Analysis of Sperm Ability in Specific Pathogen Free Miniature Pig for Production of Bio-Organ

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ABSTRACT

The purpose of this study was the analysis of sperm ability in Specific Pathogen Free (SPF) miniature pig for production of bio-organ. The collected semen was diluted with extender and stored at 17℃ for up to 7 days. The semen samples were evaluated at 0, 1, 3, 5, and 7 days of storage for analysis of sperm ability. Sperm ability was evaluated by examining viability, progressive motility, sperm abnormality and intensity of the sperm membrane. Also, the semen was processed according to the convenient freezing method, and frozen-thawed sperm was evaluated by examining viability, capacitation and acrosome reaction using chlortetracycline (CTC) staining. Motility of spermatozoa of SPF miniature pig was significantly ($P<0.05$) lower on 3 days or later compared to the Duroc, Yorkshire and Landrace in domestic boar. The percentage of abnormal spermatozoa of Landrace were significantly ($P<0.05$) higher than in SPF miniature pig, Duroc and Yorkshire that had a similar percentage on 5 or 7 days of sperm storage. The percentage of spermatozoa with coiled tail decreased during the storage period but there were no significant difference. On the other hand, viability of frozen-thawed spermatozoa had a significantly ($P<0.05$) lower in SPF miniature pig than in other domestic boars. CTC patterns had no significant difference, but SPF miniature pig had higher percentage of capacitated spermatozoa and lower percentage of acrosome-reacted it than domestic boars. Therefore, this study suggest that it is necessary to develop the suitable extender and freezing methods for the high viable rate and fertilizing ability *in vitro*.

(Key words : Sperm ability, SPF miniature pig, Bio-organ, Domestic boar, Fresh semen)

INTRODUCTION

Human organs for transplantation are insufficient in quantity, such as heart, lung, liver, bone marrow and kidney. Waiting patients for organ transplantation are constantly growing and most of them die before an organ can be found.

To supply the short organ, researches study about the xenotransplantation which uses animals as donors, but it is still highly experimental. The miniature pigs offer advantage over other species by having a renal anatomy and function very similar to human. Due to size and anatomic physiologic similarities, miniature pigs provide their organ such as heat, kidney and bone marrow to the human currently awaiting organ transplants (Fishman, 1997). However, organ transplantation between distantly related species, such as pigs and human, results in hyperaute rejection (HAR), involving the complement system. To dissolve this problem, it needs not only deleting specific animal genes but also adding specific human genes through genetic engineering (Chapman, 1995). Also condition of miniature pig must be a germ free or Specific Pathogen Free (SPF) condition to use for study. SPF is a free of disease causing microbes or pathogens, germfree is a free of viruses, bacteria, fungi, protozoa other prasites. But their quantity is very insufficient in the world, because there are not almost data about the reproduction biology and difficult to keep clean condition.

Artificial insemination to produce a piglet is an important tool in the swine industry. Fresh semen is almost exclusively used in the artificial insemination of swine. Extended semen is affected both the extender and their environment. Factors, such as pH, ionic strength, type of ions and osmotic pressure of the suspending medium, are important to subsequent sperm ability and can change during storage. A change of

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sperm ability during storage *in vitro* has influence on both fertilizing capacity and piglet production. Sperm ability is an important, so it is evaluated the viability, progressive motility, abnormal sperm, fertilization ability and intensity of sperm membrane.

To produce bio-organ for xenotransplantation, therefore, this studies have conducted analysis of sperm ability of SPF miniature pig and compared with domestic boars (Duroc, Yorkshire and Landrace).

MATERIALS AND METHODS

Semen Collection

Semen-rich fractions were collected from both domestic boars(Duroc, Yorkshire and Landrace) and SPF miniature pigs by the gloved-hand technique and filtered through cotton gauze into a pre-warm cup to remove the gel particles. Semen was extended with equal volumes of Byu-Ri extender (Sperm Gene Co., Korea). After 20 min at the room temperature, the extended semen samples were transported to the laboratory at 17℃ within 3 hours of collection. The extended semen was stored at 17℃ and evaluated the sperm ability on 0, 1, 3, 5 and 7 days of storage, respectively. Assessments at 0 day point were executed after extended semen arrived at the laboratory.

Sperm Ability

Extended semen for period of storage was evaluated viability using Hoechst 33258 staining (De leeuw *et al.*, 1991), progressive motility using Makler Counting Chamber(Self-medical Instrument, Israel), abnormal spermatozoa using Rose-bengal staining and intensity of the sperm membrane using hypo-osmotic swelling test (Vazquez *et al.*, 1997).

Cryopreservation and Thawing of Semen

Semen was processed according to the straw freezing procedure described by Westendorf *et al.* (1975) with minor modification indicated on the following (Einarsson, 1973). Diluted semen was placed at 15℃ for 2 h and centrifuged (800 ×g, 10 min), the supernatant was removed, and spermatozoa were resuspended with lactose-egg yolk extender (LEY, Almlid and Johnson, 1998) to a concentration of 1.5×10^9 cells/mL. After further cooling to 5℃ over a 2 h, two parts of LEY-extender semen were mixed with 1.5 % Orves Es Pates and 9 % glycerol to give a final freezing concentration of 1.0×10^9 cells/mLand 3 % glycerol. The diluted and cooled semen was loaded into 0.5 mL straws and placed in liquid nitrogen vapor approximately 8 cm above the level of the liquid nitrogen for 10 min. The straws were then stored in liquid nitrogen. Thawing was achieved by immersing the straws in a waterbath set at 37℃ for 30 sec. Immediately after thawing, the semen was processed for assessment of viability and chlortetracycline assay.

Chlortetracycline (CTC) Fluorescence Assessment of Spermatozoa

The state of spermatozoa capacitation and acrosome reaction were assessed using a CTC fluorescence assay (Wang *et al.*, 1995; Abeydeera *et al.*, 1997). Briefly, 8 μL of Hoechst bis-benzimide 33258 (Sigma, 100 μg/m Lin D-PBS) was added to 792 μL sperm suspension. After gentle mixing, each suspension was incubated for 3 min at room temperature in the dark, then layered onto 4 mL of 3 % (w/v) polyvinylpyrolidone (PVP-40; Sigma) in D-PBS and centrifuged at 1,400×g for 5 min. The pelleted spermatozoa were resuspended in 50 μL of washing medium and 45 μL of this suspension was added to 45 μL of CTC solution, containing 750 uM CTC (Sigma), 5 mM cysteine, 130 mM NaCl and 20 mM Tris (pH 7.8). Spermatozoa were fixed by adding 8 μL of 12.5 % (w/v) paraformaldehyde in 0.5 M Tris-HCl (pH 7.4). The CTC solution was prepared daily and kept wrapped in foil at 4℃ until just before use. Slide were prepared by placing 10 μL of the fixed sperm suspension on a slide and one drop of 0.22 M 1,4-diazabicyclo[2.2.2] octane dissolved in glycerol : D-PBS (9:1) which was then carefully mixed in order to retard the fading of fluorescence. A coverslip was sealed nail varnish. Each cell was first observed under ultraviolet (UV) illumination (excitation at 330~380 nm, emission at 420 nm) to determine the live /dead status; the sperm cells showing bright blue staining of the nucleus were consider as dead and not counted. Two hundred live sperm were then examined under blue- violet illumination (excitation at 400~440 nm, emission at 470 nm) and classified according to CTC staining patterns. The three fluorescent staining patterns identified were: F (an uncapacitated spermatozoa), with uniform fluorescence over the whole sperm head; B (a capacitated spermatozoa), with a fluorescence-free band in the post-acrosome region; and AR (an acrosome-reacted spermatozoa), with almost no fluorescence over the sperm head, except for a thin band of fluorescence in the equatorial segment (Abeydeera *et al.*, 1997).

Statistical Analysis

Each experiment was replicated three times. Statistical analysis were carried out by analysis of variance (ANOVA) and Fisher's protected least significant difference test using the SAS version 8.01 for Windows (SAS Institute, 1990). Data from each experiment are presented as the mean ± S.D. and $P<0.05$ was consi-

dered to be statistically signigicant.

RESULTS

Sperm Viability

Sperm viability of SFP-miniature pig and domestic boars during *in vitro* storage at 17℃ are shown in Fig. 1. Sperm viability were approximately 92 % at first on the day of semen collection, decreased to 69.7 % at 7 days of storage. There were no significant difference between SPF miniature pig and domestic boars. However, sperm viability of SPF-miniature pig was the lowest in four boar groups during increased periods of storage.

Progressive Motility

Progressive motility of all experimental group during *in vitro* storage boars semen at 17℃ are shown in Fig. 2. Progressive motility of all semen gradually decreased with period of storage. The progressive motility of SPF-miniature pig were significantly ($P<0.05$) lower than

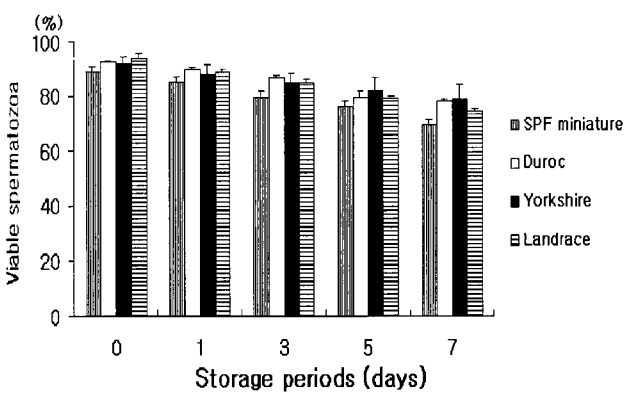

Fig. 1. Changes of viability during *in vitro* storage of spermatozoa in the pig.

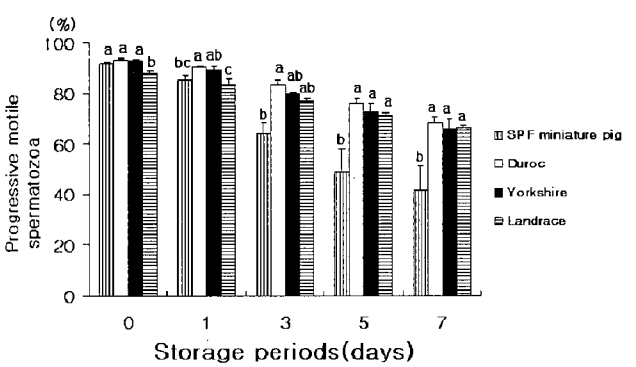

Fig. 2. Progressive motility of spermatozoa stored *in vitro* in the pig. [a,b] Different letters above the bars denote statistically significant differences ($P<0.05$).

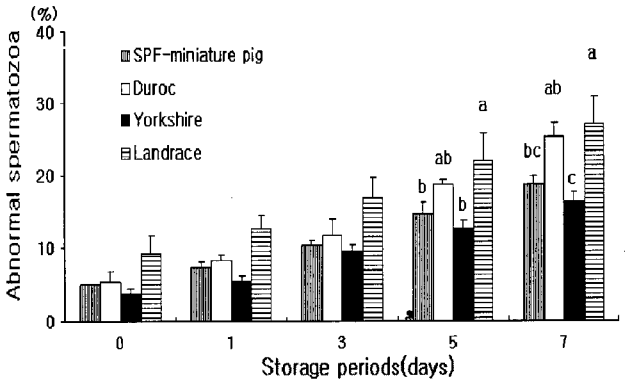

Fig. 3. Changes of abnormal rates during *in vitro* storage of spermatozoa in the pig. [a,b] Different letters above the bars denote statistically significant differences ($P<0.05$).

domestic boars on 3 days of storage, as a result, it decreased to 41.7 % on 7 days of storage.

Abnormal Spermatozoa

The percentage of abnormal spermatozoa during *in vitro* storage at 17℃ are shown in Fig. 3. Abnormal spermatozoa of all groups gradually increased from 0 day to 7 days during storage. Abnormal spermatozoa of Landrace was significantly ($P<0.05$) higher than SPF-miniature pig and Yorkshire on 5 and 7 days of storage, but there were no significant differences in the abnormal spermatozoa between Landrace and Duroc.

Hypoosmotic Swelling Test (HOST)

The percentage of coiled tail for both SPF-miniature and domestic boar groups during *in vitro* storage at 17 ℃ are shown in Fig. 4. All four boar groups, the percentage of spermatozoa showing coiled tail decreased with periods of storage, but there were no significant differences among the experimental groups. Although the percentage of coiled tail in SPF-miniature pig was higher than the domestic boars groups up to 3 days, it was lower than the other boar on 5 and 7

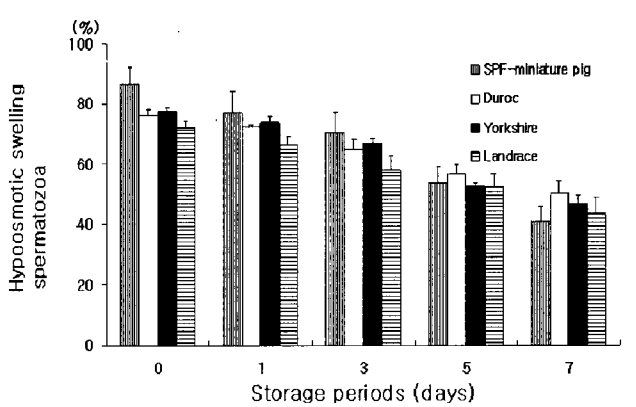

Fig. 4. Strength of boar spermatozoa stored *in vitro*.

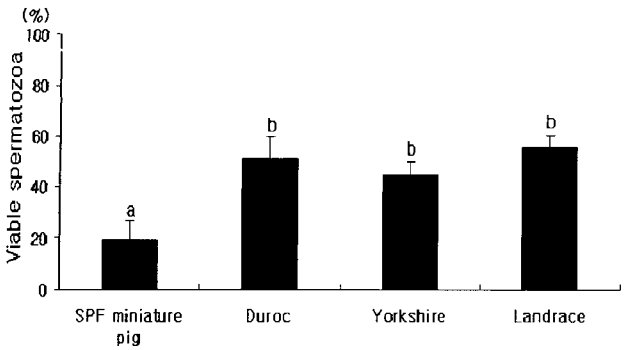

Fig. 5. Viability of spermatozoa frozen-thawed *in vitro*. [a,b] Different letters above the bars denote statistically significant differences ($P<0.05$).

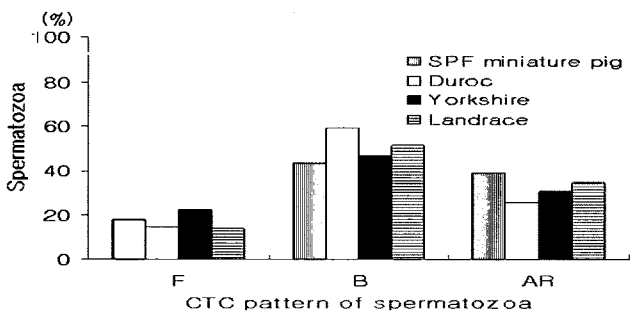

Fig. 6. Changes of CTC patterns in boar spermatozoa frozen-thawed.

days of storage.

Viability of Frozen-Thawed Spermatozoa

The viability of frozen-thawed spermatozoa for both SPF-miniature and domestic boars are shown in Fig. 5. The viability (%) of frozen-thawed spermatozoa of SPF-miniature (19.7 %) was significantly ($P<0.05$) lower than frozen-thawed spermatozoa of domestic boars(51, 45 and 56.7 % for Duroc, Yorkshire and Landrace).

Capacitation and Acrosome Reaction

The CTC patterns of frozen-thawed spermatozoa for both SPF-miniature and domestic boars are shown in Fig. 6. Although the CTC patterns were not significant differences for all boar groups. However, capacitated spermatozoa of SPF-miniature pig was lower than the domestic boars, acrosome-reacted spermatozoa was higher compared with them.

DISCUSSION

At the present time, the most widely used extender for boars is the BTS (Pursel and Johnson, 1975), Zorlesco (Gottardai *et al.*, 1980) and Androhep (Weitzw, 1990). Byu-Ri extender used for this study is based on the BSA, Glucose, Tris and Gentamicin. Extended semen was stored at 17℃ during period of storage and assessed sperm ability.

The viability of extended semen both SPF-miniature pig and domestic boars were gradually decreased with period of storage at 17℃. Its presence was also shown in BTS, Zerlesco and Androhep (Huo *et al.*, 2002). Viability of SPF-miniature pig was lower than the other boars during storage. Boar sperm are known to be particularly sensitive to their environment such as pH, ionic strength, type of ions and osmotic pressure of the extender. As a result of long-storage, capacitation involves a process of membrane distabilization resulting either in a physiological acrosome reaction or, ultimately, cell death.

Progressive motility of spermatozoa is an indicator of both unimpaired metabolism and intactness of membrane. Estimation of motility has fundamental importance in the daily quality control of the semen. Furthermore, the percentage of motile spermatozoa is used to calculate the required degree of dilution and to estimate the number of "intact" spermatozoa per insemination dose. Regular motility checks after dilution and during the holding period give information concerning the preservability of the semen of each boar of its individual peculiarities. Stored semen should be examined daily, with motility values above 60 % considered satisfactory. Progressive motility of domestic pigs gradually decreased up to 76.7 % on 7 days storage. However, progressive motility of SPF miniature pig was significantly lower than the other boars on 3 days and later. After 5 days of storage, the progressive motility showed lower than 60%, in this case, this semen should not to use IVF and artificial insemination.

Abnormal spermatozoa that there are many factor and complex reason. Boars used for commercial artificial insemination are selected to a certain degree on the basis of a low incidence of morphologically abnormal spermatozoa, so that statistical calculations concerning their correlation with fertility are not very informative. Nevertheless, the variance between the percentage of proximal and distal cytoplasmic droplets on spermatozoa in the ejaculates is sufficiently large to establish a statistical relationship with fertility. A number of studies have also shown a significant negative correlation between the percentage of spermatozoa with cytoplasmic droplets and farrowing rate and number of live born piglets (Zeuner, 1992). Abnormal spermatozoa of all four boar groups increased with period of storage. After 5 days of storage, abnormal spermatozoa of Landrace significantly higher than the other boars. Both Duroc(25%) and Landrace(27%) presented high abnormal spermatozoa at 7 days of storage.

It is very important to analyze the structural and functional intensity of the sperm membrane because

these characteristics are crucial for the viability and fertilizing ability of spermatozoa. The hypo-osmotic swelling test (HOST) can be used to evaluate functional integrity of the sperm membrane because spermatozoa with a biochemically active membrane will swell when hypo-osmotically stressed, due to the influx of water. This fact is more easily observable in the tail appears to be more loosely attached than the membrane surrounding the head. HOST has been used in several species, including man (Jeyendran *et al.*, 1984), boar (Vazquez *et al.*, 1997), dog (Kumi-Diaka, 1993), stallion (Sofickitis *et al.*, 1992) and bull (Correa *et al.*, 1994). In this study, the percentage of coiled tail spermatozoa decreased during storage in all boars grops. Although, SPF miniature pig showed high percentage of coiled spermatozoa compared with the other boar groups up to 3 days of storage.

The first calf producted from cryopreserved spermatozoa was born in 1951 (Stewart, 1951) and the technique very rapidly established itself thereafter. Successful freezing of boar semen depends on an understanding of the factors and their interactions which influence the capacity of spermatozoa the survive freezing and thawing. Viability of frozen-thawed spermatozoa in SPF miniature pig was significantly lower than domestic boars. In previous study, viability of frozen-thawed boars spermatozoa was presented approximately 57.3% under 3% glycerol level (Fisher, 1989). Straw freezing procedure has been used for domestic boar, but when this methods applied to SPF miniature pig obtained a low viability about 19.7%. It is mean that this method is short of freezing condition to keep high spermatozoa viability of SPF miniature pig. Artificial insemination of swine with frozen-thawed sperm result in farrowing rates and litter sizes 20~30% below those observed following insemination of fresh sperm (Johnson *et al.*, 2000). This is believed to be a result of spermatozoa acquiring cryoinjury, including-like reaction, during the freezing and thawing precesses. Green (2001) demonstrated that the capacitation-like reaction was induced by the cooling of boar spermatoza to 5℃ and was observed once the spermatozoa were rewarmed 39℃. Cooling causes the membrane to become unstable and fusagenic, similarly to what happens in normal capacitation (Watson, 1996). In general, the proportion of F pattern sperm decreased during periods of storage as the B pattern sperm increased. Perez *et al.* (1996) used CTC staining to demonstrate an accelerated rate of capacitation *in vitro* for frozen-thawed ram spermatozoa. After incubation for 2 h after thawing, 39 % of ram spermatozoa showed the B pattern compared with only 16 % of fresh spermatozoa incubated for the same period. In this study, there were no difference between domestic boars and SPF miniature pig at CTC pattern of post-thawed spermatozoa. Capacitation is a complex process, which renders the sperm cell capable for specific interaction with the oocyte. During capacitation, modification of membrane characteristics, enzyme activity and motility properties of spermatozoa render these cells able to penetrate oocyte investments and respective to stimuli that induce acrosome reaction prior to fertilization.

In conclusion, this study suggest that it is necessary to develop the suitable extender and freezing method for the high survival rate and fertilizing ability *in vitro.*

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