# Effect of Extenders and Temperatures on Sperm Viability and Fertilizing Capacity of Harbin White Boar Semen during Long-term Liquid Storage*

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**ABSTRACT :** In this study the effect of extenders and temperatures on sperm viability and fertilizing capacity of boar sperm during long-term storage was investigated. Acrosomal integrity, membrane integrity, motility and hypo-osmotic resistance were evaluated by fluorescence and light microscopy. An *in vitro* fertilization test was performed to assess the fertilizing capacity of stored spermatozoa. The five diluents tested were ranked according to their ability to maintain sperm functional parameters and Zorlesco (ZO) extender with BSA or with PVA instead of BSA produced the best results. Zorlesco extender substituted with PVA (ZO+PVA) was found to maintain motility both at 15 and 20°C within 5 days of storage, but the quality of semen stored at 15°C decreased thereafter as compared to semen stored at 20°C. Semen stored at 5°C demonstrated rapid loss of motility already within 24 h. Both fertilization and cleavage of semen stored at 20°C in ZO substituted with PVA instead of BSA did not change significantly until day 8 of storage. It is therefore concluded that PVA can be used to substitute for BSA and 20°C was more suitable than 15°C for boar semen storage, and *in vitro* fertilizing capacity of spermatozoa was maintained for at least 8 days in ZO+PVA at 20°C. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 11 : 1501-1508)***

**Key Words :** Boar, Semen Storage, Spermatozoon, PVA, IVF

## INTRODUCTION

One of the obstacles to the use of artificial insemination (AI) in swine is the short storage life of boar spermatozoa (Johnson et al., 2000). Although efforts have been made to improve viability of frozen-thawed sperm (Cheon et al., 2002; Yi et al., 2004a; Yi et al., 2004b), it is found that survival of cells is much greater after liquid than frozen storage of semen. Utilization of preserved semen for AI in pigs has increased approximately threefold in the past 15 years, and more than 99% of the estimated 19 million inseminations conducted worldwide are made with semen that has been extended in the liquid state and used on the same day, or stored at 15-20°C for 1 to 5 days (Johnson et al., 2000). However, as a general rule the farrowing rates were 65% to 70% when the semen was used in the first 2 days after collection, but they reduced to about 50% with 5-day-old semen (Johnson et al., 1988; Johnson, 1998). It is therefore necessary to further study methods for longer storage of boar semen in liquid state. In addition, semen quality and tolerance to long-term storage in the Harbin White boar has yet to be investigated.

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The Kiev (Plisko, 1965; Chyr et al., 1980), BTS (Beltsville thaw solution, Pursel and Johnson, 1975; Johnson et al., 1988) and Zorlesco (Gottardi et al., 1980; Cheng, 1988) are among the often-used extenders for liquid storage of boar semen, but their performance during long-term storage should be compared by *in vitro* assays. A previous study (Pursel et al., 1973) has shown that the fertility potential of extended boar semen stored at 15°C yields acceptable fertilization rates, while exposure to temperatures below 15°C would result in cold-shock and cell death. Although bovine serum albumin (BSA) was found to improve fertility when the semen was stored between 3 and 5 days (Waberski et al., 1994a), its high price will likely prohibit the commercial use of some extenders.

To test the effectiveness of various methods for semen storage, *in vitro* assays were often used. Among these assays, those focusing on sperm viability, such as motility, membrane integrity and acrosome status, are proven to be most reliable (Amann, 1989). Since it has been shown that the success of fertilization was influenced by both *in vitro* and *in vivo* aging of sperm (Waberski et al., 1994b; Soede et al., 1995) and that seminal plasma had beneficial effect on fertilization rate and accessory sperm number which was attributed to enhanced cell transport to the site of fertilization (Johnson et al., 2000), *in vitro* fertilization would be the way of choice to exclude the effect of seminal plasma and sperm aging *in vivo* on fertility of stored semen.

We wish to study, by *in vitro* assays and *in vitro* fertilization, the effect of extenders and temperatures on sperm viability and fertilizing capacity of Harbin White boar semen during long-term liquid storage.

**Table 1.** The ingredients, pH and osmotic pressure of the extenders

| Ingredients (g/100 ml) | Extenders | | | | |
|---|---|---|---|---|---|
| | Kiev | BTS | ZO | ZO-BSA | ZO+PVA |
| Glucose | 6.000 | 3.700 | 1.150 | 1.150 | 1.150 |
| Sodium bicarbonate | 0.120 | 0.130 | 0.175 | 0.175 | 0.175 |
| Tri-sodium citrate | 0.300 | 0.60 | 1.165 | 1.165 | 1.165 |
| EDTA | 0.370 | 0.130 | 0.235 | 0.235 | 0.235 |
| Tris | - | - | 0.550 | 0.550 | 0.550 |
| Citric acid | - | - | 0.410 | 0.410 | 0.410 |
| Cysteine | - | - | 0.007 | 0.007 | 0.007 |
| Potassium chloride | - | 0.400 | - | - | - |
| BSA | - | - | 0.500 | - | - |
| PVA | - | - | - | - | 0.100 |
| Streptomycin | 0.100 | 0.100 | 0.100 | 0.100 | 0.100 |
| Penicillin (IU/ml) | 1,000.00 | 1,000.00 | 1,000.00 | 1,000.00 | 1,000.00 |
| pH | 6.8 | 6.9 | 6.4 | 6.6 | 6.6 |
| Osmotic pressure (mOsm/l) | 420 | 310 | 287 | 281 | 284 |

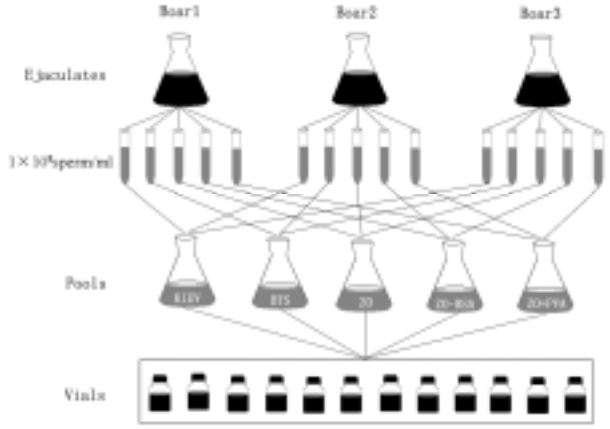


**Figure 1.** Procedure for semen dilution. Each ejaculate was split into five parts and diluted in extender Kiev, BTS, ZO, ZO-BSA and ZO+PVA to storage concentration of $1\times10^8$ sperm/ml. Equal volumes of the diluted semen were then pooled within extenders (pools Kiev, BTS, ZO, ZO-BSA and ZO+PVA). To examine the effect of extenders, each of the 5 pools was split into 12 glass vials (5 ml each) for storage at 20°C.

## MATERIALS AND METHODS

### Semen collection

Sperm-rich fractions of semen were collected by gloved-hand method from 3 boars of Harbin White breed of proven fertility, 1.5-2 years of age, and the boars were allowed at least 3 d of sexual rest between collections. The semen was transported to the laboratory at 37-38°C within 30 min and filtered through 4 layers of sterile gauze into a pre-warmed (35°C) beaker to remove gel particles.

### Extenders

The five extenders used in this study were Kiev, Beltsville thaw solution (BTS), Zorlesco (ZO), Zorlesco without BSA (ZO-BSA) and Zorlesco with PVA substituting for BSA (ZO+PVA). The ingredients, pH and osmotic pressure of the extenders were shown in Table 1. Tris, PVA and BSA were purchased from Sigma, St Louis, MO, but all other chemicals were from Shanghai Chemical Co, Shanghai, China.

### Semen dilution and storage

The procedure for semen dilution, using a split-sample technique (Paulenz et al., 2002), was illustrated in Figure 1. After filtration, an aliquot was taken from each ejaculate to measure the sperm concentration in a hemocytometer before the ejaculate was split into 5 parts and diluted in Kiev, BTS, ZO, ZO-BSA and ZO+PVA, respectively, to give a storage concentration of $1\times10^8$ sperm per ml. Equal volumes of the diluted semen were then pooled within extenders (pools Kiev, BTS, ZO, ZO-BSA and ZO+PVA). To examine the effect of extenders, each of the 5 pools was split into 12 glass vials (5 ml each) for storage at 20°C. To determine the effect of temperatures, 36 vials from the ZO+PVA pools were stored at 5, 15 and 20°C, respectively. Twelve vials from the ZO+PVA pool were stored at 20°C for the *in vitro* fertilization experiment. After a cooling procedure, the extended semen was stored at corresponding temperatures for up to 12 days. The cooling procedure predetermined by a preliminary experiment was as follows: Cooling down to 20°C was achieved by bathing the vials of extended semen in 50 ml pre-warmed (35°C) water for 1 h at 20°C, to 15°C by bathing the vials in 250 ml water for 1.25 h at 4°C and to 5°C by bathing in 250 ml water for 2 h at 4°C.

### Evaluation of semen quality

Every 24 h of storage, one vial was taken from each treatment for sperm quality evaluation. Evaluation at day 0 time point was performed before cooling.

*Subjective motility* : To assess sperm motility, 50 μl semen was taken from each vial, diluted in 1 ml incubation medium (2.18 g glucose, 0.55 g sodium pyruvate, 6.61 g sodium chloride, 0.22 g potassium chloride, 1.1 g calcium chloride, 2.42 g Tris, 0.21 g caffeine, 1.0 g BSA, distilled water to 1,000 ml) and incubated at 37°C for 20 min. Three droplets (20 μl) of diluted semen from each sample were examined using a phase contrast microscope at a 400× magnification and a temperature of 37°C. The average percentages of progressively motile sperm were recorded for further analysis.

*Osmotic resistance test* : An aliquot (50 μl) of semen was added to 1 ml pre-warmed (37°C) hypo-osmotic solution (7.35 g trisodium citrate, 13.51 g fructose, distilled water to 1,000 ml) and mixed thoroughly. After 30 min incubation at 37°C, a drop (20 μl) of the sperm mixture was placed on a hemocytometer and examined under a phase contrast microscope at a 400× magnification. A positive (osmotic resistant) response to the test was evident by coiling of the sperm tail. At least 200 spermatozoa were scored in each sample.

*Acrosome and sperm membrane integrity* : The chlortetracycline (CTC)/Hoechst 33258 staining was adopted for evaluating the integrity of acrosome and sperm membrane and the procedures were as described by Wang et al. (1995) with modifications. Fifty microliters semen was added to 1 ml prewarmed incubation medium and the sperm suspension was incubated at 37°C for 20 min. Then, 4 μl Hoechst 33258 (Sigma, 100 μg/ml in PBS) was mixed with 396 μl sperm suspension. After 3 min incubation at room temperature in the dark, the mixture was layered gently onto 1 ml 3% polyvinyl pyrrolidone-40 (PVP-40, Sigma) in PBS (w/v) and centrifuged at 500×g for 6 min. The pellet was resuspended in 50 μl PBS and 45 μl of the suspension was then mixed with 45 μl CTC solution containing 750 μM CTC (Sigma), 5 mM cysteine (Sigma) and 130 mM NaCl in 20 mM Tris-HCl buffer (pH 7.8). After incubation for 30 s in the dark, the stained spermatozoa were fixed by adding 8 μl of 12.5% paraformaldehyde (w/v) in 0.5 M Tris-HCl (pH 7.4). Ten microliters of the fixed sample were placed on a slide and mounted in the medium containing glycerol and 1,4-diazabicyclo [2,2,2] octane (Sigma) in PBS. The slides were examined with filters 1 (H3) and 2 (D) under a Leica DMLB fluorescent microscope. Spermatozoa were classified as membrane-intact only when the heads were unstained by Hoechst 33258. The membrane-intact spermatozoa were classified by CTC staining into F (uniform fluorescent head), B (fluorescence-free band on the post-acrosomal region) and AR (non-fluorescent head or a thin fluorescent band on the equatorial segment) categories and only the F and B categories were considered acrosome-intact. At least 200 spermatozoa were analyzed for acrosome or membrane integrity in each sample.

### *In vitro* fertilization

*Oocyte maturation in vitro* : Cumulus oocyte complexes (COCs) were obtained from 2-6 mm follicles of slaughterhouse ovaries and washed 3 times in Dulbecco's PBS (Hyclone, Logan, Utah) containing 0.1% PVA followed by two washes in maturation medium. The maturation medium was TCM-199 (Gibco) supplemented with 10% fetal calf serum (FCS, Gibco), 10% porcine follicular fluid (pFF), 10 IU/ml PMSG (Tianjin Huafu Co. Tianjin, China) and 10 IU/ml hCG (Tianjin Huafu Co.). After washing, COCs were placed in 100μl drops of maturation medium (15-20 COCs per drop) and cultured at 38.5C in a humidified incubator with 5% $CO_2$ and 95% air for 42 h.

*Fertilization in vitro* : Fertilization medium was prepared by adding 2 mM caffeine to the basic medium (TCM-199 supplemented with 25 mM Hepes, 0.9 mg/ml calcium lactate, 0.1 mg/sodium pyruvate and 12% FCS) and the medium for washing and pre-incubation was prepared by adjusting pH of the basic medium to 7.8 with 7.5% $NaHCO_3$. Four milliliters of the stored semen were diluted to 8 ml with the washing medium and centrifuged at 300×g for 10 min. The pellet was resuspended to 1 ml ($4\times10^8$sperm/ml) with washing medium and pre-cultured for 3 h at 38.5°C in a humidified incubator with 5% $CO_2$ and 95% air. After pre-culture, aliquots of semen (1.6-3.2 $\times10^8$ motile sperm/ml) were added to 100 μl fertilization drops containing 10-15 *in vitro* matured COCs to give a final concentration of $2\times10^6$ motile sperm/ml. Following 6 h of fertilization, oocytes were denuded of cumulus and excess spermatozoa by pipetting, transferred to modified BMOC-2 medium (Brinster, 1963) and co-cultured with porcine oviductal epithelium. Some of the oocytes were mounted for assessment of fertilization at 4 h of culture and others were examined every 24 h for development.

*Assessment of fertilization* : Oocytes mounted on a slide were fixed for 3-4 days in acetic alcohol (1:3), stained with aceto-orcein (1%) and observed under phase contrast microscopy at a magnification of 400×. Oocytes having enlarged sperm head(s) and/or male pronucleus(ei) with corresponding sperm tail(s) in the cytoplasm were considered fertilized (Nagai et al., 1984).

### Statistical analysis

We conducted three replicate trials for each treatment. Statistical analyses were carried out by ANOVA using Statistics Package for Social Sciences 8.0 (SPSS Inc. Chicago, IL, USA). The difference between treatments was evaluated by one-way variance analysis. Data were expressed as means±SEM and $p<0.05$ was considered significant.

**Table 2.** Sperm motility when boar semen was stored in different extenders at 20ºC for different time

| Days | Extenders | | | | |
|---|---|---|---|---|---|
| | Kiev | BTS | ZO | ZO-BSA | ZO+PVA |
| 0 | 92.4±5.5 $^{aA}$ | 92.5±6.0 $^{aA}$ | 92.6±3.3 $^{aA}$ | 92.4±1.1 $^{aA}$ | 92.6±2.4 $^{aA}$ |
| 1 | 89.3±5.1 $^{aA}$ | 89.3±4.4 $^{aA}$ | 88.5±2.6 $^{aA}$ | 88.8±2.5 $^{aA}$ | 89.5±2.6 $^{aA}$ |
| 2 | 82.6±2.4 $^{aAB}$ | 85.9±5.0 $^{aA}$ | 86.9±1.4 $^{aA}$ | 86.7±1.6 $^{aA}$ | 84.8±8.4 $^{aA}$ |
| 3 | 76.7±5.0 $^{aB}$ | 77.1±7.5 $^{aAB}$ | 82.1±4.5 $^{aA}$ | 81.9±8.9 $^{aAB}$ | 80.1±8.0 $^{aA}$B |
| 4 | 70.1±2.9b$^{BC}$ | 73.4±7.8$^{abB}$ | 78.8±7.7$^{aAB}$ | 74.3±4.4$^{abB}$ | 79.0±1.5$^{aA}$B |
| 5 | 65.4±3.3$^{bC}$ | 68.3±4.2$^{bBC}$ | 77.1±5.1$^{aB}$ | 69.2±5.9$^{bBC}$ | 78.1±7.9$^{aB}$ |
| 6 | 57.1±4.0$^{cC}$ | 64.1±1.2$^{bcC}$ | 76.5±6.5$^{aB}$ | 68.0±7.6$^{bBC}$ | 76.1±5.2$^{aB}$ |
| 7 | 46.8±3.5$^{cCD}$ | 59.5±4.3$^{bC}$ | 75.5±3.2$^{aB}$ | 64.4±2.5$^{bBC}$ | 73.2±4.6$^{aBC}$ |
| 8 | 35.5±6.7$^{cD}$ | 52.0±5.0$^{bCD}$ | 70.2±5.0$^{aBC}$ | 57.9±2.0$^{bC}$ | 65.9±6.6$^{aC}$ |
| 9 | 23.4±2.5$^{cD}$ | 43.7±7.5$^{bDE}$ | 65.4±6.5$^{aC}$ | 49.1±4.9b$^{C}$ | 59.2±5.9$^{aC}$ |
| 10 | 11.7±1.3$^{cE}$ | 34.5±1.3$^{bE}$ | 60.3±9.7$^{aC}$ | 40.2±1.8$^{bCD}$ | 53.7±5.3$^{aC}$ |
| 11 | 5.6±4.6$^{dE}$ | 21.1±1.7$^{cE}$ | 53.6±1.2$^{aCD}$ | 32.4±7.5$^{bD}$ | 49.4±1.4$^{aCD}$ |
| 12 | 0.8±5.3$^{dE}$ | 12.5±5.6$^{cE}$ | 45.5±4.7$^{aD}$ | 26.9±9.5$^{bD}$ | 41.5±2.9$^{aD}$ |

$^{a, b, c, d}$ Values with common lowercase letters in the superscripts in the same row did not differ significantly ($p>0.05$) among different extenders.
$^{A, B, C, D}$ Values with common uppercase letters in the superscripts in the same column did not differ significantly ($p>0.05$) among different time points.

**Table 3.** Percentages of osmotic resistant spermatozoa during storage in different extenders at 20°C

| Days | Extenders | | | | |
|---|---|---|---|---|---|
| | Kiev | BTS | ZO | ZO-BSA | ZO+PVA |
| 0 | 98.1±3.5 $^{aA}$ | 98.0±4.7 $^{aA}$ | 98.0±1.5 $^{aA}$ | 98.0±5.0 $^{aA}$ | 98.0±2.2 $^{aA}$ |
| 1 | 96.7±5.3 $^{a A}$ | 96.7±1.6 $^{a A}$ | 96.6±6.4 $^{a AB}$ | 95.5±0.2 $^{a A}$ | 95.7±4.3 $^{aA}$ |
| 2 | 90.7±4.2$^{aA}$ | 92.6±3.7 $^{aA}$ | 95.7±8.6 $^{a AB}$ | 92.8±4.7$^{aA}$ | 93.5±0.9 $^{aAB}$ |
| 3 | 85.5±4.5 $^{aAB}$ | 89.3±4.5 $^{aAB}$ | 93.8±7.5 $^{aAB}$ | 92.6±5.2 $^{aAB}$ | 89.2±5.5$^{aAB}$ |
| 4 | 80.0±7.5$^{bB}$ | 83.4±6.0$^{abB}$ | 90.2±5.0$^{aAB}$ | 91.1±1.8$^{aAB}$ | 86.5±7.6$^{aB}$ |
| 5 | 74.3±4.0$^{bBC}$ | 77.7±5.5$^{abBC}$ | 89.2±9.1$^{aAB}$ | 89.9±3.8$^{aAB}$ | 85.0±9.4$^{aB}$ |
| 6 | 69.9±5.2$^{bC}$ | 72.3±2.0$^{bC}$ | 87.5±4.4$^{aB}$ | 82.5±4.6$^{aB}$ | 81.5±5.6$^{aBC}$ |
| 7 | 60.5±5.3$^{cC}$ | 66.2±1.5$^{bcCD}$ | 82.9±0.5$^{aBC}$ | 72.6±1.9$^{bC}$ | 79.8±1.9$^{abBC}$ |
| 8 | 52.2±9.0$^{cCD}$ | 60.9±6.9$^{bcD}$ | 79.4±1.7$^{aBC}$ | 65.6±8.5$^{bCD}$ | 75.6±7.2$^{aBC}$ |
| 9 | 46.5±1.5$^{cD}$ | 55.5±4.5$^{bD}$ | 76.3±4.8$^{aC}$ | 56.9±7.0$^{bD}$ | 72.5±5.3$^{aC}$ |
| 10 | 36.3±0.5 $^{cE}$ | 47.2±9.0$^{bD}$ | 71.5±2.0$^{aC}$ | 48.8±7.5$^{bDE}$ | 68.2±1.8$^{aC}$ |
| 11 | 27.1±7.5 $^{cE}$ | 33.5±4.7$^{bcE}$ | 67.0±3.5$^{aCD}$ | 37.6±4.9$^{bE}$ | 64.1±2.6$^{aC}$ |
| 12 | 15.7±0.9$^{cF}$ | 19.2±3.5$^{cF}$ | 59.5±6.1$^{aD}$ | 28.2±2.1$^{bE}$ | 56.5±4.8$^{aC}$ |

$^{a, b, c}$ Values with common lowercase letters in the superscripts in the same row did not differ significantly ($p>0.05$) among different extenders.
$^{A, B, C, D, E}$ Values with common uppercase letters in the superscripts in the same column did not differ significantly ($p>0.05$) among different time points.

## RESULTS

### Sperm viability of semen stored at 20ºC in different extenders for different time

*Subjective motility* : There was no significant difference in sperm motility among the 5 extenders tested during the first 3 days of storage (Table 2). Thereafter, sperm motility declined in all extenders but more rapidly in the Kiev, BTS and ZO-BSA. A motility of more than 40% was maintained for 7 days in Kiev, 9 days in BTS, 10 days in ZO-BSA and 12 days in ZO and ZO+PVA.

*Osmotic resistance* : The difference in the percentage of osmotic resistant spermatozoa was not significant among the 5 extenders during the first 3 days of storage. A rate of more than 50% osmotic resistant spermatozoa was maintained for 8 days in Kiev, 9 days in BTS and ZO-BSA, but 12 days in ZO and ZO+PVA (Table 3).

*Membrane integrity* : The 5 extenders did not differ significantly in the percentage of Hoechst 33258 unstained spermatozoa up to 4 days of storage. A rate of more than 50% unstained spermatozoa was maintained for 9 days in Kiev and BTS, 10 days in ZO-BSA, but 12 days in ZO and ZO+PVA (Table 4).

*Acrosomal intactness* : Up to 5 days of storage, no significant difference in sperm acrosome intactness was found among the five extenders (Table 5). More than 70% spermatozoa were maintained acrosome-intact for 8 days in Kiev, 9 days in ZO-BSA, 10 days in BTS and 12 days in both ZO and ZO+PVA.

### Effects of cooling and storage temperature on sperm motility

The purpose of this experiment was to test the performance of the ZO+PVA extender at different storage temperatures. To determine the effect of the cooling procedure we used, sperm motility was assessed soon after

**Table 4.** Percentages of membrane-intact spermatozoa during storage at 20°C in different extenders as revealed by Hoechst 33258 staining

| Days | Extenders | | | | |
|---|---|---|---|---|---|
| | Kiev | BTS | ZO | ZO-BSA | ZO+PVA |
| 0 | 98.5±2.9 $^{aA}$ | 98.7±6.1 $^{aA}$ | 98.5±3.8 $^{aA}$ | 98.4±7.2 $^{aA}$ | 98.5±1.0 $^{aA}$ |
| 1 | 96.0±4.4 $^{aAB}$ | 96.0±2.8 $^{aA}$ | 96.9±1.1 $^{aAB}$ | 94.7±2.1 $^{aA}$ | 95.6±3.3 $^{aAB}$ |
| 2 | 90.5±5.8 $^{aAB}$ | 91.9±1.9 $^{aAB}$ | 96.0±0.5 $^{aAB}$ | 95.5±4.4 $^{aAB}$ | 95.1±5.3 $^{aAB}$ |
| 3 | 87.2±6.4$^{aB}$ | 89.5±4.6$^{aAB}$ | 94.7±4.0$^{aAB}$ | 92.1±7.5$^{aAB}$ | 88.6±5.0$^{aAB}$ |
| 4 | 83.5±0.2$^{aBC}$ | 85.5±7.3$^{aB}$ | 89.7±5.6$^{aAB}$ | 85.8±1.6$^{aB}$ | 87.9±4.2$^{aB}$ |
| 5 | 76.3±8.0$^{bBC}$ | 79.9±5.4$^{abBC}$ | 88.1±2.5$^{aB}$ | 87.6±3.5$^{aB}$ | 87.0±1.5$^{aB}$ |
| 6 | 70.3±4.3$^{bC}$ | 74.1±5.3$^{bC}$ | 86.5±2.3$^{aB}$ | 82.4±2.4$^{abB}$ | 86.2±4.8$^{aBC}$ |
| 7 | 64.3±2.8$^{bCD}$ | 66.5±5.0$^{bCD}$ | 82.1±1.9$^{aBC}$ | 71.8±1.1$^{bC}$ | 82.1±8.6$^{aBC}$ |
| 8 | 60.2±6.7$^{bD}$ | 61.2±4.9 $^{bD}$ | 81.4±8.9$^{aBC}$ | 67.1±0.8$^{bCD}$ | 80.0±5.9$^{aBC}$ |
| 9 | 54.4±9.2$^{cD}$ | 57.7±3.2 $^{bD}$ | 78.5±9.5$^{aBC}$ | 58.5±2.3$^{bD}$ | 76.8±7.3$^{aC}$ |
| 10 | 40.0±1.7$^{cE}$ | 47.5±2.5$^{bcE}$ | 73.4±5.0$^{aC}$ | 50.7±4.5$^{bDE}$ | 70.2±4.1$^{aC}$ |
| 11 | 32.6±0.5$^{cEF}$ | 36.7±1.4$^{bcF}$ | 69.9±5.8$^{aC}$ | 42.8±2.5$^{bEF}$ | 67.5±9.6$^{aC}$ |
| 12 | 25.9±2.6$^{cF}$ | 29.5±3.0$^{bcF}$ | 58.5±2.0$^{aD}$ | 37.4±7.6$^{bF}$ | 54.7±0.9$^{aD}$ |

$^{a, b, c}$ Values with common lowercase letters in the superscripts in the same row did not differ significantly ($p>0.05$) among different extenders.
$^{A, B, C, D}$ Values with common uppercase letters in the superscripts in the same column did not differ significantly ($p>0.05$) among different time points.

**Table 5.** Percentages of acrosome-intact spermatozoa during storage in different extenders at 20°C

| Days | Extenders | | | | |
|---|---|---|---|---|---|
| | Kiev | BTS | ZO | ZO-BSA | ZO+PVA |
| 0 | 99.1±5.0$^{aA}$ | 99.0±4.5 $^{aA}$ | 99.0±3.8 $^{aA}$ | 99.1±6.8 $^{aA}$ | 99.2±3.5 $^{aA}$ |
| 1 | 98.5±6.3 $^{aA}$ | 98.5±4.2 $^{aAB}$ | 99.1±1.5 $^{aAB}$ | 98.7±2.5 $^{aA}$ | 98.5±2.3 $^{aAB}$ |
| 2 | 96.3±7.2 $^{aA}$ | 95.9±7.6 $^{aAB}$ | 98.2±8.9 $^{aAB}$ | 97.7±7.6 $^{aA}$ | 96.7±4.6 $^{aAB}$ |
| 3 | 92.5±4.2 $^{aAB}$ | 93.5±4.3 $^{aAB}$ | 97.9±4.7 $^{aAB}$ | 96.0±8.6 $^{aA}$ | 95.1±7.9 $^{aAB}$ |
| 4 | 89.5±3.0$^{aAB}$ | 90.9±6.5$^{aAB}$ | 94.4±4.9$^{aAB}$ | 90.3±4.2$^{aAB}$ | 94.5±1.0$^{aAB}$ |
| 5 | 85.2±5.9$^{aB}$ | 89.5±4.9$^{aAB}$ | 93.6±1.3$^{aAB}$ | 85.8±2.0$^{aB}$ | 93.1±4.2$^{aAB}$ |
| 6 | 80.5±7.3$^{bBC}$ | 88.6±8.9$^{abB}$ | 92.2±4.9$^{aA}$ | 81.2±3.9$^{bBC}$ | 92.2±1.3$^{aAB}$ |
| 7 | 77.6±6.4$^{bBC}$ | 85.2±5.6$^{abB}$ | 89.9±8.9$^{aAB}$ | 78.4±4.6$^{bBC}$ | 89.9±7.6$^{aAB}$ |
| 8 | 71.0±5.0$^{bC}$ | 80.4±4.3$^{abB}$ | 88.4±8.5$^{aB}$ | 74.3±7.6$^{bC}$ | 88.4±1.5$^{aB}$ |
| 9 | 65.5±7.7$^{cCD}$ | 75.3±1.9$^{bC}$ | 85.6±7.6$^{aB}$ | 70.5±4.0$^{bcCD}$ | 85.5±2.5$^{aB}$ |
| 10 | 60.8±4.5$^{cD}$ | 70.7±5.7$^{bC}$ | 83.9±4.9$^{aBC}$ | 63.7±8.2$^{bcD}$ | 83.2±0.3$^{aB}$ |
| 11 | 57.2±2.6$^{cD}$ | 65.9±5.3$^{bCD}$ | 78.6±1.3$^{aBC}$ | 60.9±2.5$^{bcD}$ | 78.8±9.6$^{aBC}$ |
| 12 | 52.9±0.2 $^{cD}$ | 60.2±1.2 $^{bD}$ | 74.5±0.9$^{aC}$ | 56.5±0.9$^{bcD}$ | 70.5±5.4$^{aC}$ |

$^{a, b, c, d}$ Values with common lowercase letters in the superscripts in the same row did not differ significantly ($p>0.05$) among different extenders.
$^{A, B, C, D}$ Values with common uppercase letters in the superscripts in the same column did not differ significantly ($p>0.05$) among different time points.

cooling. The sperm motility after cooling down to 20°C and 15°C was 91.3% and 89.6%, respectively, not different significantly from those before cooling. After cooling down to 5°C, however, the sperm motility reduced significantly to 75.6%. When semen was stored at 5°C, sperm motility declined markedly to 35.5% within 24 h and by day 5 of storage it went down to 0.2% (Figure 2). When semen was stored in the same extender at 15°C and 20°C, sperm motility (92.5-69.2% and 92.0-78.1%, respectively) was not different significantly up to 5 days of storage, but during the following days of preservation sperm motility became significantly higher at 20°C (74.1-41.5%) than at 15°C (62.6-31.5%).

### *In vitro* fertilization

When the *in vitro* matured COCs were inseminated with spermatozoa that were stored in ZO+PVA extender at 20°C for different days and examined for fertilization and development, both fertilization and cleavage rates did not change significantly until day 8 of semen storage, and rate of 4-16 cell embryos did not change until day 11. Two and one morulae were obtained with spermatozoa from day 2 and day 7 of storage, respectively (Table 6). This indicated that sperm fertilizing capacity declined slowly during storage but normal fertilization and development up to 4-16 cell stage can be maintained at least for 8 days in our system.

## DISCUSSION

Sperm motility is crucial in facilitating passage through the cervix and uterotubal junction and even more important, it makes possible the actual penetration of the cumulus and

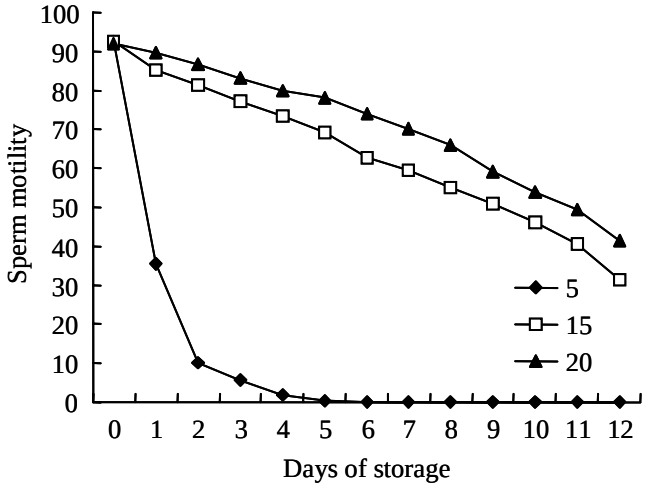


**Figure 2.** Sperm motility during semen storage in the ZO+PVA extender at different temperatures.

zona pellucida of the ovum (Hafez, 1987). Estimation of motility, therefore, has fundamental importance in the daily quality control of the semen (Johnson et al., 2000). However, motility, while an essential feature of healthy spermatozoa, is not necessarily indicative of fertilizing capacity (Hafez, 1987). Polge (1956) noticed that boar spermatozoa that survived freezing could be motile but did not necessarily fertilize. The morphology and functional integrity of the acrosome in ejaculated human spermatozoa are of fundamental importance in attachment, species-specific binding and zona penetration (Schill et al., 1988; Yi et al., 2004b). Integrities of sperm membrane and acrosome are therefore important parameters of sperm fertility. The osmotic resistance test (or hypo-osmotic swelling test, HOST, Jeyendran et al., 1984; Vazquez et al., 1997) and Hoechst 33258 staining (De Leeuw et al., 1991) are among the most sensitive methods for assessment of sperm membrane integrity. The CTC fluorescence assay, first described by Saling and Storey (1979), was later successfully used in assessment of sperm capacitation and acrosome status (Ward and Storey, 1984; DasGupta et al., 1993; Fraser et al., 1995; Wang et al., 1995). In the present study, subjective motility, osmotic tolerance test, Hoechst staining, CTC fluorescence assay and *in vitro* fertilization were adopted for evaluation of sperm viability and fertilizing capacity during long term semen storage.

Sperm motility is retained for only a few hours unless the ejaculate is diluted to reduce the metabolic activity and to low the temperature as well (Johnson et al., 2000). The Kiev (Plisko, 1965; Chyr et al., 1980), BTS (Pursel and Johnson, 1975; Johnson et al., 1988) and Zorlesco (Gottardi et al., 1980; Cheng, 1988) are among the often-used extenders for liquid storage of boar semen, but their performance during long-term storage should be compared by *in vitro* assays. Although BSA was found to improve fertility when the semen was stored (Waberski et al., 1994a), its high price will likely prohibit the commercial use of some extenders. We therefore compared the effect of the Kiev, BTS, ZO (Zorlesco), ZO-BSA (Zorlesco without BSA) and ZO+PVA (Zorlesco with PVA in place of BSA) extenders on sperm motility, membrane integrity, acrosome intactness and *in vitro* fertilization rate during semen storage for up to 12 days. The results showed that ZO and ZO+PVA were superior to other extenders in maintaining the sperm viability parameters and the difference between ZO and ZO+PVA was insignificant. Cheng (1988) also found a superiority of the ZO+PVA over the Kiev extender in the maintenance of both sperm motile life and intact acrosome percentages, but the difference between ZO and Kiev was not significant in their experiment. These results indicated that addition of either BSA or PVA to extenders conduced to the maintenance of sperm motility, membrane integrity and acrosome intactness, and that PVA could be used to substitute for BSA in semen dilution.

Excessive dilution, especially when using pure

**Table 6.** *In vitro* fertilizing capacity of porcine spermatozoa during semen storage at 20°C in ZO+PVA extender

| Days of semen storage | Oocytes insemi-nated | Fertilization (%) | | Embryo development (%) | | | |
|---|---|---|---|---|---|---|---|
| | | Oocytes examined | Oocytes fertilized | Oocytes cultured | Oocytes cleaved | 4-16 cell | Morulae |
| 0 | 55 | 15 | 7 (46.6) [a] | 40 | 14 (35.0) [a] | 10 (71.4) [ab] | 0 |
| 1 | 58 | 16 | 7 (43.7) [a] | 42 | 14 (33.3) [a] | 10 (71.4) [ab] | 0 |
| 2 | 59 | 17 | 7 (41.2) [a] | 42 | 14 (33.3) [a] | 9 (64.3) [ab] | 2 (14.3) |
| 3 | 56 | 15 | 6 (40.0) [ab] | 41 | 13 (31.7) [ab] | 10 (76.9) [a] | 0 |
| 4 | 65 | 20 | 8 (40.0) [ab] | 45 | 14 (31.1) [ab] | 11 (78.6) [a] | 0 |
| 5 | 50 | 18 | 7 (38.9) [ab] | 32 | 10 (31.2) [ab] | 7 (70.0) [ab] | 0 |
| 6 | 61 | 21 | 8 (38.1) [ab] | 30 | 9 (30.0) [ab] | 7 (77.8) [a] | 0 |
| 7 | 65 | 14 | 5 (35.7) [ab] | 51 | 15 (29.4) [ab] | 9 (60.0) [ab] | 1 (13.3) |
| 8 | 66 | 17 | 6 (35.3) [ab] | 49 | 14 (28.5) [ab] | 8 (57.1) [ab] | 0 |
| 9 | 60 | 19 | 6 (31.9) [b] | 29 | 7 (24.1) [b] | 4 (57.1) [ab] | 0 |
| 10 | 67 | 14 | 4 (28.5) [bc] | 53 | 11 (20.7) [b] | 7 (63.6) [ab] | 0 |
| 11 | 62 | 13 | 3 (23.1) [bc] | 49 | 7 (14.2) [bc] | 4 (57.1) [ab] | 0 |
| 12 | 60 | 15 | 3 (20.0) [c] | 45 | 4 (8.8) [c] | 0 (0%) [c] | 0 |

[a, b, c, d] Values with common lowercase letters in the superscripts in the same column did not differ significantly ($p>0.05$) among different time points.

electrolyte media, caused considerable loss of sperm viability (Johnson et al., 2000). Watson (1995) considered this so-called dilution effect a consequence of loss of intracellular components and/or dilution of a protective agent in seminal fluid. Harrison et al. (1982) proposed that the dilution effect is due to the absence of proteinaceous motility stimulants from seminal plasma and they showed that serum albumin could stimulate motility in a reversable manner. Waberski et al. (1989; 1994a) found that BSA stimulated the motility and improved fertility of spermatozoa when the semen was stored *in vitro*. The mechanism by which PVA protects spermatozoa during semen storage is still unknown.

In this experiment, even with BSA removed, the ZO-BSA extender worked still better than the Kiev and BTS extenders, especially during the last part of storage. This might be due to the fact that ZO-BSA contained less and much less glucose than the BTS and Kiev, respectively. Although the high glucose content of most boar semen diluents was considered to cause a considerable reduction in intracellular pH below 6.0 and enable spermatozoa to survive storage of some days at ambient temperature (Johnson et al., 2000), our results were consistent with the possibility that too much glucose in an extender would go against long-term storage of boar semen at 20°C.

Boar spermatozoa tolerate a relative wide range of osmolality between 240 and 380 mosM, but it seems that isotonic or slightly hypotonic media offer better preservation of fertilizing capacity than hypertonic diluents (Weitze, 1990). In this study, the osmotic pressure of the Kiev extender was the highest (420 mosM), while the other extenders were slightly hypotonic (from 281 to 310 mosM). This might be another reason why the performance of the Kiev extender was inferior to others.

In this study, when semen was stored in the ZO+PVA extender at 5°C, sperm motility declined markedly to 35.5% within 24 h and by day 5 of storage sperm motility went down to 0.2%. However, when semen was stored in the same extender at 15 and 20°C, sperm motility was improved markedly. Sperm motility did not differ significantly during the first 5 days of storage, but during the following days it became significantly higher when semen was stored at 20°C than at 15°C. This indicated that boar sperm motility was much better maintained during semen storage at 20 and 15°C than at 5°C, and the temperature of 20°C was more suitable for preservation in ZO+PVA. It is known that boar spermatozoa are very susceptible to cold shock. When freshly ejaculated boar spermatozoa are cooled quickly from body temperature to temperatures below 15°C, an increasing number of spermatozoa loss viability, especially when cooling is quickly continued to 1-2°C (Johnson et al., 2000). The high sperm motility in the 15 and 20°C treatments when examined soon after cooling and at 24 h of storage suggested that the cooling procedures we adopted for these two treatments were suitable but the sharp decline in sperm motility in the 5°C treatment at these time points suggested an inappropriate cooling that caused cold shock to spermatozoa.

Sperm fertilizing capacity after boar semen storage was usually assessed by artificial insemination (Johnson et al., 1988; Johnson, 1998). It was found, however, that pre-infusion of seminal plasma increased the percentage of embryos and the number of accessory spermatozoa per zona (Johnson et al., 2000). This beneficial effect of seminal plasma on fertilization rate and accessory sperm number was attributed to the enhanced cell transport to the site of fertilization. When inseminations were performed with semen stored for long periods, a combined effect of *in vitro* and *in vivo* ageing of spermatozoa caused decreased fertilization rates (Waberski et al., 1994b). While *in vivo* fertilization is the all-round test for sperm fertilizing potential, it can neither eliminate the effect of seminal plasma, nor differentiate between effects of *in vivo* and *in vitro* aging on sperm fertilizing potential. In this experiment, the *in vitro* matured oocytes were inseminated with spermatozoa that had been stored in ZO+PVA extender for different days. Both fertilization and cleavage rates did not changed significantly until day 8, and morulae were obtained with spermatozoa from day 2 and day 7 of semen storage. The cleavage rates (35.0-28.5%) we obtained with semen stored up to 8 days were similar to those obtained by Mattioli et al. (1989) and Naito et al. (1989) with fresh semen. Our fertilization rate was similar to but cleavage rate was lower than those achieved by Yi et al. (2004a) using frozen-thawed spermatozoa. The low cleavage rate in our experiment could not simply be attributed to sperm quality, because oocyte quality and the IVF system used might also affect embryo development. Although our results indicated that the Zorlesco diluent with PVA substituting BSA maintained sperm viability and fertilizing capacity after 8 days of semen storage, further *in vitro* and *in vivo* experiments are needed to prove embryo development. In this study, oocytes were inseminated with estimated equal number of motile sperm in each treatment. This might be the reason why the fertilization and cleavage rates did not decline as sharply as sperm motility during semen storage and it implies that sperm motility was still highly correlated with fertilizing capacity after long-term semen storage.

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