Relationship between Stress Gene Polymorphisms and Litter Size by AI in Pigs

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ABSTRACT

This study was performed to investigate the relationship between PSS-HSP70 gene polymorphism and artificial insemination (AI) reproductivity in the pigs. The RFLP polymorphism of PSS and the SSCP polymorphisms of HSP70 K1, K3 and K4 PCR product were detected different patterns. In the experiment for AI of fresh semen, spring and fall season showed higher litter size born of 10.89 head than 10.47 head of summer season. Landrace was showed higher litter size of 9.96 head than that of Duroc and Yorkshire($p<0.05$). Stress relating PSS and HSP70 polymorphism of PSS-Normal, HSP70 K1-BB, K3-AB, K4-AA showd a highest litter size born of 10.97 head and litter size born alive of 10.69 head than that of the other polymorphisms($p<0.05$). In the experiment for AI of frozen semen, effects of season and pig breeds were not showed for litter size born. The stress relating polymorphism of PSS-Carrier, HSP70 K1-BB, K3-BB, K4-AB showed highest litter size born of 11.29 head and litter size born alive of 10.82 head and PSS-Normal, HSP70 K1-BB, K3-AB, K4-AA showed the lowest litter size born of 8.48 head and litter size born alive of 7.33 head than that of the other polymorphisms($p<0.05$). These results suggest that AI litter size born for the stress of forzen thawed semen may be affected by PSS and HSP70 polymorphism in pigs.

(Key words : HSP70 gene, PSS, AI, pig)

INTRODUCTION

The temperature increase of testis reduces the spermatogenesis in most of mammals (Chowdbury and Steinberger, 1964; Vandemark and Free, 1970). The phenomenon indicates that the formation of sperm cells is highly sensitive to heat stress. Although the amount of sperm and weight did not change under heat stress, the sperm motility and the normal sperm ratio have been reduced with the increase of abnormal and aged acrosome sperm proportion (Christenson *et al.*, 1972; Malmgren and Larsson, 1984; Wettemann *et al.*, 1976).

The heat stressed sires reduced the pregnancy rate of the dams when natural mating and artificial insemination (Wettemann *et al.*, 1976; Wettemann and Desjardins, 1979), and the growth of sperm cells was suppressed, which consequently reduced the numbers of ejaculated sperms and the insemination capacity (Wettemann and Desjardins, 1979).

In pigs, the PSS is the genetic disease causing muscle cramp, respiratory disorder, malignant hyperthermia, sudden death, production of low grade pork and economical losses by the high sensitivity of pigs to surrounding environmental factors of high temperature, small area breeding and slaughterhouse transportation. The major genetic cause of PSS was reported as the nucleotide point mutation in the ryanodine receptor 1(RYR1) of 6 chromosome (Fujii *et al.*, 1991). The SNP of RYR1 gene related to PSS is the substitution of Arginine with Cysteine in 1843th cDNA in Exon 17 functional region which controls the Ca^{2+} ion transport from inside of sarcoplasmic reticulum of skeletal muscle and the transition of the 18,618th coding sequence DNA from cytosine base to thymine base.

Single cell or multi cell tissue commonly respond to heat or other types of stresses by increasing or decreasing the synthesis of specific protein group that is regarded as HSPs (Lindquist, 1986; Lindquist and Craig, 1988; Welch, 1992). Especially, the HSP70 with 70kDa of molecular weight is fully expressed in several tissues and confirmed to have an important role in

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acquiring the resistance to exterior high temperature (Li and Laszlo, 1985; Lindquist, 1986; Nover, 1991; Sanchez *et al.*, 1992; Sanchez and Lindquist, 1990; Subjeck와 Shyy, 1986), and it was also confirmed to act as a biomarker of cellular temperature resistance (Craig and Gross, 1991; Leung *et al.*, 1996).

HSPs may contribute to mucosal protection and ulcer healing by regulating the activity of enzymes such as cyclooxygenase and nitric oxide synthase (Rokutan, 2000; Tsukimi and Okabe, 2001), as well as by increasing mucosal blood flow (Shichijo *et al.*, 2003).

The HSP 70 gene of pigs was classified as the swine major histocompatibility complex (MHC) class Ⅲ and present in 7th chromosome (Rothschild and Ruvinsky, 1998).

Sarge (1995) showed that the expression of HSP 70 in reproductive cells of male was initiated at lower temperature in *in-vitro* incubation than the temperature of somatic cells. This suggests that the reproductive cells of male respond more sensitively to heat than somatic cells.

The heat stress of sires during summer time was reported to decrease the sperm quality (Cheng and Wung, 1974; Kennedy and Wilkins, 1984; Koh *et al.*, 1976; Kuo *et al.*, 1997; Liu *et al.*, 1994), and the report was made that the sperm quality of sires during the summer time in sub-tropical region of Taiwan generally decreased, which consequently reduced the reproductivity (Cheng and Wung, 1974; Liu *et al.*, 1994; Kuo *et al.*, 1997).

So, screen of stress related gene polymorphism are required to improve the reproductivity. The present was conducted to reveal the effect of stress related PSS and HSP 70 gene mutagenic genotype of sires on the artificial insemination conception rate and the off spring size of liquid and frozen semen.

MATERIALS AND METHODS

1. Animal and Genotype

Forty three heads of sire were experimented from the National Institute of Animal Science (NIAS) and inseminated semen to Landrace×Yorkshire crossbred of dams at pig farm that were bred from 1997 to 2000.

Genomic DNA was isolated from both whole blood samples, and purified according to the method of Sambrook *et al.* (1989). The isolated DNA was diluted to a final concentration of 50 ng/ml.

Detection of PSS were analyzed according to PCR-RFLP method of Jin *et al.* (2006). Polymorphism of HSP70 gene screened according to PCR-SSCP method of Jin *et al.* (2005).

2. Preparation of Liquid Semen

As the preserving solution, Beltsville Thawing Solution (BTS) was basically used to slow conduct 2～3 times of dilution of collected semen at constant temperature to reach the sperm concentration of 3.0×10^9/80 ml. The diluted semen was dispensed into 100 ml of plastic bottles to be used by storing at the 17℃ adjusted exclusive liquid semen container for pigs. The sperm concentration was measured by using a photoelectric colorimeter (Spectronic-20, USA).

3. Preparation of Frozen Semen

The concentrated semen collected by using the gloved-hand technique was diluted with BTS preserving solution in 1:1 ratio and transported to a laboratory to slowly cool its temperature to 22～25℃ from room temperature for 2 hours, and dispensed to 15 ml test tubes to centrifuge at 1,500 rpm for 15 minutes.

The frozen preserving solution was prepared by using the lactose-egg yolk (LEY) method reported by Richter *et al.* (1975) and the dilution of precipitated concentrated semen was done by adding the primary frozen preserving solution (lactose hydrate 11.0 g, egg yolk 25.0 ml, distilled water up to 100.0 ml) that is equivalent to 2/3 of the finally diluted amount to prepare the sperm concentration of 1.5×10^9/ml.

After diluting with the primary preserving solution, it was cooled down to 5℃ for 2 hours by using the low temperature processing unit of sperm (FHK, FA 112, Japan), and the secondary frozen preserving solution (lactose hydrate 11.0 g, egg yolk 25.0 ml, glycerol 6.0%, orvus es paste; Nova Chemical Sales - USA 1.0%, distilled water up to 100.0 ml) that is equivalent to 1/3 of the treated amount of sperm was added by dividing in 4 fractions with 15 minutes of interval.

The final glycerol concentration and the sperm concentration was adjusted as 2.5% and 1.0×10^9/ml, respectively. The finally diluted sperm was injected into the 5ml maxi-straw (Minitüb, Gmbh, Landshut, Germany) and the both ends were sealed with metallic balls.

After packing the preparation by using the Cryogenic-vials (Corning, Cambridge, MA 02140, USA), the horizontal freezing was done for 20 minutes at the surface where was placed 5 cm above the liquid nitrogen (for the purpose of the experiment) and stored them by placing at −196℃ of liquid nitro-

gen storage tank.

The dissolution of semen was done by placing the liquid nitrogen stored Maxi-straw in a 52℃ water bath for 45 seconds, and used by diluting with 80ml BTS 36℃ preserving solution.

4. Artificial Insemination and Offspring Size

The estrus confirmed sows 5～7 days after the weaning have been observed for 2 times with 12 hour interval for a day, and the first insemination of liquid semen was made 12 hours after the estrus as soon as the approval for boar was confirmed. The second insemination was made 12 hours after the first insemination. The insemination of frozen semen was done 10～12 hours later than the liquid semen for 2 times with 6～12 hours of interval. After the artificial insemination, the relapse of estrus was checked and the parturition rate and offspring size were investigated.

5. Statistical Analysis

The effects of PSS and HSP70 genotypes on littter size were evaluated by analysis of variance using GLM procedure of SAS/STAT. The assumed linear model was as follows:

$Y_{ijklm} = \mu + B_i + S_j + F_k + G_l + e_{ijklm}$

where Y = the vlue of the m^{th} animal

μ = population mean

B_i = fixed effect of the i^{th} breed

S_j = fixed effect of the j^{th} season

F_k = random effect of the k^{th} farm

G_l = random effect of the l^{th} genotyping

e_{ijklm} = random error

Differences in least squares means were analysed by Fisher's least significant difference test (SAS, 1989) with a comparison error rate of 0.05.

RESULTS

1. Effect of Litter Size on Fresh Semen AI

In the artificial insemination of fresh semen, the factors of season, breed, PSS and HSP70 genotypes that affect on total offspring size and actual offspring size were summarized in Table 1 and Table 2.

In the offspring sizes of artificial insemination by using fresh semen, the total offspring size and actual offspring size in spring and fall recorded 10.89 and 10.02, respectively. The result was larger than the 10.47 of total and 9.71 of actual offspring sizes recorded in summer. In the pig breeds, the Landrace breed resulted with the largest offspring size by recording 10.83 of total and 9.96 of actual offspring sizes ($p<0.05$).

The result was followed by Yorkshire breed that recorded 10.64 of total and 9.87 of actual offspring sizes, and Duroc breed that recorded 10.57 of total and 9.77 of actual offspring sizes. Incase of recessive homo PSS genotype, the offspring sizes were lowest by recording 9.09 of total and 8.68 of actual offspring sizes. In case of latent genotype, the total offspring size was 10.82 without showing reduction compared to normal offspring sizes of 10.7～10.97. In overall, the genotype comprised by PSS-Normal, HSP70 K1-BB, K3-AB and K4-AA resulted with the largest offspring size by recording 10.97 of total and 10.96 of actual offspring sizes.

2. Effect of Litter Size on Frozen Semen AI

In the artificial insemination of frozen semen, the factors of season, breed, PSS and HSP70 genotypes that affect on total and actual offspring sizes were summarized in Table 3 and Table 4.

The seasonal effect of the frozen semen on offspring size

Table 1. Effect of season and breeds on litter size after artificial insemination using fresh semen in the pigs

Sources		No. of boar	No. of mating	Litter size born(head)	Litter size born alive(head)
Season*	Spring, fall	19	2,967	10.89±0.17[a]	10.02±0.15[a]
	Summer	16	884	10.47±0.17[b]	9.71±0.16[b]
Breeds	Duroc	1	1,525	10.57±0.18[b]	9.77±0.16[b]
	Landrace	9	1,757	10.83±0.18[a]	9.96±0.16[a]
	Yorkshire	8	2,430	10.64±0.17[b]	9.87±0.15[ab]

[a,b] : LSM±SE with different superscripts in the same column are significantly different ($p<0.05$).

* : Spring, fall; March to May, September and Octber, Summer; June to August.

Table 2. Effects of PSS and HSP70 genotype on litter size after artificial insemination using fresh semen in the pigs

PSS*	HSP70			No. of boar	No. of sow	Litter size born(head)	Litter size born alive(head)
	K1	K3	K4				
A	BB	BB	AA	1	5	9.09±1.00^{b}	8.68±0.90^{b}
C	BB	BB	AB	2	370	10.82±0.12^{ab}	10.00±0.11^{ab}
N	AB	AB	AB	3	916	10.81±0.08^{b}	9.94±0.08^{ab}
N	AB	BB	AA	1	115	10.58±0.22^{b}	9.75±0.20^{b}
N	BB	AB	AA	1	21	10.97±0.49^{a}	10.69±0.45^{a}
N	BB	BB	AA	5	1,195	10.70±0.07^{b}	10.03±0.06^{ab}
N	BB	BB	AB	12	1,673	10.78±0.06^{b}	9.97±0.06^{ab}

^{a,b} : LSM±SE with different superscripts in the same column are significantly different($p<0.05$).
* : A; affected, C; carrier, N; Normal.

Table 3. Effects of season and breeds on litter size after artificial insemination using frozen semen in the pigs

Sources		No. of boar	No. of sow	Litter size born(head)**	Litter size born alive(head)**
Season*	Spring	23	264	10.02±0.39	9.25±0.34
	Fall	21	199	9.74±0.41	9.28±0.36
Breeds	Duroc	4	98	9.51±0.59	8.98±0.52
	Landrace	13	173	10.09±0.42	9.49±0.37
	Yorkshire	11	192	10.04±0.38	9.32±0.34

* : Spring; March to May, Fall; September and October.
** : LSM±SE.

Table 4. Effects of PSS and HSP70 genotype on litter size after artificial insemination using frozen semen

PSS*	HSP70			No. of boar	No. of sow	Litter size born(head)	Litter size born alive(head)
	K1	K3	K4				
C	BB	BB	AB	2	21	11.29±0.66^{a}	10.82±0.58^{a}
N	AB	AB	AB	3	85	10.28±0.43^{ab}	9.74±0.38^{b}
N	AB	BB	AA	1	25	8.93±0.75^{b}	8.74±0.67^{bc}
N	BB	AB	AA	1	5	8.48±1.38^{ab}	7.33±1.23^{c}
N	BB	BB	AA	4	61	10.35±0.41^{ab}	9.48±0.37^{b}
N	BB	BB	AB	7	95	9.95±0.37^{b}	9.47±0.33^{b}

$^{a\sim c}$: LSM±SE with different superscripts in the same column are significantly different($p<0.05$).
* : C; carrier, N; normal.

was not confirmed. In the pig breeds, even if the Landrace breed resulted with the largest offspring size by recording 10.09 of total and 9.49 of actual offspring sizes, the significant difference was not found compared to the following breeds of Yorkshire that recorded 10.04 of total and 9.32 of actual offspring sizes, and Duroc breed that recorded 9.51 of total and 8.98 of actual offspring sizes.

In reviewing the stress related genotype, the genotype com-

prised by PSS-Carrier, HSP70 K1-BB, K3-BB and K4-AB resulted with the largest offspring size by recording 11.29 of total and 10.82 of actual offspring sizes, which showed the significant difference to the genotype comprised by PSS-Normal, HSP70-K1-BB, K3-AB and K4-AA that recorded 8.48 of total and 7.33 of actual offspring sizes ($p<0.05$).

DISCUSSION

The present reviewed the fertility rates and offspring sizes of fresh and frozen semen by RFLP and SSCP genotypes of the PSS and HSP70 genes to acquire the related offspring sizes of artificial insemination.

The HSP is one of protective mechanisms against the exterior stresses. If its expression regulation procedure screening or related gene screening is achieved, the capacity debilitation related to reproductivity is expected to be prevented. The human HSP70-1 gene is comprised by exon 1, 2 and 3 regions. If the partial deletion of exon 2 region was occurred by DNA base mutations during the expression procedure, the expression only at the regions of 1 and 3 without the expression of exon 2 region was reported to increase stress sensitivity and reduce the disease resistance (Shoichi *et al.*, 1999).

At the present experiment, the seasonal factor in artificial insemination of pigs resulted with significantly high offspring size and survived offspring size when the artificial insemination was conducted in fall and spring compared to the result acquired in summer ($p<0.05$).

The comparison of the results acquired by using frozen semen was only available for spring and summer due to the absence of artificial insemination records conducted in summer, however, no significant difference was found between spring and fall. In reviewing the factor of breed, the Landrace breed that recorded 10.83 of offspring size was significantly higher than Duroc and Yorkshire breeds that recorded 10.57 and 10.64 offspring sizes, respectively ($p<0.05$).

However, in case of frozen semen, the significant difference was not found between breeds. Considering the reports of Huang *et al.* (2000) that stated the HSP70 gene expression is higher in winter than in summer and the increase of its expression resulted with better mobility with higher sperm concentration without showing the HSP70 expression difference between breeds, it was possible to confirm the lower offspring size in artificial insemination conducted in summer by the using liquid semen that could be considered to show lower HSP 70 expression.

In addition, the offspring size by breeds did not show significant difference in frozen semen, but liquid semen revealed the difference by showing the Landrace breed to record the largest offspring size of 10.83.

At the time of artificial insemination, the effect of stress related genotype to litter size resulted to show the significantly lower offspring sizes by showing 9.09 of total and 8.68 of actual offspring sizes in case of the PSS genotype of liquid semen was recessive homo type. In case of the hetero genotype, the total offspring size was 10.82 which was found to have no effect on the offspring size reduction by comparing to the normal genotype's total offspring size of 10.70～10.97.

In overall, the genotype comprised by PSS-Normal, HSP70 K1-BB, K3-AB and K4-AA resulted with the largest offspring size by recording 10.97 of total and 10.96 of actual offspring sizes.

However, seeing the effect of PSS and HSP70 genotypes on offspring size in frozen semen, the genotype comprised by PSS-Carrier, HSP70 K1-BB, K3-BB and K4-AB resulted with the largest offspring size by recording 11.29 of total and 10.82 of actual offspring sizes, and the genotype comprised by PSS-Normal, HSP70-K1-BB, K3-AB and K4-AA resulted with the smallest offspring size by recording 8.48 of total and 7.33 of actual offspring sizes ($p<0.05$).

As shown in above, the one with the genotype of PSS-Normal, HSP70-K1-BB, K3-AB and KA-AA resulted with the largest offspring size by using liquid semen, but the offspring size fell down to the smallest at the time of using frozen semen confirming the largest functional debilitation in low temperature.

According to the report of Peña *et al* (1997), the 3 different semen treatment groups of Oxytocin (4IU) diluted, Oxytocin (4IU) injected through the vulva lips mucosa and Oxytocin (4IU) untreated in the artificial insemination of pigs, the Oxytocin (4IU) diluted group in summer time around July and August turned out to have the highest effect by showing the 10.77 of total offspring size and delivery rate of 73.02% compared to the records of Oxytocin (4IU) untreated and Oxytocin (4IU) injected through the vulva lips mucosa, which respectively recorded total offspring sizes of 10.45 and 8.53 with the delivery rate of 56.25% and 54.39% ($p<0.05$). Although the offspring size in winter around January to March resulted to show the significantly high numbers by recording 12.2 in Oxytocin (4IU) diluted group compared to other types of treatment results of

10.76 and 10.12 ($p<0.001$), the report did not find significant difference at the delivery rate. As stated in above, the artificial insemination of pigs in summer is important factor in considering the offspring size and delivery rate, and the performance is expected to be improved by and genetic factor and by other treatments.

Huang *et al.* (2000) reported the high expression level of HSP70 gene in winter time in the pig breeds of Duroc, Landrace and Yorkshire with increased mobility and high normal sperm rate, but reported reduced HSP70 gene expression and high abnormal sperm rate in summer time.

Based upon these results, the studies on high temperature stress function to reduce the sperm function debilitation are considered to be necessary. In addition, the present study was able to confirm that the specific PSS-HSP70 genotype maintained the high offspring size at the time of artificial insemination of liquid semen, but the offspring size was reduced, which confirmed the necessity to conduct the study on the low temperature stress function.

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