0.1 M cacodylate buffer, then postfixed in 1% osmium tetroxide (EMS, Hatfield, PA, USA) for 1 h. After washing three times in 0.1 M cacodylate buffer, samples were dehydrated through a graded ethanol series and critical point dried. The coverglasses were mounted on stubs and coated with gold prior to examination using an SEM (JEOL JSM-35CF; JEOL Ltd., Tokyo, Japan).

Sperm preparation for transmission electron microscopy (TEM)

Fresh and cooled-preserved semen samples were fixed in 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer overnight at 4°C and washed three times in 0.1 M cacodylate buffer before being postfixed in 1% osmium tetroxide (EMS) for 1 h at 4°C and washed three times in 0.1 M cacodylate buffer for 30 min. Dehydration was carried out through a graded series of ethanol. Sperm cells were individually embedded in araldite resin 502 (EMS) and blocks were allowed to polymerize for 2 d at 45°C and then 2 d at 60°C. Ultrathin sections of 75 nm were prepared and collected onto copper grids. Sections were stained with uranyl acetate (EMS) and lead citrate (EMS) and examined using an H-7500 Hitachi TEM. Ultrastructural changes were examined, interpreted and compared to the fresh control.

Statistical analysis

Each experiment was replicated five times. Differences among means of percentage of sperm motility were compared by one-way analysis of variance (ANOVA; SPSS 11.5 software package 020245, Thailand), followed by Tukey's test. Differences among proportions of ultrastructural alteration data in experimental groups were analyzed using the Chi-square test. Values with $P < 0.05$ were considered to be significant.

RESULTS

The mean volume and concentration of fresh semen was 214.4±33.3 mL and 117±0.08×10^6 cells/mL, respectively. The

percentage of sperm motility after storage for each of the three different semen extenders (BTS, Merck III and Androhep) is shown in Table 1. The percentage of sperm motility did not differ significantly among extenders at days 0 to 3, although there was a significant reduction in the percentage of sperm motility from days 5 to 7 in BTS and Merck III. At days 5 and 7, there were differences in the percentage of sperm motility between the semen extenders. Androhep showed a significantly higher motility percentage than BTS and Merck III.

Morphological assessment by SEM

Normal spermatozoa in samples at day 7 from the control (fresh) and from cooled-preserved storage were examined under SEM and showed an intact acrosomal membrane with a smooth covering and a prominent apical ridge (Figures 1A & 1B). Spermatozoa with plasma membrane and acrosomal changes were observed, including plasma membrane disruption, but intact acrosomes (Figure 1C) and disruption of both plasma membrane and acrosome (Figure 1D). The amounts of membrane-damaged spermatozoa stored in BTS, Merck III and Androhep were 30% (9/30), 30% (9/30) and 16.7% (5/30), respectively.

Morphological assessment by TEM

The effect of cooled-preservation on membrane and acrosomal alterations as revealed by TEM is shown in Table 2. The percentage of normal spermatozoa with intact plasma and acrosomal membrane in cooled-preserved semen in all extenders was significantly lower than in fresh semen. There were no significant differences among groups in the percentage of spermatozoa with plasma membrane damage but with the acrosome intact. However, the percentage of spermatozoa with plasma and acrosomal membrane damage was significantly higher ($P<0.05$) in BTS and Merck III extender than in Androhep and the fresh control. Figure 2 shows a TEM micrograph of spermatozoa in the fresh semen control, with the normal spermatozoan head and intact plasma and the acrosomal membrane. Ultrastructure alterations of the cooled-preserved spermatozoa after storage for 7 d were observed, including extension of the outer plasma membrane (Figure 3A), fusion of the inner plasma membrane and acrosomal membrane (Figure 3B), rupture of the plasma membrane and acrosomal membrane and loss of the acrosomal contents (Figures 3C and 3D).

DISCUSSION

In this study, the effects of storage time on cooled-preserved boar semen in three different extenders (BTS, Merck III and Androhep) were determined. The results indicated that Androhep maintained sperm motility better than BTS and Merck III from days 5 to 7. A low concentration of potassium in BTS is believed to play a role in maintaining the intracellular concentration of this ion at physiological levels during storage (Johnson

Table 1 Percentage of sperm motility after storage in BTS, Merck III and Androhep for a time period up to 7 d.

Extenders	Percentage of sperm motility (Mean ±SD)				
	Day 0	Day 1	Day 3	Day 5	Day 7
BTS	93±4.5 [aA]	73±2.7 [aA]	60.9±6.5 [aA]	46.5±12.8 [aB]	25.8±7.2 [aC]
Merck III	93±4.5 [aA]	81±4.2 [aA]	70±7.3 [aA]	44.5±7.6 [aB]	23.3±9.0 [aC]
Androhep	93±4.5 [aA]	79±4.2 [aA]	72.3±5.8 [aA]	67.3±6.5 [bA]	43.3±9.6 [bB]

[aA] = Means followed by different superscripts (capitals for rows and lowercase for columns) within each time period are significantly different (ANOVA, *P <0.05*).
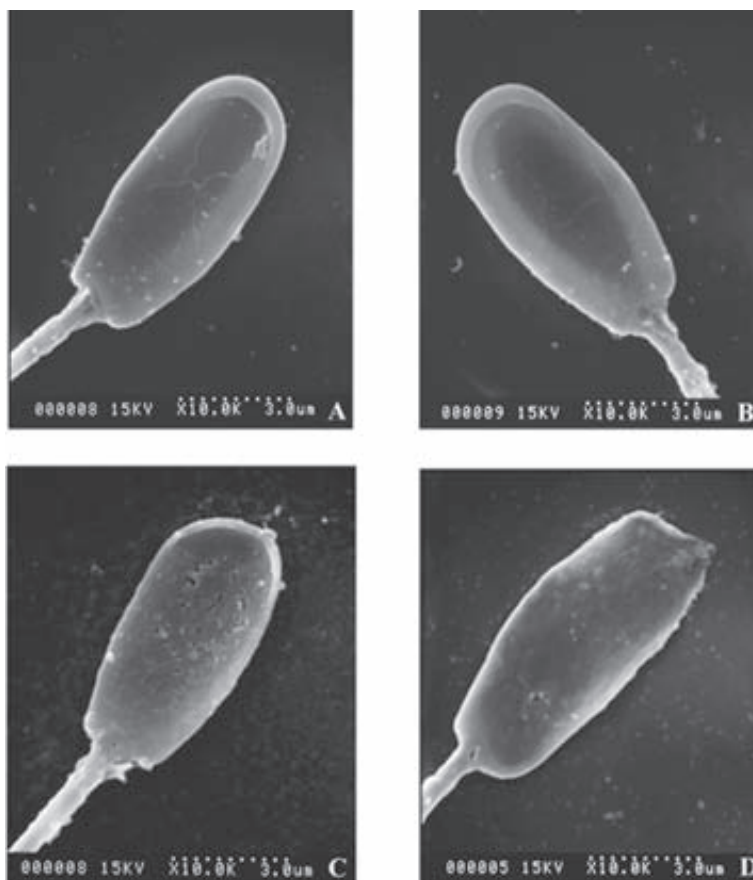
Figure 1 SEM photographs of normal spermatozoa in fresh (A) and cooled-preserved semen (B) at day 7 showed an intact acrosomal membrane with a smooth covering and a prominent apical ridge. SEM photographs of cooled-preserved spermatozoa after storage for 7 d showed plasma membrane disruption, but the acrosome intact (C) and disruption of plasma membrane and acrosome (D) (x10,000).

Table 2 Ultrastructural alterations of plasma membrane and acrosome.

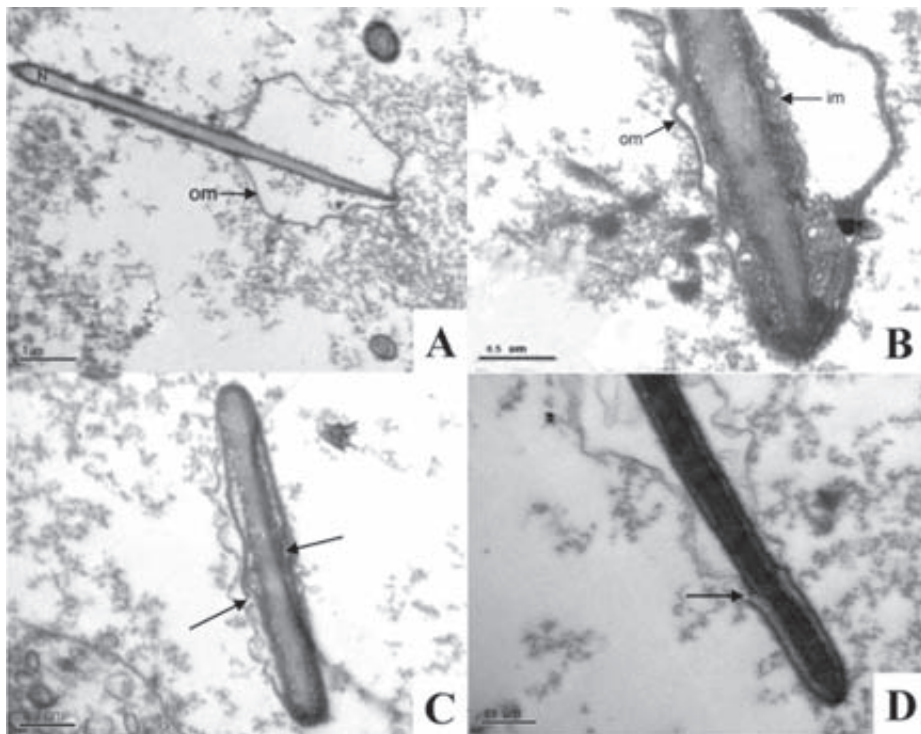|	Fresh spermatozoa (%)	Cooled-preserved spermatozoa at day-7 storage		
		BTS (%)	Merck III (%)	Androhep (%)
Intact plasma and acrosomal membrane	64/72 (88.9)[a]	63/104 (60.1)[b]	60/102 (58.8)[b]	86/112 (76.8)[c]
Plasma membrane damage but intact acrosomal membrane	3/72 (4.2)[a]	12/104 (11.5)[a]	14/102 (13.7)[a]	9/112 (8.0)[a]
Plasma and acrosomal membrane damage	5/72 (6.9)[a]	25/104 (24.0)[b]	26/102 (25.5)[b]	16/112 (15.4)[ab]

The percentage of spermatozoa with intact or damaged plasma and acrosomal membrane in fresh semen and cooled-preserved semen in each extender were observed by TEM.

Values with different superscripts (a, b, c) within each row differ significantly (Chi-square test, *P<0.05*).
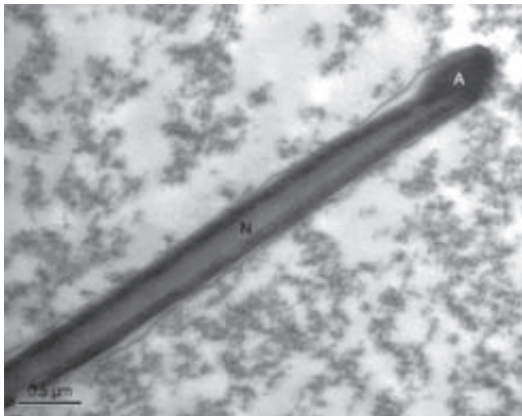
Figure 2 TEM micrograph of the normal cephalic part of fresh spermatozoa. *A*= acrosome, *N*= nucleus (x30,000).

et al., 2000). Androhep diluent contains BSA and HEPES, which reduce storage-dependent aging processes. The addition of BSA to the medium was proposed by Harrison *et al.* (1982) to stimulate motility in a reversible manner. Waberski *et al.* (1989; 1994a; 1994b) found that BSA stimulated the motility of spermatozoa and improved fertility during a six-day storage test. Buhr (1990) used BSA in an attempt to overcome the fluidity of the membrane and therefore the deleterious effect of lysophospholipids and free fatty acids, produced by cooling. The study of Alvarez and Storey in 1995 confirmed that BSA is a very potent inhibitor of lipid peroxidation in sperm and this type of damage reduces sperm motility, as shown in the

Figure 3 TEM micrographs of cooled-preserved spermatozoa after storage for 7 days. (A) Spermatozoa with extension of outer plasma membrane (x15,000). (B) Spermatozoa with fusion of inner plasma membrane and acrosomal membrane (x40,000). (C) Spermatozoa with rupture of plasma membrane and acrosomal membrane (arrow) (x30,000). (D) Spermatozoa with rupture of acrosomal membrane (arrow) (x40,000). *N* = nucleus, *om* = outer membrane, *im* = inner membrane.

sea urchin. HEPES is known as capture heavy metals and intracellular pH (Crabo *et al.*, 1972). The present work supported other reports that Androhep would be better for preserving sperm and the fertilizing capacity of long-term storage semen (Waberski *et al.*, 1994a, b; Dube *et al.*, 2004)

According to the results observed under SEM and TEM after storage for 7 d, it was demonstrated that plasma membrane and acrosome modification were induced by storage at 15°C in all extenders. The decreased motility of preserved spermatozoa may result from the ultrastructural changes observed with SEM and TEM. The plasma membrane modifications may be induced by low temperature (15-20°C) (Holt *et al.*, 1999). The plasma membrane of the spermatozoa is responsible for maintaining cellular integrity, is an integral part of the acrosome reaction and is involved in fertilization, so that its effective functional preservation is of high importance during and following preservation.

CONCLUSIONS

Although sperm motility decreased at all intervals in all extenders, Androhep maintained sperm motility better than BTS and Merck III from day 5 to 7. At day-7 storage, the ultrastructural observations correlated well with motility data. The lower level of damage of the plasma and acrosomal membrane of spermatozoa preserved in Androhep confirmed a higher efficiency in maintaining an intact plasma membrane.

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