Correlations between Sperm Motility, SCSA (Sperm Chromatin Structure Assay), Reproductive Performance and Heterospermic Fertility in Boars

In Cheul Kim[1], Jae Weon Ryu[1,†], Kyu Ho Cho[1], Joon-Ki Hong[1], Eun Ji Choi[1], Bong-Hwan Choi[1], Jun Cheol Park[1], Hong-Kil Moon[1] and Jung-Ho Son[2]

[1]*National Institute of Animal Science, RDA, Cheonan 330-801, Korea*

[2]*Noah Biotech Inc., Cheonan 330-714, Korea*

ABSTRACT

The objective of this study was two folds: to investigate the relationship between paternal identification rate and sperm quality parameters such as motility and sperm chromatin structure assay after heterospermic insemination; to see if mutual complement between tests and development of useful technique to enhance the fertility in artificial insemination. In individual boar's fertilizing ability, 3 high fertility boars showed significantly high fertility (p<0.05) compared to 3 low fertility boars, but there was no difference in litter size between two groups. Sperm motility test in pooled and individual semen using computer assisted sperm analysis (CASA) revealed that no significant difference among boars. The high fertile boar showed tendency of low %Red (High red fluorescence/green+red fluorescence) in sperm chromatin structure assay (SCSA) but paternal identification rate from piglets did not differ after heterospermic insemination. The correlation coefficient between individual or pooled semen function test and farrowing rates were well correlated as follows: %Red with litter size (r=−0.53, p=0.03); %Red with paternal identification rates (r=−0.51, p=0.03); paternal identification rates with litter size (r=0.57, p=0.02). These results indicate that sperm chromatin structure assay and sperm quality parameter test in pooled semen are useful method to predict and evaluate the fertilizing capacity after heterospermic insemination in boars.

(Key words : Heterospermic insemination, Artificial insemination, Boar, Fertility)

INTRODUCTION

As artificial insemination (AI) has been widely practiced in the world, AI in swine has increased the importance of determining fertility of a semen sample before it is used. Semen volume, sperm concentration, motility, viability and morphological abnormality examination are generally accessed for semen quality test for AI. Although general sperm quality test described above could detect extremely poor fertilizing capacity of semen, it is not enough for differentiating the subtle difference for sub-fertile (Woelders, 1991). The analysis of conception rates, farrowing rates and litter size through AI are useful method to access detail information for evaluating the fertilizing capacity (Galli *et al.*, 1988; Hammitt *et al.*, 1989; Gadea *et al.*, 2000). However, fertility evaluation through AI takes many hours and financial burden and misleading could happen by herd management type, raising environment and operator's skill and therefore discrepancy would exist between fertility and sperm function test.

In pig, heterospermic insemination is very useful technique for evaluating boar semen fertility. Fertility evaluation after heterospermic insemination is considered to be highly reliable method for minimizing the variation of each individual recipient's status, season, herd management and AI skill. In this study, we conducted the experiment to determine the correlation among sperm motility, sperm chromatin structure assay (SCSA), reproductive performance results from individual semen AI and paternal identification rates from heterospermic insemination with the hope to see if mutual complement between tests and development of useful technique to enhance the fertility in AI.

MATERIALS AND METHODS

Animals

Six duroc boars raised in National Institute of Animal Science were assigned by two groups; 3 high and 3 low fertility based on their farrowing rates from individual semen AI. Thirty two recipient sows were used for heterospermic AI. Boars and sows were fed

† Corresponding author : Phone: +82-41-580-3450, E-mail: ryujj@rda.go.kr

according to the guide line of National Institute of Animal Science.

Semen Collection, Preparation and Artificial Insemination

Sperm rich-fraction from each ejaculate was collected by gloved hand technique. Immediately following collection, semen was delivered to the lab and sperm motility was evaluated by computer assisted sperm analysis (CASA). Ejaculated semen from six boars were pooled and the mixture contained equal numbers of motile sperm from each boar (5×10^8 motile sperm per boar). Pooled semen was added to Beltsville Thawing Solution to produce AI doses of 30×10^8 motile sperm in 100 ml of extended semen. Aliquots of 2 ml semen were preserved in liquid nitrogen tank for the analysis of sperm chromatic structure assay.

For estrus synchronization, sows were received 5 ml of PG 600 (400 IU PMSG + 200 IU HCG, Intervet, Holland) 24 hr after weaning and 80 hr later 50 μg of GnRH (Gonadon, Dongbang Korea) was treated. Estrus was detected 2 times (8 AM & 5 PM) everyday and sows were inseminated twice (12 hr interval) at 24 hr after showed standing estrus.

Analysis of Sperm Motility

Semen samples were stored at 17℃ for 4 days. The sperm movement characteristics were measured using CASA system for 4 times in 24 hr interval. CASA was determined as Zeng *et al.* (2001). In brief, 1.5 ml semen sample was incubated for 30 min at 37℃ water bath and 10 μl of semen were applied to pre-warmed Makler counting chamber (Sefi-Medical, Israel) and were analyzed by SAIS II system (Medical Supply Co. Ltd., Korea) connected with CCD camera (Toshiba, Japan) on microscope (Olympus, Japan) with a warm plate (37℃).

Sperm Chromatin Structure Assay (SCSA)

Frozen semen samples were thawed at 37℃ for 15 sec and diluted PBS. An aliquot of thawed semen were centrifuged and were diluted to a concentration of 1×10^6/ml. The SCSA was performed according to the procedure described by Evenson (1990). Sperm were analyzed by FACS Aria (Becton Dickinson, San Jose, CA, USA) flowcytometer. After passing a 560 nM short pass filter, the green fluorescence was collected through 525 band pass filter. The red fluorescence passed through a 640 nM long pass filter and then collected through 675 long pass filter. Before recording the data, debris was removed and percentage of green- (double stranded DNA) and red-fluorescence (single stranded DNA) was recorded as described by Bochenek *et al.* (2001).

Parental Identification of Piglets

Genomic DNA Isolation

DNA was extracted from 10 ml of blood from six boars, twenty one sows and 179 offspring using Wizard genomic DNA purification kit (Promega Co. USA). For whole blood cell extraction, 30 ml of cell lyses solution was added for 10 min to lysis the red blood cells. White blood cells were collected by centrifugation at 2,000 × g for 10 min and resuspended with 10 ml of nuclei lysis solution. After nuclei lysis, 20 μg/ml of RNase A was treated at 37℃ for 15 min and vortexed vigorously for 20 sec after adding 3.3 ml of protein precipitation solution. Cell lysis were purified by centrifugation at 2,000 × g for 10 min and DNA was precipitated by isopropanol and washed dry by 70% ethanol and were stored in TE buffer (pH 8.0) for PCR application.

Table 1. Microsatellite markers used for paternal analyses of piglets after heterospermic insemination

MS marker	Chromosome	Locus	Size	No. of Allele	T_m[a]	Forward	Reverse
S0112	1	121.3	140~165	7	55	AATCCTGAGTATCCTTAATCAGGC	TTGACATGATGCAGAGAAGGAGTC
S0301	4	27.1	252~268	7	55	CCGTCTTACTTAGGATGTTT	TGATGTGTTTATGTGTTTGA
SW707	X	108.6	0~101	5	55	ACGTGCTTTTCTTTGAGCTG	AAAAACGCTAAAGAACAAAGCG
SW1495	13	58	146~168	8	58	AGACGCTGCTTGGTGTTAGG	TGGGTCTGTATCCCTGAAGG
SW607	6	165.7	152~172	3	58	AGCACCTGGCACAGGATAAC	GCAAGAACTGGTTTTCCAGC
SW1680	6	153.9	118~158	10	65	AGCCACCTAAATGTCCATCG	CATGTTGCTGCTAGTGGCAG
s0002	3	102.2	190~216	5	62	GAAGCCCAAAGAGACAACTGC	GTTCTTTACCCACTGAGCCA
sw1426	X	71.7	90~96	5	60	TGGTTGTCACAGTTTATTGGG	TCCCTATCTTCTAAATGCTAGTAGG
sw2406	6	21.4	220~256	7	58	AATGTCACCTTTAAGACGTGGG	AATGCGAAACTCCTGAATTAGC

[a] Annealing temperature (℃).

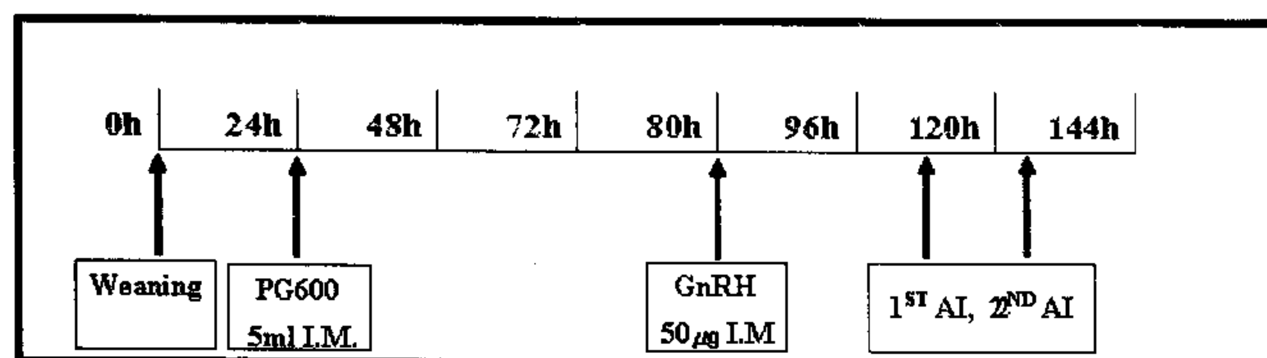

Fig. 1. Synchronization methods of estrus followed artificial insemination in sows.

Selection of Microsatellite Marker

Microsatellite marker for pig genotyping was provided by U.S Pig Genome Coordinator (Max F. Rothschild, Table 1).

PCR

For PCR amplification, reaction mixture (total of 10 μl) was consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM dNTPs, 3 pmol fluorescent dye labeling primer pairs, 10 ng of template DNA and 0.5 U Taq DNA polymerase (TaKaRa Shuzo Co., Shiga, Japan). For PCR amplication, the reactions were heated for 5 min at 94℃, denaturation at 94℃ for 30 sec, annealing at 55~65℃ for 40 sec and extension at 72℃ for 1 min (35 cycles) with a final extension step of 10 min at 72℃. Electrophoresis was performed in 2% gel stained with EtBr to determine proper PCR condition and to see if allele is in the proper range under UV illuminator.

Genotyping of Parents and Piglets

PCR products were diluted with distilled water and mixed with DNA, formamide and standard (Genescan-350 TAMRA) as the ratio of 1 μl: 12 μl: 0.5 μl volumes. After mixing, PCR product was denatured at 95℃ for 3 min and analyzed by ABI 310 Analyzer (Applied Biosystems, USA). The volume and size of DNA fragmentations were collected by GeneScan software (Applied Biosystems, USA, version 2.1). For electrophoresis, performance optimized polymer 4 (POP4) (Applied Biosystems, USA) and 10× buffer with EDTA were mixed 1:1 ratio and run for 22 min. Genotyping was conducted using software (Applied Biosystems, USA, version 2.5).

Statistical Analysis

Analysis of variance using GLM procedures of SAS (SAS Inst. Inc., Cary, NC) was used to rank the boars on the number of offspring in a litter sired by either boar. Least squares means were calculated for all semen characteristics assessed in the ejaculates from each boar. The correlations between boar fertility, individual parameters and paternal identification rates were calculated using the SAS package.

RESULTS

Fertilizing capacity was higher ($p<0.05$) in 3-high fertility group (56.5%) compared to 3-low fertility group (41.5%). Sperm motility evaluated by CASA among boars did not differ between high- and low-fertility groups based on farrowing rates (Table 3). Sperm motility on elapsed time after preservation was not difference between two groups (Table 4). % Red as de-

Table 2. Comparison of semen characteristics and reproductive performance between 2 boar groups with different farrowing rates

Fertility groups	Boars	Semen characteristics			Farrowing			
		Volume (ml)	Concentration (×10⁸)	Motility (%)	No. of head	Rate (%)	Litters	
							Total	Alive
Low	A	175.0	4.0	95.4	5	38.5	10.4	9.4
	B	185.0	5.6	92.1	5	41.7	9.0	6.6
	C	150.0	3.8	89.1	8	44.4	8.1	7.4
	Mean	170.0	4.5	92.2^a	6.7	41.5^b	9.2	7.8
High	D	160.0	5.8	93.5	9	69.2	9.7	8.4
	E	95.0	5.0	90.2	9	56.3	8.7	7.7
	F	150.0	6.0	94.0	7	58.3	10.3	8.9
	Mean	135.0	5.6	92.6^a	8.3	61.3^a	9.6	8.3
Pooled semen		-	-	91.6	21	100	8.5	7.1

^{a,b} Means with different superscript in the column were significantly differ($p<0.05$).

Table 3. Comparison of sperm movement characteristics between 2 boar groups with different farrowing rates

Fertility groups	Boars	Motility (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	ALH (µm)	STR (%)
Low	A	89.8	212.7	87.9	112.7	41.3	9.0	77.9
	B	88.8	206.0	80.9	101.9	39.7	8.1	79.5
	C	81.0	216.0	90.1	109.4	41.6	8.6	81.9
High	D	89.4	196.5	74.2	94.7	37.8	7.8	78.3
	E	92.1	217.8	87.2	111.5	39.9	9.1	78.3
	F	91.1	204.9	87.6	106.1	42.9	8.5	82.6

[1] Means.

Table 4. Comparison of sperm motility among 3 boar groups with different farrowing rates according to days of semen storage

Fertility groups	Motility (%)[1]			
	Day 0	Day 1	Day 2	Day 3
Low	94.9±0.9	89.5±1.3	87.6±1.6	86.5±1.8
High	94.2±0.9	88.9±1.3	85.3±1.6	83.6±1.8
Pooled semen	91.6±1.5	91.5±2.2	87.9±2.7	86.4±3.2

[1] Means±SE.

Table 5. Comparison of SCSA parameters among 3 boar groups with different farrowing rates

Fertility groups	%Red[2]
Low	3.4±0.2[1]
High	2.5±0.2
Pooled semen	2.9±0.3

[1] Means±SE.
[2] Sperm DNA fragmentation index as determined by High red fluorescence/green + red (fluorescence).

termined by sperm chromatin structure assay in high- and low-fertility groups, or pooled semen were 2.5, 3.5 and 2.9, respectively. High fertility group showed a tendency of less DNA fragmentation rates but there was no statistical difference (Table 5). Paternal identification rates after heterospermic insemination were not difference between high and low fertility groups (51.0 vs 49.0%, Table 6). Correlations between fertilizing capacity after AI using individual or pooled semen and sperm function test was correlated with % Red and litter size ($r=-0.53$, $p=0.033$), % Red and paternal identification rates ($r=-0.51$, $p=0.031$) and litter size and paternal identification rates ($r=0.57$, $p=0.026$).

Table 6. Results of paternal identification rates of piglets after heterospermic insemination

Fertility groups	Boars	Paternal identification	
		No. of piglets	Rate (%)
Low	A	42	31.9±3.9
	B	19	12.2±3.9
	C	13	7.4±3.9
	Total	74	51.0
High	D	36	23.9±3.9
	E	12	9.5±3.9
	F	23	15.1±3.9
	Total	71	49.0

[a,b] Means±SE.

DISCUSSION

Fertilization is very complicated mechanism by which sperm and egg undergoes serial process. After sperm deposit into cervix, sperm swim to oviductal am-

Table 7. Correlation coefficients between motility, %Red, reproductive performance after individual semen AI and paternal identification rates according to heterospermic AI results in pig

	Motility	%Red	Farrowing rate	Litter size	Paternal identification rates
1	-	0.06	−0.14	−0.03	0.38
2		-	0.03	−0.53*	−0.51**
3			-	−0.11	0.10
4				-	0.57***
5					-

* p=0.033.
** p=0.031.
*** p= 0.026.
1 Motility, 2 % Red, 3 Farrowing rate, 4 Litter size, 5 Paternal identification rates.

pulla where sperm can encounter with egg and penetrates zona pellucida result in male pronucleus formation and thereafter zygote is developed by fusion with female pronucleus.

To evaluate the sub-fertility, proper evaluation method for sperm and egg is needed at fertilization step (Amann, 1989). In this study, we investigate the correlations among farrowing rates after individual semen AI, sperm motility, SCSA and paternal identification rates after heterospermic insemination.

Farrowing rates after individual semen AI was significantly different between two groups but there was no difference in litter size. Data obtained from this study agreed with Gadea *et al.* (1998) and Clark *et al.* (1989)'s report that sperm motility and viability test based on farrowing rates can differentiate the fertility difference between high- and low-fertility group, but it was not enough to recognize the difference between high- and sub-fertility group. The most effective evaluation method for boar semen fertility is detecting the pregnancy after insemination and recording the offspring at delivery but in reality, delivery record was not clear in many aspects so new method is required for detecting infertility and low fertilizing capacity sperm (Hammerstedt, 1996). ·

Relationship between sperm motility and fertility has variation and depends on experimental conditions. To eliminate those disadvantages, CASA was developed for more objective evaluation of sperm motility and their usefulness has been recognized from many domestic animals.

In this study, individual and pooled semen motility on elapsed time after preservation was not different in high- and low- fertility groups. This result is similar as Gadea's report (1998) that sperm motility analysis from 3 fertility groups, high (>80%), medium (60~80 %) and low (<20%), was useful in evaluating the poor fertility group but not in high- and sub-fertility groups.

Sperm motility test is depends on ejaculation situation and was affected by many factors so it is not suitable method for evaluating the boar's fertilizing capacity (Trish, 2001). Therefore new reliable method is required for sperm function test for boar's fertility and analysis of factors that could affect sperm parameter change.

Fertility is also predicted by hypo-osmotic swelling test (HOST, Pérez-Llano *et al.*, 2001), *in vitro* penetration test (IVP, Gadea *et al.*, 1998) and sperm acrosome status. These tests are better than general sperm function test (Waberski *et al.*, 2005), but microscopic analysis can measure limited numbers of sperm within a population and can be subjective. To minimize the variation and to detect wide spread of sperm quality, flowcytometer has been adopted. Flowcytometer can measure many number of spermatozoa (20,000~30,000/sample) accurately and rapidly. Sperm viability (Garner *et al.*, 1995; Nagy *et al.*, 2003), mitochondrial membrane potential (Evenson *et al.*, 1982; Petit *et al.*, 1995; Thomas *et al.*, 1998; Boilard *et al.*, 2002; Marchetti *et al.*, 2002), acrosome status (D'Cruz *et al.*, 1996; Carver-Ward *et al.*, 1997) and sperm chromatin structure assay (Ward *et al.*, 1990; Boissenault, 2002) can be assessed by flowcytometer.

The sperm DNA denaturation status can be objectively and precisely determined by SCSA in frozen stored samples (Evenson *et al.*, 2000). SCSA is used for determining of human (Evenson *et al.*, 1999), bull (Bochenek *et al.*, 2001), horse (Love *et al.*, 2005, 2002) and boar's fertility (Gillan *et al.*, 2005). Sperm chromatin structural changes could attribute by spermatogenesis process (Karabinus *et al.*, 1991), by deficiency of chromatin condensation (Evenson *et al.*, 2000) or by environmental factors during sperm preservation after ejaculation (Karabinus *et al.*, 1997). On the other hand, SCSA data on semen collection frequency show many variations in low fertile animal (Larson, 2000).

In this study, DNA fragmentation rates determined by SCSA were low in high fertility boar group but there was no statistical difference. Correlation analysis between sperm function test on fertilizing ability and sperm DNA integrity after individual or heterospermic insemination results showed negative correlation as fellows: DNA fragmentation rates with litter size from individual semen; litter size from individual semen insemination with paternity rates from heterospermic insemination; DNA fragmentation rates with paternity rates from heterospermic insemination, respectively. These results are similar as Collins's (2008) report of positive correlation between litter size from individual semen insemination with paternal identification rates from heterospermic insemination and fertility from individual semen insemination with litter size after heterospermic insemination. Hammmitt *et al.* (1989) report that individual boar paternal identification rates were significantly higher than heterospermic AI from two boars.

Sperm acrosome status and fertility after heterospermic insemination was not related (Stahlberg, 2000). Sperm motility and morphological abnormality from 2 individual samples were not different from general semen quality test. In accordance with Stahlberg's (2000) finding, conception rates were not different between two semen samples but paternal identification rates were significantly different after heterospermic insemination. Litter size after individual semen insemination and paternal identification rate after heterospermic insemination was all negatively related. Data obtained from this study showed similar result of Evenson *et al.* (2000) as sperm DNA fragmentation rate and fertilizing capacity is well-correlated. Other investigators also mentioned that sperm DNA integrity and fertility has been shown to correlate (Schmid *et al.*, 2003; Bielecki *et al.*, 2001; Saleh *et al.*, 2002).

In conclusion, sperm chromatin structure assay would be very useful method for accessing to predict the sperm quality after heterospermic insemination in boars. Moreover, for successful fertilization, sperm requires to complete a number of steps. Other variables not assessed may have affects on sperm fertility, such as sperm transport, acrosome reaction and the ability to fuse with the oocyte. A more complete assessment of fertility potential may require a large battery of tests. It will be important to determine which tests are most efficacious as predictors of sperm fertility.

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(Received; 4 June 2008/ Accepted: 12 June 2008)