Development of Semen Extenders by Assessment of Sperm Viability in Miniature-Pig Semen

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ABSTRACT

The purpose of this study was to assess sperm quality during *in vitro* storage of miniature-pig semen in order to determine which extender should be used and how extender can be diluted for *in vitro* storage of miniature-pig semen. Freshly ejaculated miniature-pig's semen was diluted with same volumes of Beltsville Thawing Solution (BTS), Androhep, Modena, Mulberry Ⅲ and modified-Modena extenders. Sperm quality was evaluated by examining viability, motility, abnormality, acrosome intactness, intensity and capacitation status by chlorotetracycline (CTC) staining. Sperm motility decreased with storage period prolonged and differences among BTS, Androhep, Modena and Mulberry Ⅲ were apparent. On Day 1, approximately 80% of the sperm were motile, but motility decreased to 40% at Day 7. During the 7 days of storage, sperm survival in modified-Modena B extender was higher than another extenders. However, it was not differ significantly among other extenders. The percentage of F and B patterns were not differ significantly among the extenders. However, F pattern in modified-Modena B extender was slightly higher until 3 days of storage than that of Modena extender, modified-Modena A extender and modified-Modena C extender. The percentage of AR patterns in modified-Modena B extender was slightly lower, but did not differ significantly among other extenders. The results of present study suggest that modified-Modena B was effective as new extender for *in vitro* storage of miniature-pig semen.

(Key words : Miniature-pig, Semen extender, Sperm viability Hoechst 33258, CTC pattern)

INTRODUCTION

The first attempts on artificial insemination (AI) of pigs were made in 1926~1927 by Ivanov, which were continued between 1930 and 1936 by Milovanov *et al.*, (cited by Serdiuk, 1970). The early diluents (glucose-sulphate and glucose-tartrate) for boar semen were proposed by Milovanov in the years of 1931~1933 (Milovanov, 1962).

Since these earliest studies of semen storage in the pig, it has been understood that only a portion of spermatozoa in the original semen sample survives preservation of any type. It is also well known that survival of cells is much greater after liquid than frozen storage of semen. As temperature declines, there is an inevitable reduction in the proportion of spermatozoa that maintain normal membrane integrity, ultrastructure and biochemical components.

Utilisation of preserved semen for AI in pigs has increased approximately threefold in the past 15 years. 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℃ for 1 to 5 days. Eighty-five percent of all inseminations are conducted on the day of collection or on the following day. Virtually all AI with liquid-stored semen is used for market hog production.

Sperm motility and survival are a fundamental factor to the success of reproduction because it allows the sperm to reach the site of fertilization. So, development of miniature-pig semen extenders is very important for liquid stored. The volume of miniature-pig semen is less than semen of other pigs. Because, sperm motility and survival are very important that for liquid stored. During liquid storage, miniature-pig semen extender is necessitate that for improvement of sperm motility and survival.

The purpose of this study was to assess sperm quality in extended miniature-pig semen during *in vitro* storage in order to determine which extender should be used and how extender can be diluted for *in vitro* storage of miniature-pig semen.

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MATERIALS AND METHODS

Semen Collection and Processing

Miniature-pig semen was collected using a gloved-hand technique and filtered through cotton gauze to remove the gel particles. The fresh collected semen was extended with same volume of extenders (Table 1) ; Beltsville Thawing Solution (BTS), Androhep, Modena and Mulberry Ⅲ (VMD, Inc.). After maintenance at room temperature for 20 min, the semen samples were transported to the laboratory at 17℃ within 3 hours. The extended semen was stored at 17℃, and was used for examination of semen characteristics at 1, 3, 5 and 7 days of storage.

Chlorotetracycline Assessment of Spermatozoa

The methods used for chlorotetracycline (CTC) assessment were the same as described by Wang *et al.*, (1995) and Mattioli *et al.*, (1995). Briefly, 4 μL Hoechst 33258 (Sigma, 100 μg/mL in PBS) was mixed 200 μL boar semen and 196 μL PBS. After 3-min incubation at room temperature in the dark, the mixture was layered onto 1 mL of 3% polyvinyl pyrrolidone-40 (PVP-40, Sigma) in PBS (w/v) and centrifuged at 500 × g for 6 min. The pellet was suspended in 200 μL PBS, then mixed with 200 μL CTC solution containing 750 μM CTC, 5 μM cysteine and 130 μM NaCl in 20 mM Tris-HCl buffer (pH 7.8), and incubated for 30 sec in the dark. The stained spermatozoa were fixed by adding 36 μL of 12.5% paraformaldehyde (w/v) in 0.5M Tris-HCl (pH 7.4). The slides were prepared by placing 10~15 μL of the sample on a slide and mounted in the medium with 1,4-diazabicyclo octane. The B (capacitated, acrosome intact), F (uncapacitated, acrosome intact) and AR (acrosome reacted) patterns were examined with filter 2 (D) under a fluorescent microscope as described. Both capacitated and viable spermatozoa were defined as capacitated spermatozoa in this study.

SYBR-14/PI Staining

Both SYBR-14 and PI were obtained from the live/dead sperm viability kit (L-7011, Molecular Probes, Inc., Eugene, OR). The stock solutions were first prepared as 0.02 mM SYBR-14 in DMSO and 2.4 mM PI in D_2O, respectively. The staining solution contained 0.187 micro-mole SYBR-14 and 38.4 micro-mole PI. Fifty microliters of boar semen was incubated with the staining solution at 37℃ for 30 min under light-proof conditions. The stained spermatozoa were then examined woth filter under a fluorescent microscope. Viable spermatozoa show a bright green fluorescence over the sperm head when stained with SYBR-14 (Thomas *et al.*, 1997).

Table 1. Composition of Beltsville thawing solution (BTS), Androhep, Modena and Modified-Modena extenders

Ingredients(g/L)	Extenders			Modified-Modena		
	BTS[1)]	Androhep[2)]	Modena[3)]	A	B	C
Glucose	37.0	26.0	25.0	30.0	30.0	25.0
EDTA	1.25	2.40	2.25	2.25	2.25	2.25
Sodium citrate	6.00	8.00	6.90	6.90	6.90	6.90
Sodium bicarbonate	1.25	1.20	1.00	1.00	1.00	1.00
Potassium chloride	0.75	-	-	-	-	-
Tris	-	-	5.65	4.50	5.00	5.65
HEPES	-	9.00	-	-	-	-
Citric acid	-	-	2.00	2.20	2.50	2.00
Cysteine	-	-	0.05	0.05	0.05	-
Taurine	-	-	-	-	-	6.255
BSA	-	2.50	3.00	3.00	4.00	3.00
Gentamicin sulfate	0.30	0.30	0.30	0.30	0.30	0.30
pH	7.00	6.80	7.80	7.00	7.00	7.80

[1)] Beltsville Thawing Solution(BTS) : Pursel and Johnson(1975), Johnson and Garner (1984).
[2)] Androhep : Weitze (1990).
[3)] Modena : Moretti (1981).

Semen Evaluation

Sperm characteristics of boar semen stored at 17℃ was evaluated at days 1, 3, 5 and 7 of storage, respectively. For examination, semen volumes were determined by an electron weighing beam. Sperm concentrations were estimated by a hemocytometer. Sperm viability was determined using by Makler Counting Chamber. Sperm abnormality was determined by Rose-Bengal staining methods. Sperm acrosome intactness was determines using by Coomassie Briliant Blue staining. Sperm intensity was determines by hypoosmotic swelling test (Neild *et al.*, 1999).

Statistical Analysis

Data are presented as mean ± standard deviation (S.D). Statistical analysis were conducted using SAS Version 8.01 for Windows. Statistical significance was regarded when $p<0.05$. Multiple comparisons were made with the protected Fisher's least significant difference (LSD) test. The Duncan's multiple range test was used to determine the significance of difference between the mean.

RESULTS

Sperm motility during storage in extenders is shown in Table 2. Sperm motility decreased with storage and differences Beltsville Thawing Solution (BTS), Androhep, Modena and Mulberry Ⅲ were apparent. On Day 1, approximately 80% of the sperm were motile, but motility decreased to 40% at Day 7. Motility did differ significantly between BTS and Mulberry Ⅲ during on 3 day of storage.

Sperm viability were assessed by Hoechst 33258 and SYBR-14/PI staining Hoechst 33258 and SYBR-14/PI were

Table 2. Spermatozoa motility in different extenders during *in vitro* storage*

Spermatozoa motility (%) in different extenders	Storage periods (days)			
	1	3	5	7
BTS	80±6.1	65±3.5[a]	47±4.4	37±6.7
Androhep	83±4.4	67±5.7[ab]	49±2.2	36±5.4
Modena	85±3.5	70±3.5[ab]	54±4.1	41±7.1
Mulberry Ⅲ	87±1.5	76±4.1[b]	55±5.0	44±5.4

* At the least three times sperm motility were evaluated for each groups.
[a,b] Values with different superscripts within the same column are significantly different ($p<0.05$).

Table 3. Viability of spermatozoa assessed by Hoechst 33258 and SYBR-14/PI staining in miniature-pig semen extended with different extenders during *in vitro* storage*

Periods of storage (days)	Methods assessed	Sperm viability (%) in semen extender with :·			
		BTS	Androhep	Modena	Mulberry Ⅲ
1	Hoechst 33258	85.8±1.5	85.4±1.6	87.6±0.8	88.1±1.2
	SYBR-14/PI	81.6±1.8	81.6±1.0	84.3±1.3	85.0±2.2
3	Hoechst 33258	78.9±0.9	80.7±2.3	81.6±1.5	80.1±1.3
	SYBR-14/PI	75.7±1.0	76.1±1.0	76.3±0.7	77.1±1.6
5	Hoechst 33258	59.5±3.2[†]	68.9±2.1	70.8±3.9	70.0±4.1
	SYBR-14/PI	54.9±1.7[†]	66.0±0.8	70.0±0.8	66.0±1.4
7	Hoechst 33258	48.9±2.6[†]	58.0±1.4	60.4±2.0	59.4±1.7
	SYBR-14/PI	43.6±2.1[†]	54.5±2.8	59.9±1.0	57.0±1.7

* At the least three times sperm ability were evaluated for each groups.
[†] Values with different superscripts within the same row are significantly different ($p<0.05$).
The each row data was evaluated among different extenders.

used to evaluate the viability of miniature-pig semen extended with four different extenders during storage. As shown in Table 3, spermatozoa viability were higher in Hoechst 33258 method than in SYBR-14/PI method during the sperm storage with different extenders. However, the sperm viability of semen extended in BTS extender was lower during on day 3 or later compared to the other three extenders. Viability decreased obviously for all four extenders. Sperm viability was 48.9 % for BTS extender, 58.0% for Androhep extender, 60.4 % for Modena extender and 59.4% for Mulberry III extender on day 7 of storage.

In evaluation of the viability by SYBR-14/PI staining, the viability in BTS extender was 81.6%, while the viability in the other extenders was high on day 1 of storage. The viability remained over 70% at 3 days for all three extenders. Viability then decreased obviously for all four extenders. Sperm viability was 43.6% for BTS extender, 54.5% for Androhep extender, 59.9% for Modena extender and 57.0% for Mulberry III extender on day 7 of storage. Sperm viability in BTS extender was significantly ($p<0.05$) low than in another extenders within 5 or 7 days of storage periods. The rank order of four extenders for maintaining viability was as fo-

Table 4. Sperm characteristics of miniature-pig semen at 1, 3, 5 and 7 days of storage in modified Modena*

Item assessed (%)	Storage periods(days)	Semen extender			
		Modena	Modified A	Modified B	Modified C
Motility	1	90.2±1.5	88.3±2.4	92.1±1.3	90.3±2.2
	3	83.4±2.2	78.3±2.0	85.3±3.7	80.1±0.8
	5	70.5±3.4	65.2±3.8	75.2±2.5	70.0±1.3
	7	60.5±1.8	55.4±0.8	60.5±4.1	60.4±3.5
Survival	1	90.5±1.2	91.4±1.5	94.2±0.8	91.2±1.6
	3	83.2±0.8	82.5±0.4	86.3±0.2	83.1±0.9
	5	73.3±0.8	74.3±1.3	76.6±0.6	71.5±0.3
	7	64.2±0.9	65.0±1.2	67.4±1.4	63.6±1.3
Viability	1	85.2±0.8	80.4±1.1	89.1±1.4	83.6±1.4
	3	64.4±1.8	65.2±0.9	77.3±1.2†	64.2±1.6
	5	49.2±0.9^{ab}	50.6±1.5^{ab}	56.5±0.5^{a}	44.4±0.8^{b}
	7	42.3±1.2	41.5±1.5	45.1±0.7	40.2±0.4
Abnormality	1	7.2±0.4	8.4±0.2	5.1±0.5	8.4±0.4
	3	10.3±0.6	11.0±0.5	10.2±0.6	13.6±0.4
	5	14.1±0.2	16.3±0.4	15.6±1.2	19.5±0.8
	7	18.0±1.2	20.1±0.4	19.2±0.4	22.1±1.2
Intactness	1	81.6±0.5	78.1±0.2	83.1±0.8	80.0±0.4
	3	73.2±0.2	70.0±0.6	73.6±0.2	72.5±0.6
	5	67.4±1.2	62.4±0.2	64.4±0.5	62.4±0.2
	7	58.3±0.7	55.2±1.4	56.3±0.4	51.2±1.0
Intensity	1	82.2±0.4	83.1±0.2	86.4±0.7	82.3±0.4
	3	70.1±0.6	71.6±0.5	72.2±0.2	74.0±1.2
	5	62.5±0.4	62.1±1.3	61.7±1.5	63.1±0.4
	7	53.2±1.2	51.2±0.7	50.4±0.6	52.6±0.8

* At the least three times sperm characteristics were evaluated for each groups.

† Values with different superscripts within the same row are significantly different ($p<0.05$).

Table 5. The CTC patterns of miniature-pig spermatozoa extended with different extenders*

CTC patterns	Storage periods (days)	No. of spermatozoa examined	Spermatozoa (%) in different extenders			
			Modena	Modified A	Modified B	Modified C
F	1	100	82	81	85	84
	3	100	75	72	78	76
	5	100	72	69	72	71
	7	100	64	65	65	62
B	1	100	10	10	7	8
	3	100	15	14	10	12
	5	100	17	16	14	16
	7	100	19	19	18	22
AR	1	100	8	9	8	8
	3	100	10	14	12	12
	5	100	11	15	14	13
	7	100	17	16	17	16

* At the least three times spermatozoa were evaluated for each groups.

llows: BTS, Androhep, Modena and Mulberry Ⅲ.

As shown in Table 4, sperm characteristics is evaluated with 4 extenders. The motility, survival, viability, intensity and intactness miniature-pig sperm decreased with periods prolonged in Modena, modified-Modena A, modified B and modified C. Sprm viability in modified-Modena B extender was significantly ($p<0.05$) higher than in another extenders within 3 or 5 days of storage periods. However, there was not differences significantly within the same storage periods except viability in 3 or 5 days of sperm storage. On the other hand, the abnormality of spermatozoa increased with storage periods. However, there was not differences significantly within same storage periods storage among the extenders.

Capacitation and acrosome reaction were determined with chlorotetracycline (CTC) patterns by CTC/Hoechst 33258 staining. As shown inn Table 5, the proportions of F pattern decreased with storage periods prolonged, but B and AR patterns increased with storage periods in all extenders.

DISCUSSION

The purpose of this study was to assess sperm quality in extended miniature-pig semen during *in vitro* storage in order to determine which extender should be used and how extender can be diluted for *in vitro* storage of miniature-pig. Sperm quality of semen stored at 17℃ was evaluated at 1, 3, 5 and 7 of days after storage, respectively.

This study indicated the sperm viability and motility during periods of storage that semen extender was used BTS, Androhep, Modena and Mulberry Ⅲ. Mulberry Ⅲ extender maintained better motility than BTS, Androhep and Modena extenders essentially throughout storage. It was suggested that main difference between BTS, Androhep, Modena and Mulberry Ⅲ is the putative presence of ingredients in Mulberry Ⅲ. however, we did not knew what ingredients of Mulberry Ⅲ extender. So, we bought it. Selection of semen extender was using Modena extender. Modified-modena extender was using base for develop of new extender.

In this study, the viability of miniature-pig spermatozoa was assessed with Hoechst 33258 and SYBR-14/PI staining. The results from Hoechst 33258 and SYBR-14/PI staining were assessed similar to viability. However, Hoechst 33258 staining was higher than that from SYBR-14/PI. Assay of sperm viability did not differ significantly hoechst 33258 and SYBR-14/PI staining.

The another experiment assessed the sperm characteristics during periods of storage that semen extender was used Modena and modified-Modena. Modena and modified-Modena were made from composition of Moretti (1981) reported and are proprietary products. The exact chemical composition of the extender is therefore unknown. However, Weitze (1991) reported the following recipes : for Modena (in g/L) 25.0 glucose, 2.25 EDTA, 6.90 sodium citrate, 1.00 sodium bicarbonate,

5.65 tris, 2.00 citric acid, 0.05 cysteine, 3.00 BSA and 0.30 gentamicin sulfate ; for modified-Modena A (in g/L) 30.0 glucose, 4.50 tris and 2.20 citric acid ; for modified-Modena B (in g/L) 30.0 glucose, 5.00 tris, 2.50 citric acid and 4.00 BSA ; for modified-Modena C (in g/L) 6.255 taurine 50mM. In this study, the chemical composition of the extender is similar with Zorlesco extender. Weitze (1991) found that BSA in both Androhep and Zorlesco extenders could selectively bid to the plasma membrane surrounding th mid-piece and principal piece of boar spermatozoa. It has been shown that BSA can neutralize the metabolic byproducts from spermatozoa and bacteria, and may have antiperoxidative activity. In present study, the chemical composition of BSA was treated : modified-Modena B (in g/L) 4.00 BSA. Additionally, Zorlesco extender was the Tris-based extender (Roca *et al.*, 2000). Tris-buffered extenders have been shown to be effective for preserving viability and fertilizing capability of rabbit spermatozoa stored at 15℃. So, the chemical composition of Tris was treated : modified-Modena A (in g/L) 4.50 Tris ; modified-Modena B (in g/L) 5.00 Tris.

There were differences in maintaining sperm viability, motility, abnormality, acrosome intactness, intensity and CTC patterns. The rank order of four extenders for maintaining sperm characteristics was as follows : Modena, modified-Modena A, modified-Modena B and modified-Modena C. However, the percentage of sperm viability, sperm motility, sperm abnormality, acrosome-intact spermatozoa, sperm intensity in semen extended with Modena, modified-Modena A and modified-Modena C extenders were similar, and were lower than that for semen extended with modified-Modena B extender. However, did not differ significantly among other extenders.

In the CTC fluorescence assay, the proportion of F pattern sperm decreased with time of storage as the B pattern sperm increased, however, it is difficult to discuss which extender better preserved the sperm. In the present study, the CTC patterns was similar among the experimental groups. Vredenburgh-Wilberg and Parrish (1995) reported that the intracellular pH of bovine sperm increased during capacitation. The increase in B pattern during sperm storage could be explained by variations in sperm pH that would induce on-regulated capacitation-like modifications. However, this was not evaluated in the present study. Modena extender and modified-Modena C extenders are pH 7.8, but, modified-Modena A and modified-Modena B are pH 7.0 in this study.

In conclusions, characteristics of semen extended did not remakable differences among the extenders. However, modified-Modena B can choose as extender for liquid storage of miniature-pig semen and artificial in semination. So, further research is necessary to develop of semen extender and freezing methods to improve sperm ability in miniature-pig.

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