J. Emb. Trans. (2010) Vol. 25, No. 4, pp. 263~266

Effects of Sperm Motility on *In Vitro* Production of Embryo and Correlation with Mitochondria Amount in Pig

Ki-Hwa Chung[1], In-Cheul Kim[2] and Jung-Ho Son[3,*]

[1]Department of Animal Resources Technology, Jinju National University, Jinju 660-758, Korea
[2]Swine Science Division, National Institute of Animal Science, RDA, Cheonan 330-801, Korea
[3]Noah Biotech Inc, Cheonan 330-714, Korea

ABSTRACT

Prediction of semen's fertilizing ability used in artificial insemination (AI) is one of very important factors on pig reproductive performance. *In vitro* fertilization (IVF) has been used for indirect evaluation of sperm's fertilizing ability and it has been showed as highly correlated index. In swine industry, increasing interest in preservation of boar semen raises questions on the sperm motility from semen used in commercial AI centers. Mitochondria in sperm mid-piece generate the energy to support motility and could be an explanation of impaired fertility. Objective of this study was to suggest usable sperm motility to farms in measuring the effect of sperm motility and sperm abnormality on *in vitro* production of embryo in which sperm's fertilizing ability can be determined indirectly. Semen samples were provided from local AI center and used within 3 days after collection. Semen samples were divided by 4 different motile groups (>70%; 61~70%; 51~60%; <50%) using CASA (computer-assisted sperm analysis) on the days of IVF. Developmental rate to the blastocyst stage from over 61% motile sperm group showed significantly higher rate than below 60% motile sperm group (16.5 ± 0.7~18.4 ± 0.8% vs 6.3 ± 0.8~11.5 ± 0.7%, $p<0.05$). In experiment to determine the relationship between sperm motility and viability and abnormality, over 61% motile sperm groups showed significantly higher viability rate compared to below 60% motile sperm groups (84.8 ± 4.0~88.1 ± 4.0% vs 69.1 ± 4.0~74.2 ± 4.0%, $p<0.05$). On the other hand, morphological sperm abnormality showed significantly higher in over 70% motile sperm group (10.2 ± 2.2 vs 16.0 ± 2.2~21.0 ± 2.2%, $p<0.05$). In experiment to find the correlation between sperm motility of 4 different motile groups and amount of mitochondria, lower motility group also showed lower level of mitochondria ($p<0.05$). The mitochondria parameter used in this study showed another possibility to differentiate the sperm motility. Taken together, because below 60% motile semen used in AI reduce the fertility, AI centers should provide the over 60% motile sperm to the farms at the time of AI.
(Key words : sperm motility, viability, mitochondria, AI, IVF)

INTRODUCTION

As of 2008, 1,729 doses of boar semen are provided by 52 artificial insemination (AI) centers and AI service rate is over 90% in Korea (Kim *et al.*, 2008). As time passes, delivered and preserved semen's motility drop gradually until used in AI and it attributes to lower the fertility and liter size. Often disputes occur when fertility problems raise between provider and user in which user claim semen quality problem but provider do not agree with semen quality but rather than sow's management problem.

The prediction of sperm fertilizing ability is one of important factors in reproductive performance of pig when AI is used (Gadea, 2005). Livestock breeding managers could predict reproductive performance in combining the physical evaluation of boar as well as general sperm parameters such as concentration, morphology and motility (Gibson, 1989). Evaluation of semen's fertilizing ability can be done by testing the sperm motility, sperm viability, sperm morphology and sperm DNA status, and sperm-zona pellucida binding test, oocyte penetration assay (Berger *et al.*, 1996), *in vitro* fertilization (IVF) assay (Sells *et al.*, 2003) and sperm-oviduct binding assay (Foxcroft *et al.*, 2008) for sperm function evaluation can be used. Sperm motility is the most absolute factors affecting the fertility and the

† This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ005952)" Rural Development Administration, Republic of Korea.
‡ This work was supported by Jinju National University Grant.
* Correspondence : E-mail : jhson@noahbio.com

simple way of predicting fertility in fertilizing process of sow's reproductive tract in which sperm moves to oviduct, binds to zona pellucida, and fertilizes the egg in the ampulla. Flowers (1997) reported that sperm motility is the very useful index of fertilizing ability and if sperm's motility is over 60%, there is no difference in sperm penetration rate *in vitro*, farrowing rates, and litter size. Sperm motility, however, is not an absolute standard in evaluation of sperm's fertilizing ability (Foxcroft *et al.*, 2008). IVF is the one of highly correlated index and indirect evaluation method of sperm's fertilizing ability (Parinaud *et al.*, 1996; Gadea, 2005). Zhang *et al.*, (1997) reported that cleavage and blastocyst formation rates and fertility are highly correlated with IVF in bovine semen.

Mitochondria in sperm mid-piece generate the energy to support motility and could be an explanation of impaired fertility (Pena *et al.*, 2003). Flowcytometry, using fluorescent dye in boar semen, is a useful tool to evaluate the mitochondrial function because of its objectivity, high accuracy to detect small differences between samples, and speedy count as fast as 5,000 cells/sec. Mitochondria function was found to correlate well with the viability using SYBR-14 assessment and microscopic estimation of motility (Lindsay *et al.*, 2005).

Objective of this study was to suggest the usable sperm motility to farms in measuring the effect of sperm motility and sperm abnormality on *in vitro* production of embryo in which sperm's fertilizing ability can be determined indirectly.

MATERIALS AND METHODS

1. Sperm Preparation

Semen sample were provided by local AI center and used within 3 days after collection. Semen sample were divided by 4 different motile groups (>70%; 61～70%; 51～60%; <50%) using CASA (Medical Supply Co. Ltd., Korea). For the analysis of sperm motility, CASA was used as Zeng *et al.* (2001). In brief, 1.5 ml semen sample was incubated in water bath for 30 min at 37℃, 10 μl of semen samples were applied to pre-warmed Makler counting chamber (Sefi-Medical, Israel), and then sperm motility on a warm plate (37℃) was analyzed by SAIS II system (Medical Supply Co. Ltd., Korea) connected with CCD camera (Toshiba, Japan) to microscope (Olympus, Japan).

2. Oocyte Collection and *In Vitro* Maturation

Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO, USA). Ovaries were collected from local slaughter house and were transported to the laboratory within 2～4 hr of collection in thermos bottle containing sterile 0.9% physiological saline solution with antibiotics. Ovaries were washed 2～3 times with physiological saline solution before aspiration and cumulus-oocyte-complexes (COC's) were aspirated from follicles with a diameter of 2～8 mm using a 10 ml syringe fitted with an 18 gauge needle. The COC's were washed with TL-Hepes-PVA three times and only COC's with a uniformly dark cytoplasm and with at least 3-fold compact layers of cumulus cells were selected under the microscope. Final concentration of 10 i.u. hormones of equine chorionic gonadotropin (eCG, Intervet America Inc. DE) and human chorionic gonadotropin (hCG, Intervet America Inc. DE) were added to the culture drops containing oocytes and culture for 22 hr. After culture fro 22 hr, oocytes were washed three times and then culture in M199 medium without hormones for another 22 hr at 39℃, 5% CO_2 in air.

3. *In Vitro* Fertilization

Fertilization medium was prepared using a modified Tris-Buffered Medium (mTBM) containing 1 mM caffeine and 0.4% BSA. After 42 hr of *in vitro* maturation, cumulus mass were removed by vortexing and washed three times with mTBM and then transferred to 50 μl drop of fertilization medium. Sperm (0.5×10^6 /ml) and oocytes were incubated with for 6 hr. After fertilization, the oocytes were washed with sperm free medium consisted of NCSU-23 containing 0.4% BSA media and each 20 oocytes were then transferred to 50 μl droplet at 39℃ under 5% (v/v) CO_2 in air for further incubation.

4. Analysis of Sperm Viability and Abnormality

For sperm viability and abnormality test, Fast green FCF (2%, v/v) and Eosin B (0.8%, v/v) were solved in PBS and filtered before staining the sperm. Ten micro liter of sperm were applied to the slide and equal volume of dye were added, smeared with cover glass, and dried quickly to avoid live sperm stain as dead. If sperm head stains with dye, it determines as dead. Total of 100 sperm from 25 each of 4 different compartments were counted for both viability and abnormality test.

5. Analysis of Mitochondria by Flowcytometer

Aliquots of 1 ml of semen were stained with Rhodamin-123 for 10 min at room temperature and 20,000 spermatozoa were counted by flowcytomer (Becton & Dickinson, San Jose CA, USA). Double staining of green fluorescence of Rhodamine-

123 of live and motile cells that passed through a 575/26 BP filter and red fluorescence of Propidium Iodide of dead cells that passed through 610/20 BP were compared. Small particles and debris from semen sample were eliminated using threshold function to minimize the spillover of fluorescence signal.

6. Statistical Analysis

Data were analyzed using the general linear models of SAS (SAS ver 8.2, SAS Inst. Inc., Cary, NC). The Duncan's new multiple range test were used for group mean differences. In all studies, statistical significance was determined at $p<0.05$.

RESULTS

1. Effects of Sperm Motility on Embryo Development *In Vitro*

The results of the study to determine the effect of 4 different sperm motile groups on *in vitro* production of embryo were shown in Table 1. The embryo developmental rate to the stage of cleavage, morula, and blastocyst were significantly higher in >61% motile sperm group compared to <60% motile sperm group ($p<0.05$).

2. Effect of Sperm Viability and Abnormality on Sperm Motility

Sperm viability rate showed significant higher in >61% motile sperm group compared to <60% motile sperm group ($p<0.05$). Sperm abnormality rate showed significant higher in <60% motile group compared to >61% motile group ($p<0.05$) suggesting, there is a positive relationship between sperm motility and sperm viability.

3. Relationship between Sperm Motility and the Amount of Mitochondria in Sperm

Sperm motility determined by CASA analysis were compared to sperm mitochondria level measured by flowcytomer. There was a positive relationship ($p<0.05$) between sperm motility and mitochondria level. As motility decreases that measured by CASA, mitochondria level also decreased (Table 3), suggested that measurement of mitochondria can be used as an alternative expression of sperm motility.

DISCUSSION

Sperm's fertilizing ability measured by IVF is useful recognition method of various biochemical and biophysical changes. The production of blastocyst stage embryo means the powerful index to see if series of capacitation, acrosome reaction, sperm-

Table 1. Effects of sperm motility on *in vitro* production of pig embryo

Sperm motility (%)	No. of oocytes	Developmental stage (%)		
		2~4 cells	Morula	Blastocyst
>70	898	79.2 ± 1.1^a	41.3 ± 1.1^a	18.4 ± 0.8^a
61~70	960	78.0 ± 1.0^a	38.3 ± 1.1^a	16.5 ± 0.7^a
51~60	1,100	72.6 ± 1.0^b	32.6 ± 1.0^b	11.5 ± 0.7^b
<50	820	66.0 ± 1.1^c	27.1 ± 1.2^c	6.3 ± 0.8^c

[a~c] Means with different superscripts in the column are significantly differ ($p<0.05$).

Table 2. Effect of sperm motility on sperm viability and abnormality

	Sperm motility (%)			
	>70	61~70	51~60	<50
Viability(%)	88.1 ± 4.0^a	84.8 ± 4.0^{ab}	74.2 ± 4.0^{bc}	69.1 ± 4.0^c
Abnormality(%)	10.2 ± 2.2^b	16.0 ± 2.2^a	16.5 ± 2.2^a	21.0 ± 2.2^a

[a~c] Means with different superscripts in the row are significantly differ ($p<0.05$).

Table 3. Comparison of mitochondria amount with sperm motility

	Sperm motility (%)			
	>70	61~70	51~60	<50
Mitochondria amount (%)	80.03 ± 3.64^a	77.23 ± 1.96^a	70.58 ± 1.51^b	62.48 ± 4.20^{bc}

[a~c] Means with different superscripts in the row are significantly differ ($p<0.05$).

zona binding, sperm-oocyte binding and penetration, and sperm condensation has been done properly. Flowers (1997) reported that sperm motility is very useful index of fertilizing ability and the reproductive performance such as sperm penetration rate (%), farrowing rate, and number born alive were significantly lower in below 52.4% motile sperm group. However, there was no difference in reproductive performance in over 66.2% motile sperm group.

IVF is most valid fertilizing ability test due to it represents all of sperm function comprehensively during fertilization process (Parinaud *et al.*, 1996; Gadea, 2005). But because male pronuclear formation rate occurring during fertilization showed 12～17% variation (Ruiz-Sanchez *et al.*, 2006) compared to *in vivo* fertility, the author mentioned that test should be done carefully at well setting lab of *in vitro* maturation (IVM)/IVF technique to minimize the variation.

In our experiment, sperm abnormality increase at below 70% motile sperm, and it would speculate that sperm viability decreases after secondary sperm abnormality occur.

Because morphologically abnormal sperm has been shown negative relationship with fertility (Gadea and Matas, 2000), if sperm abnormality is over 20%, pig farms should not use that semen to prevent from lowering the fertility.

In routine microscopic estimation used to evaluate semen motility was mostly subjective and not correlated highly with fertilizing capacity and sperm parameters. The use of variety fluorochromes has enabled a more widespread analysis of sperm parameters in conjunction with the flowcytometer. In an experiment to compare the CASA analysis of porcine semen motility with the sperm mitochondria level measured by flowcytomer, we confirmed that sperm motility showed higher correlation (Table 3, $p<0.05$) with mitochondria amount in sperm.

Taken together, because below 60% motile semen used in AI reduce the fertility, AI centers should provide the higher than 60% motile semen to pig farms and farm managers also aware that over 60% motile semen should be used at the time of AI.

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(접수: 2010. 11. 17 / 심사: 2010. 11. 18 / 채택: 2010. 11. 30)