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Comparison of Viability, ATP and *In vitro* Fertilization of Boar Sperm Stored at 4°C in the Three Different Diluents

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ABSTRACT : This study was designed to develop a method of liquid storage of boar sperm at 4°C by using the modified Beltsville F5 (BF5) diluent with bovine serum albumin (BSA) and N-acetyl-D-glucosamine. Boar sperm were stored in lactose-egg yolk and N-acetyl-D-glucosamine (LEN), BF5 and Golden-Pig liquid 4 (GPL4) diluents at 4°C for 5 days and were examined for sperm viability, adenosine triphosphate (ATP) and *in vitro* fertilization (IVF). The percentage of sperm viability in GPL4 diluent was higher than in LEN and BF5 diluent from 1 to 5 days of storage at 4°C. The percentage of sperm viability steadily declined from 1 to 5 days of storage in the three different diluents. Sperm ATP in GPL4 diluent was higher than in LEN and BF5 diluents from 1 to 5 days of storage. Sperm ATP rapidly declined after 5 days of storage in the three different diluents. Porcine oocytes matured *in vitro* were inseminated with different sperm concentrations of liquid semen stored for 3 days in GPL4 diluent. The percentage of monospermic oocytes did not show any differences from 2.5 to 20×10^5 sperm/ml. However, the percentage of polyspermic oocytes in the sperm concentration of 2.5×10^5 sperm/ml was lower than in concentrations of 5, 10 and 20×10^5 sperm/ml. The percentage of blastocysts from the cleaved oocytes at 2.5×10^5/ml sperm concentration was significantly lower than at 5, 10 and 20×10^5 sperm/ml concentrations. In conclusion, GPL4 diluent can be stored at 4°C for 5 days and showed higher sperm viability and sperm ATP concentration compared with LEN and BF5 diluents. Also, we found that GPL4 diluent can be used for IVF of porcine oocytes. (**Key Words :** Fertilization, Liquid Semen, Oocyte, Porcine)

INTRODUCTION

The storage technologies of boar semen can be divided into two major types: the liquid storage and the cryopreservation. The use of liquid semen will increase on a worldwide basis because countries continue to improve their transport methods, thus, liquid semen can be quickly delivered from a boar stud to a pig farm both within and between countries. According to the range of temperature, the liquid storage also can be divided into the normal temperature storage (15-25°C, the short term storage) and the low temperature storage (0-5°C, the long term storage). Because of their peculiar plasma membrane composition, boar sperm are extremely sensitive to cooling, freezing and thawing (White, 1993). This sensitivity to low temperatures requires storage at moderately reduced temperatures (16-20°C), which restricts the sperm's storage capacity because cell metabolism cannot be slowed down and because microbiological conditions may not be as effectively controlled as at lower temperatures. So it is worthy to develop a method of storage of boar sperm at 4°C in refrigerator.

The primary action of BSA in an extended sperm suspension is surely a stimulation of the sperm motility, which appears to be reversible. In this aspect, BSA is more effective than other commercially available macromolecular substances, such as α-lactalbumin, β-lactalbumin, β-lactglobulin, ovalbumin or γ-globulin (Weitze, 1991). N-acetyl-D-glucosamine is a major component in the exoskeletons of insects and crustanceans. Therefore, we hypothesized that soluble N-acetyl-D-glucosamine would protect sperm cell after freezing and thawing. Many different parameters have been described as a measure for semen quality. Various methods have been employed for the evaluation of motility, acrosome integrity, sperm viability and sperm ATP concentration. Selles et al. (2003) reported

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Table 1. The composition of LEN, BF5 and GPL4 diluents for storage at 4°C

Ingredient	LEN	BF5	GPL4
Lactose hydrate (g)	11.0	-	-
Glucose (g)	-	3.5	3.5
TES (g)	-	1.2	1.2
Tris (g)	-	0.2	0.2
KCl (g)	-	0.08	0.08
N-acetyl-D-glucosamine (g)	0.05	-	0.05
Egg yolk (ml)	20.0	20.0	20.0
Bovine serum albumin (g)	-	-	0.3
Penicillin G (g)	0.06	0.06	0.06
Streptomycin (g)	0.1	0.1	0.1
Distilled water (ml)	100.0	100.0	100.0

that the *in vitro* fertilization (IVF) system was a good tool to evaluate the quality of frozen-thawed boar semen previous to its commercial utilization and a good way to modulate new sperm freezing procedures, as it was the more precise evaluating method in estimating the potential fertilizing capacity. Successful IVF could be induced effectively when both fresh boar ejaculate (Nagai et al., 1984; Hamano and Toyoda, 1986) and epididymal sperm (Nagai et al., 1984) were preincubated at a high density in a suitable medium before IVF. In this study, we tried to develop a method of liquid storage of boar sperm at 4°C by using the modified BF5 diluent with BSA and N-acetyl-D-glucosamine (GPL4 diluent), and evaluate the liquid sperm quality by *in vitro* fertilization of porcine oocytes.

MATERIALS AND METHODS

Semen collection

Semen was collected from adult Duroc boars 15-22 months of age. Boars were housed at pig farm of Chungnam National University in Daejeon. Semen was collected one time per week from each of four Duroc boars. The sperm-rich fraction (30 to 60 ml) of ejaculate was collected into an insulated vacuum bottle by grasping and pressing the glans penis with a gloved hand. The sperm-rich fractions of ejaculates with greater than 85% motile sperm and normal acrosome were used.

Processing of liquid semen stored at 4°C

Semen was slowly cooled to room temperature (20 to 23°C) by 2 h after collection. Semen was transferred into 15 ml tubes, centrifuged at room temperature for 10 min at 800×g, and supernatant solution was poured off. The concentrated sperm was resuspended with 10 ml of LEN, BF5 or the modified BF5 with BSA and N-acetyl-D-glucosamine (GPL4) diluents using for frozen boar semen processing to provide 1.0×10^9 sperm/ml at room temperature. The composition of the above diluents was shown in Table 1. The resuspended semen was cooled in a refrigerator to 4°C and preserved for 5 days.

Fluorescent staining to assess sperm viability

Sperm viability was assessed using SYBR-14 and propidium iodide (PI) kit according to the manufacturer's protocol (Live/Dead Sperm Viability Kit, Molecular Probes, Eugene, OR, USA). One μl SYBR-14 of 1 mM solution in dimethylsulphoxide (DMSO) was diluted with 49 μl HEPES-buffered saline solution (10 mM HEPES, 150 mM NaCl, pH 7.4) containing 10% BSA. Five μl SYBR-14 was added into 1 ml sperm samples containing 2×10^7 sperm/ml in HEPES-buffered saline solution. Sperm samples were incubated for 10 min at 38.5°C. Then, 5 μl PI was added into the samples and sperm were incubated for 10 min at 38.5°C. Stained sperm cells were placed on microscope slides and covered with coverslips. One hundred sperm cells per slide were examined in random fields, using an epifluorescence microscope (Olympus BX51, Korea) equipped with blue and green excitations for SYBR-14 and PI, respectively. The nucleus of the SYBR-14-stained cells fluoresced bright green while the dead sperm nuclei exhibited red fluorescence (PI). The fluorescent staining of sperm was monitored and photographed by DP controller (Olympus) equipped with fluorescent image analyzer (Figure 1).

Measurement of adenosine triphosphate (ATP)

Semen was adjusted to concentration of 2×10^6 sperm/ml and washed with 10 ml TL-HEPES-PVA medium at 37.5°C, twice (120×g, 10 min). After washing, the supernatant were discarded and resuspended with buffer (50 mM Tricine, 10 mM $MgSO_4$, 2 mM EDTA, pH 7.8; Ford and Leach, 1998). The buffers were heated for 10 min before the samples were added. Sperm samples were boiled in the water bath for 3 min at 100°C, transferred to an ice bath, and centrifuged at 5,000×g for 30 min at 4°C. The supernatant was used for determination of total adenosine triphosphate (ATP). ATP was determined using a luciferase reaction kit according to the manufacturer's protocol (Enliten® ATP Assay System, Promega, Madison, WI, USA). Standards were prepared from ATP standard (F203A, Promega) using serial dilutions to obtain concentrations of 1×10^{-7}, 5×10^{-8}, 1×10^{-8}, 5×10^{-9}, 1×10^{-9}, 5×10^{-10} and 1×10^{-10}. Aliquots of the ATP stock solution were stored at -20°C until use and standard curve dilutions were prepared for each assay. Bioluminescence was measured with a HTS multi label reader (Perkin Elmer Inc., Boston, MA, USA) after addition of 50 μl sample and 50 μl luciferin-luciferase reagent.

Collection and *in vitro* maturation of porcine oocytes

Ovaries were collected from prepubertal gilts at a local slaughter house and transported to the laboratory in 0.9%

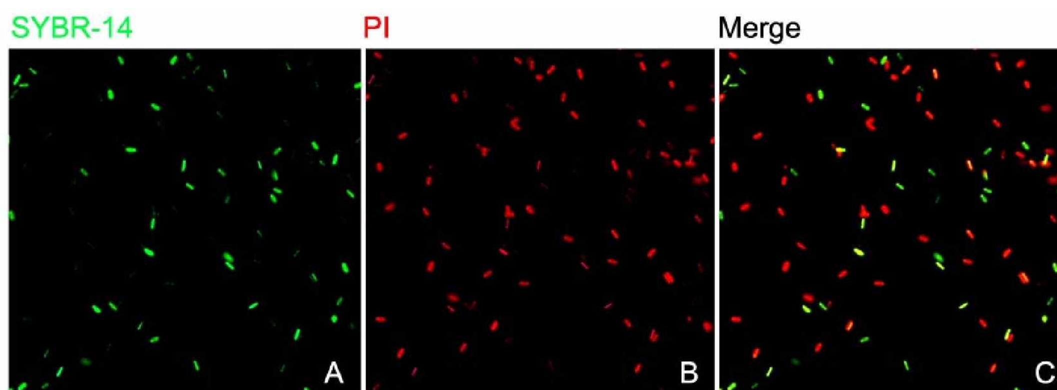

Figure 1. Fluorescent staining of boar sperm. Boar sperm were stained by using SYBR-14 (living sperm, A) and PI (dead sperm, B).

saline at 30-35°C. Follicular fluid and cumulus-oocyte complexes (COCs) were aspirated from follicles of 2-6 mm in diameter using an 18-gauge needle fixed to a 10 ml disposable syringe. The follicular contents were pooled into 50 ml tubes and allowed to sediment, the sediment was placed into HEPES buffered Tyrode-lactate medium (TL-HEPES-PVA; 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 mM NaH_2PO_4, 12 mM sorbitol, 10 mM sodium lactate, 2 mM $CaCl_2{\cdot}2H_2O$, 0.5 mM $MgCl_2{\cdot}6H_2O$, 10 mM HEPES, 0.2 mM sodium pyruvate, 65 μg/ml penicillin G, and 25 μg/ml gentamycin) containing 0.1% (w/v) polyvinyl alcohol (PVA). COCs were selected and washed with TL-HEPES-PVA and then washed twice with the maturation medium. The basic media used for *in vitro* maturation (IVM) was tissue culture medium (TCM) 199 supplemented with 26.19 mM sodium bicarbonate, 3.05 mM glucose, 0.91 mM sodium pyruvate, 75 μg/ml sodium penicillin G, 50 μg/ml streptomycin sulfate and 0.1% PVA. COCs were cultured in 2 ml maturation medium containing the 0.5 μg/ml LH, 0.5 μg/ml FSH, 10 ng/ml epidermal growth factor (EGF), 10% porcine follicular fluid (pFF) and 0.57 mM cysteine in the presence of 2 follicle shells (Cong et al., 2007). After 22 h of maturation, oocytes were cultured without cysteine and hormones for 22 h at 38.5°C, 5% CO_2 in humidified air.

In vitro fertilization (IVF) and culture of oocytes

After 44 h of maturation, cumulus cells of oocyte were removed by 0.1% hyaluronidase in TL-HEPES-PVA and washed twice with TL-HEPES-PVA and modified Tris-buffered media (mTBM), respectively (Abeydeera and Day, 1997). Thereafter, 30-40 oocytes were transferred into each well of a 4-well multidish containing mTBM that had been covered with mineral oil and equilibrated at 38.5°C, 5% CO_2 in air for 4 h. The dishes were kept in a CO_2 incubator until spermatozoa stored for 3 days in GPL4 diluent were added for insemination. For IVF, 0.5 ml liquid semen was washed twice in TL-HEPES-PVA and then resuspended with mTBM to adjust sperm concentrations (2.5, 5, 10 and 20×10^5 sperm/ml, respectively). Oocytes and sperm were co-incubated in 500 μl mTBM for 6 h. After insemination, oocytes were transferred into 500 μl PZM-3 medium containing 0.4% BSA for further culture.

Evaluation of oocytes and embryos produced _in vitro_

At 12 h after IVF, oocytes were fixed in 2% formaldehyde for 40 min to examine sperm penetration, polyspermic oocytes and pronucleus formations. On 6-7 days after IVF, embryos were fixed with 2% formaldehyde for 40 min at room temperature. Fixed oocytes and embryos were washed with PBS, permeated with PBS containing 0.1% Triton X-100, and stained with 2.5 μg/ml DAPI (Molecular Probes). Sperm penetration, monospermic oocytes, polyspermic oocytes, the cleavage rate, blastocysts and cell number per blastocyst were observed under epifluorescence microscope (Olympus).

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Statistical analysis

Analyses of variance (ANOVA) were carried out using the SAS package in a completely randomized design. Duncan's multiple range test was used to compare values of individual treatment, when the probability value was lower than 0.05.

RESULTS

Comparison of sperm viability in the three different diluents according to storage period

The percentage of sperm viability by using SYBR-14 and PI in the three different diluents according to storage period is presented in Figure 2. The percentage of sperm viability in GPL4 diluent was higher than that in LEN and BF5 diluent from 1 to 5 day of storage at 4°C. The percentage of sperm viability steadily declined from 1 to 5

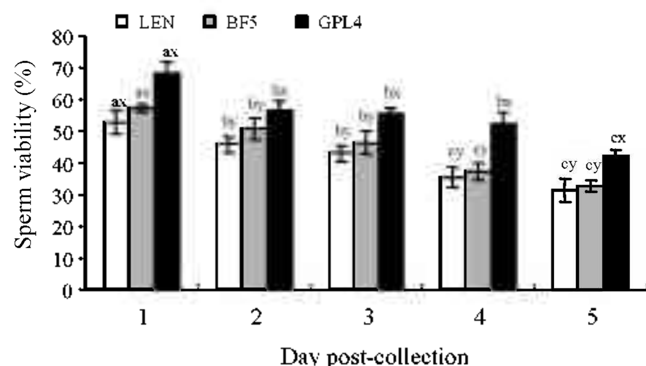

Figure 2. Comparison of sperm viability (%) by using SYBR-14 and propidium iodide in the three different diluents according to storage period. Experiments were repeated 6 times. Graph showing mean percentages±SE. Superscripts a, b and c in figures denote a significant difference at $p<0.05$ among storage periods. Superscripts x and y in figures denote a significant difference at $p<0.05$ among LEN, BF5 and GPL4 diluents, respectively.

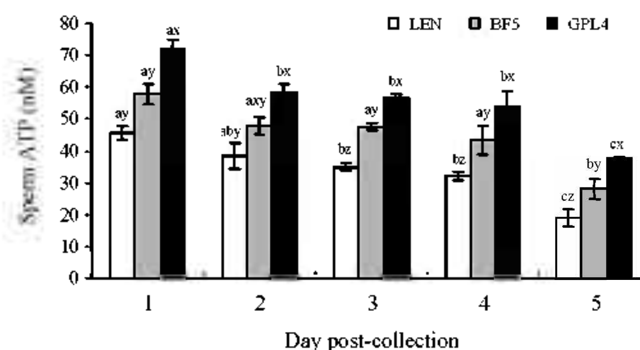

Figure 3. Comparison of sperm ATP (nM/2×10^6 sperm) in the three different diluents according to storage period. Experiments were repeated 6 times. Graph showing mean percentages±SE. Superscripts a, b and c in figures denote a significant difference at $p<0.05$ among storage periods. Superscripts x, y and z in figures denote a significant difference at $p<0.05$ among LEN, BF5 and GPL4 diluents, respectively.

day of storage in the three different diluents, respectively.

Comparison of sperm ATP in the three different diluents according to storage period

As shown in Figure 3, sperm ATP in GPL4 diluent was higher than that in LEN and BF5 diluents from 1 to 5 day of storage. Sperm ATP rapidly declined after 5 day of storage in the three different diluents, respectively.

***In vitro* fertilization of porcine oocytes and subsequent development of embryos by different concentrations of sperm stored for 3 days at 4°C in GPL4 diluent**

As shown in Table 2, the percentage of monospermic oocytes with one sperm head after *in vitro* fertilization for 6 h did not show any differences from 2.5 to 20×10^5 sperm/ml. However, the percentage of polyspermic oocytes with two or more sperm head after *in vitro* fertilization for 6 h in the sperm concentration of 2.5×10^5 sperm/ml was lower than that in the sperm concentrations of 5, 10 and 20×10^5 sperm/ml. The mean number of sperm per oocyte significantly increased from 20×10^5 sperm/ml sperm concentration.

As shown in Table 3, the percentage of cleaved oocytes at 2.5×10^5 sperm/ml sperm concentration was significantly higher than that 10 and 20×10^5 sperm/ml sperm concentrations. However, the percentage of blastocyst from the cleaved oocytes at 2.5×10^5/ml sperm concentration was significantly lower than that at 5, 10 and 20×10^5 sperm/ml sperm concentrations. The cell number of per blastocyst at 5 and 10×10^5 sperm/ml sperm concentrations was higher than

Table 2. Fertilization parameters of porcine oocyte by different concentration of sperm stored at 4°C in GPL4 diluent

Sperm concentration ($\times10^5$ sperm/ml)	No. of oocytes inseminated	% of monospermic oocytes[1]	% of polyspermic oocytes[1]	Mean no. of sperm penetrated in oocyte[1]
2.5	148	45.7±5.6	18.9±1.5[b]	2.5±0.1[b]
5	155	41.2±3.1	39.6±7.0[a]	2.5±0.2[b]
10	150	44.2±3.3	45.1±5.8[a]	2.7±0.2[ab]
20	140	40.3±3.9	45.2±7.8[a]	3.0±0.2[a]

[1] Means±SE. Experiments were repeated six times.

[a, b] Means±SE in the same column with different letters differ significantly ($p<0.05$).

Table 3. Subsequent development of embryos by different concentrations of sperm stored at 4°C in GPL4 diluent

Sperm concentration ($\times10^5$ sperm/ml)	No. of embryos cultured	% of oocytes cleaved[1]	% of blastocyst[1]	Cell no. per blastocyst[1]
2.5	109	96.0±0.9[a]	15.7±4.3[b]	26.4±1.7[b]
5	117	95.2±1.2[ab]	30.8±3.4[a]	30.1±1.7[ab]
10	115	94.6±0.8[b]	33.6±4.0[a]	33.7±1.3[a]
20	113	86.3±5.5[b]	37.1±4.0[a]	26.8±1.2[b]

[1] Means±SE. Experiments were repeated four times.

[a, b] Means±SE in the same column with different letters differ significantly ($p<0.05$).

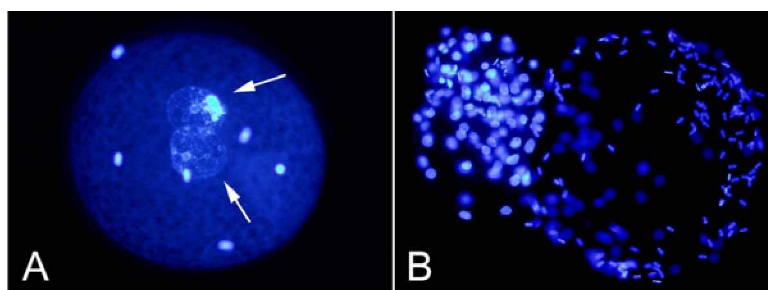

Figure 4. Fluorescence pictures of embryo and blastocyst. Porcine oocytes were inseminated with 1×10^6 sperm/ml for 6 h. Arrows indicate pronucleus formations after IVF for 12 h (A). Hatched blastocyst was observed after culture for 144 h (B).

that at 2.5 and 20×10^5/ml sperm concentrations (Figure 4).

DISCUSSION

Our results clearly demonstrated that the modified BF5 diluent with N-acetyl-D-glucosamine and BSA (GPL4) is efficient for storage of boar sperm at 4°C for 5 days. The low fertility results associated with deep frozen semen techniques have meant that the very small amount of frozen semen used to date has been confined mainly to the transfer of genes between national breeding pyramids (Reed, 1985). Fresh semen may be able to be stored for up to five days with minimal loss in fertility. Therefore, frozen semen will ever replace the use of liquid semen on an extensive basis. The most widely used diluents for deep freezing are BF5 developed by Pursel and Johnson (1975) for freezing boar sperm in the pellet form and LEY developed by Westendorf et al. (1975) for freezing boar sperm in straw. Yi et al. (2002) developed LEN diluent modifying the LEY diluent. The LEN diluents containing 0.05% N-acetyl-D-glucosamine for boar sperm freezing had a beneficial effect on acrosome morphology and motility. This finding is significant because it offers the possibility of using N-acetyl-D-glucosamine for boar sperm preservation. The polysaccharide chitin is a homopolymer of N-acetyl-D-glucosamine in β (1→4) linkage and the major organic element in the exoskeleton of insects and crustacean. Lehninger (1975) reported that many polysaccharides served primarily as structural elements in cell walls and coats, intercellular spaces, and connective tissue, where they gave shape, elasticity, or rigidity to plant and animal tissue as well as protection and support to unicellular organisms.

Harrison et al. (1982) proposed that the dilution effect is due to the absence of proteinaceous motility stimulants from seminal plasma and showed that serum albumin could stimulate motility in a reversable manner. When spermatozoa were washed in the absence of albumin, an increased tendency to stick to glass surfaces indicated surface membrane changes. Waberski et al. (1989) found that BSA stimulated the motility of spermatozoa during a six day storage test. The effect of BSA and various zwitterionic buffers (MOPS and HEPES etc.) was investigated *in vitro* and *in vivo*. Five various BSA types, PSA and ovalbumin were tested in view of their effects on sperm motility and acrosome integrity for 144 h at 15°C and then in a thermo-resistance test at 38°C. All the diluents containing albumin showed a positive effect on motility compared with the media without albumin. Buhr (1990), on the other hand, 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. It was assumed that changes in membrane lipid fluidity could affect trans-membrane movement of Ca^{2+}, which is essential in the capacitation process but deleterious during storage. However, BSA induced only a temporary decrease in membrane fluidity which was difficult to interpret. Nevertheless, BSA improved fertility when the semen was stored between 3 and 5 days (Waberski, 1994). In this study, GPL4 diluent with BSA showed higher sperm viability and sperm ATP concentration compared with LEN and BF5 diluents.

Diluents for boar sperm commonly have contained buffers, sugars, egg yolk, and the surfactant sodium and triethanolamine lauryl sulphate (Pursel and Johnson, 1975; Westendorf et al., 1975; Hu et al., 2006; Yi et al., 2008). Glucose, fructose and lactose are the main sugars added to diluents. They act mainly by their osmotic properties as extracellular cryoprotectants. The addition of egg yolk in the diluents contributes only slightly to improve the maintenance of normal acrosomes, but the proportion of spermatozoa with severely damaged acrosome is higher when media without egg yolk are used (Visser and Salamon, 1974). The mechanisms by which egg yolk provides protection during freezing are not known with precision but could be mediated either by a less intense cellular dehydration (Courtens and Paquignon, 1985) or by stabilization of the plasma membrane (Paquignon, 1985).

The reduction of motility which occurs during storage has long been the main parameter used to judge the decrease of fertilizing ability. The loss of ATP and cAMP, as well as reduced calcium uptake, are characteristic of

decreased motility. An important indicator of storage-related membrane damage is a change in membrane permeability, such as increased permeability to stains and a release of intracellular substances. In relation to this permeability, a distinction must be made between the acrosomal and plasma membranes of sperm (Petzoldt and Nehring, 1988).

Coy et al. (1999) reported that the penetration rate and number of sperm cells per oocyte were dependent on the boar, maturation system and sperm concentration, but the rate of male pronuclear formation seemed to be influenced only by the boar and the maturation system but not by sperm concentration. Wang et al. (1991) reported that high penetration rates (85-89%) and increased incidence of polyspermy were obtained at 25-100$\times 10^6$ sperm/ml by frozen-thawed ejaculated sperm. Nagai et al. (1984) reported that sperm concentration at insemination by epididymal or ejaculated boar sperm was 2×10^6 sperm/ml. Abeydeera and Day (1997) reported that insemination with 1×10^5 sperm/ml by frozen-thawed sperm resulted in a 40% sperm penetration rate of oocytes with 16% polyspermy. Mean number of sperm per oocyte was 1.2±0.1. At 5×10^5 sperm/ml and 1×10^6 sperm/ml, penetration rate (84-87%) and polyspermy (57-64%) increased, with no difference between the two concentrations. Normal cleaved embryos at 2.5×10^5 sperm/ml showed further low competence to form blastocyst, which might be due to the part degeneration of blastomeres of embryos during the long incubation. However, further studies are required to demonstrate the exact mechanism on the degeneration of blastomeres of embryos at 2.5×10^5 sperm/ml. Mean number of sperm per oocyte increased with increasing sperm concentration. In this study, we found out that the sperm concentrations of 5, 10 and 20×10^5 sperm/ml showed higher percentages of blastocyst from the cleaved oocytes than that of 2.5×10^5 sperm/ml. However, mean number of sperm per oocyte increased from 10×10^5 sperm/ml sperm concentration.

In conclusion, we confirmed that GPL4 diluents protected boar semen at 4°C for 5 days and maintained higher sperm viability and sperm ATP concentration compared with LEN and BF5 diluents. Also, GPL4 diluents supported semen viability for *in vitro* fertilization of porcine oocytes.

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