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Effect of Using Supernatant for Post-thawing Solution and Semen Extender prior to Insemination on Sow Reproductive Performance

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Original Article

Effect of Using Supernatant for Post-thawing Solution and Semen Extender prior to Insemination on Sow Reproductive Performance

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Abstract

The objective of the present study was to determine the effects of using supernatant (seminal plasma: extender, 50:50 v/v) as post-thawing solution and semen extender on sow fertility. Fifteen sows were allocated into the following experimental groups: Groups A (control), sows (n=5) were inseminated with fresh semen, using a dose of $4x10^9$ spermatozoa in 60 ml of Modena™ extender; Group B, sows (n=5) were inseminated with frozen semen (supplemented with 10 mM of *L*-cysteine), using a dose of $2x10^9$ spermatozoa in 60 ml of supernatant (50% v/v of seminal plasma plus Modena™ extender); Group C, sows (n=5) were inseminated with frozen semen (supplemented with 10 mM of *L*-cysteine), using a dose of $2x10^9$ spermatozoa in 60 ml of Modena™ extender. All sows were inseminated twice using an intrauterine catheter depending on their weaning to oestrous interval (WOI). Pregnancy rate (PR), farrowing rate (FR), total number of piglet born (TNB) and number of piglet born alive (NBA) were recorded. In group A, the PR and FR were 100%, TNB and NBA were 7.8±3.9 and 7±3.9, respectively. For frozen semen, PR, FR, TNB and NBA in group B were higher than group C (100% versus 60%, 60% versus 0%, 6.0±2.7 versus 0, 6.0±2.7 versus 0, respectively). In conclusion, using supernatant (50% v/v of seminal plasma plus Modena™) as post-thawing solution and semen extender for artificial insemination in field condition improve sow fertility.

Keywords: artificial insemination, boar semen, cryopreservation, supernatant

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บทคัดย่อ

ผลของการใช้สารละลายส่วนใสที่ได้จากการปั่นเหวี่ยง (Supernatant) สำหรับเป็นสารละลายเจือจางน้ำเชื้อแช่แข็งและเป็นสารละลายเจือจางน้ำเชื้อก่อนการผสมเทียมต่อประสิทธิภาพในการสืบพันธุ์ของสุกรนาง

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การศึกษาผลของการใช้สารละลายส่วนใสที่ได้จากการปั่นเหวี่ยง (Supernatant ที่มี Seminal plasma ร้อยละ 50 โดยปริมาตร) สำหรับเป็นสารละลายเจือจางน้ำเชื้อแช่แข็งและเป็นสารละลายเจือจางน้ำเชื้อต่อประสิทธิภาพในการสืบพันธุ์ของสุกรนาง ทำการศึกษาในสุกรนางทั้งหมด 15 ตัว โดยแบ่งสุกรออกเป็น 3 กลุ่ม คือกลุ่ม A เป็นกลุ่มควบคุมถูกผสมเทียมด้วยน้ำเชื้อสด ที่มีจำนวนตัวอสุจิที่เคลื่อนที่ได้ $4x10^9$ ตัว ปริมาตรน้ำเชื้อ 60 มล. และทำการเจือจางด้วยสารละลายเจือจางน้ำเชื้อ Modena™ กลุ่ม B ถูกผสมเทียมด้วยน้ำเชื้อแช่แข็ง (เติม *L*-cysteine, 10 mM) ที่เจือจางด้วยสารละลายส่วนใสที่ได้จากการปั่นเหวี่ยง (Supernatant = Modena™+seminal plasma ร้อยละ 50 โดยปริมาตร) ที่มีจำนวนตัวอสุจิที่เคลื่อนที่ได้ เท่ากับ $2x10^9$ ตัว ปริมาณน้ำเชื้อ 60 มล. และ กลุ่ม C ถูกผสมเทียมด้วยน้ำเชื้อแช่แข็ง (เติม *L*-cysteine, 10 mM) ที่เจือจางด้วยสารละลายน้ำเชื้อ Modena™ ที่มีจำนวนตัวอสุจิที่เคลื่อนที่ได้ $2x10^9$ ตัว ปริมาตรน้ำเชื้อ 60 มล. สุกรนางทุกกลุ่มจะถูกผสมด้วยวิธีการผสมเทียมแบบสอดท่อลึกเข้าไปภายในตัวมดลูก (Intrauterine insemination, IUI) จำนวน 2 ครั้ง ระยะเวลาการผสมขึ้นอยู่กับระยะเวลาจากหย่านมถึงเป็นสัด จากนั้นเก็บข้อมูล อัตราการการตั้งท้อง อัตราเข้าคลอด จำนวนลูกสุกรต่อครอก และจำนวนลูกสุกรมีชีวิตต่อครอก ซึ่งพบว่าสุกรนางในกลุ่ม A มีอัตราการตั้งท้องและอัตราเข้าคลอด 100% ให้จำนวนลูกสุกรต่อครอกและจำนวนลูกสุกรมีชีวิต 7.8±3.9 และ 7±3.9 ตามลำดับ ในขณะที่สุกรนางในกลุ่มน้ำเชื้อแช่แข็ง กลุ่ม B มีอัตราการตั้งท้อง อัตราเข้าคลอด จำนวนลูกสุกรต่อครอกและจำนวนลูกสุกรมีชีวิตมากกว่าสุกรนางในกลุ่ม C (100% เทียบกับ 60%, 60% เทียบกับ 0% ตามลำดับ; 6±2.7 ตัว เทียบกับ 0 ตัว และ 6±2.7 ตัว เทียบกับ 0 ตัว ตามลำดับ)

จากการศึกษาครั้งนี้แสดงให้เห็นว่าการใช้สารละลายส่วนใสที่ได้จากการปั่นเหวี่ยง (Supernatant ที่มี seminal plasma ร้อยละ 50 โดยปริมาตร) สำหรับเป็นสารละลายเจือจางน้ำเชื้อแช่แข็งและเป็นสารละลายเจือจางน้ำเชื้อสำหรับการผสมเทียมในภาคสนามเป็นวิธีที่ช่วยเพิ่มประสิทธิภาพทางการสืบพันธุ์ของสุกรนางได้

คำสำคัญ: การผสมเทียม น้ำเชื้อพ่อสุกร น้ำเชื้อแช่แข็ง สารละลายส่วนใสที่ได้จากการปั่นเหวี่ยง

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Introduction

Cryopreservation of boar spermatozoa offers an effective means of long-term storage of important genetic materials (Okazaki et al., 2009), transportation this genetic line across the countries and also minimize boar transportation (Biley et al., 2008). Generally, frozen-thawed boar semen has a short survival time in the reproductive tract compared with extended fresh semen and insemination with frozen-thawed sperm usually results in inferior farrowing rates and litter sizes compared to insemination with fresh sperm (Johnson et al., 2000).

Cryopreservation process damage the sperm plasma membrane and lead to a decrease of sperm viability and fertilizing ability, and other effects of this process on sperm have also been reported, e.g. cold-shock, osmotic stress and oxidative stress from reactive oxygen species (ROS) (Watson., 2000; Aitken and Krausz, 2001). *L*-cysteine is an antioxidant in which supplemented in the freezing extenders of frozen boar semen in order to minimize the effect of ROS which occurred during the freezing process (Gadea et al., 2005). Supplementation of lactose-egg yolk (LEY) extender with *L*-cysteine had pronounced effect in improving frozen-thawed boar semen qualities (Kaeoket et al., 2008; Chanapiwat et al., 2009; Kaeoket et al., 2010).

Seminal plasma is the liquid component of an ejaculate, containing a combination of fluids secreted by the male accessory sex glands (i.e., in boar, mainly secreted by seminal vesicle) during an ejaculation. There is evidence that seminal plasma is

able to arrest or reverse cryoinjury and perhaps extend sperm longevity by inhibiting or reversing capacitation and inhibiting acrosome reaction (Suzuki et al., 2002). In addition, seminal plasma seems to play a significant role in the female reproductive tract after insemination, e.g. diminish the inflammatory response in the uterus of the sow which may influence the chances of conception (Rozeboom et al., 1999) and its component such as hormone estrogen may also resulted in a release of prostaglandins from the porcine endometrium to the plexus of blood and lymph vessels, so called the utero-ovarian veins and lymphatic vessels which in turn decrease duration time from standing oestrous to ovulation in gilts (Clause et al., 1987, 1990; Weitze et al., 1990), Therefore, it seems likely that seminal plasma constituents (both the estrogen and the protein fraction) have an effect on ovulation time in sows (Waberski et al., 1995; Kaeoket and Tummaruk, 2002).

Generally, limitations to achieve a high reproductive performance in swine arise from a failure of sows to express estrus, failure to accurately determine onset of estrus for artificial inseminations and failure to determine the ovulation time after standing oestrous. The best predictor for time of ovulation is frequent detection of oestrous, because time of ovulation occurs approximately 38 to 48 hrs after onset of oestrous (Anderson et al., 1993; Weitze et al., 1994; Soede et al., 1995). The effects of the timing of insemination relative to ovulation on fertilization rate has been study by Soede et al.(1995). Targeting insemination within 24 hrs before ovulation seems optimal to achieve a high farrowing rate and large little size (Nissen et al., 1997). Insemination from 24 to 0 hr before ovulation results in high fertilization rates and consequently, a low number of repeat mating and a larger litter size (Kemp et al., 1996; Kaeoket et al., 2002, 2005).

Therefore, the aim of this study was to investigate the effect of using supernatant (50% seminal plasma V/V plus Modena™) as post-thawing solution and semen extender on sow fertility.

Materials and Methods

Animals: Duroc boar (ID = 4648), having proven fertility and being using for routine AI from a commercial herd was used. The boar was kept in individual pens in an evaporative housing system system. Water was provided *ad libitum* via water nipple. Feed was given approximate 3 kg/day (twice a day).

Evaluation of spermatozoa: Sperm motility: Sperm motility was subjectively evaluated by the same person throughout the study, at 37°C under phase contrast microscope at 100x and 400x magnification (Berger et al., 1985). Progressive motility was expressed as the percentage of motile spermatozoa.

Sperm concentration: Sperm concentration was assessed by Neubauer hemocytometer after dilution (1:100 v/v). A drop of diluted semen sample was placed into the chamber and the number of spermatozoa was evaluated under the phase contrast microscope 400x magnifications. Sperm concentration was expressed as spermatozoa $x10^6$ sperm/ml.

Assessment of sperm viability: The sperm viability was evaluated with SYBR-14/Ethd-1 (Ferilight®; Sperm Viability Kit, Molecular Probes Europe BV, The Netherlands). Ten micro liters of diluted spermatozoa were mixed with 2.7 µl of the user solution of SYBR-14 and 10 µl of EthD-1. After incubation at 37°C for 20 min, altogether 200 spermatozoa were assessed (x400) under fluorescence microscope (Carl Zeiss, Inc./Axoskop 40, Germany). The nuclei of spermatozoa with intact plasma membrane stained green with SYBR-14, while those with damaged membranes stained red with EthD-1 (Kaeoket et al., 2008). Spermatozoa were classified into three patterns: having intact plasma membrane (stained with green), "live"; having damaged plasma membrane but an intact acrosome (stained with red and green), "damaged" and having both plasma membrane and acrosome damaged stained with red, "dead". The result is recorded as the percentage of non-alive (damage and dead spermatozoa) and live spermatozoa.

Assessment of acrosome integrity: The integrity of sperm acrosome was evaluated with FITC-PNA staining. 10 µl of diluted semen with 140 µl phosphate buffered saline (PBS) was mixed 10 µl of Ethd-1 and incubated at 37°C for 15 min. Then, 5 µl of the mixtures was smeared on glass slide and fixed with 95% ethanol for 30 sec and air dry. The next step, 50 µl FITC-PNA (dilute Fit C-PNA with PBS 1:10 v/v) was spread over the slide and incubated in a moist chamber at 4°C for 30 min. After incubation, the slide was rinsed with cold PBS and air dried. A total of 100 spermatozoa were assessed under fluorescence microscope at 1000x magnification and classified to intact acrosome (stained green with FITC-PNA) and non-intact acrosome, which consist of loose and acrosome reacted (stained red with EthD-1) as percentage.

Preparation of boar spermatozoa before freezing: Sperm ejaculated from a Duroc boar (ID= 4648) was collected by using the gloved-hand technique (Kaeoket et al., 2002; Kaeoket et al., 2005). During collection the semen was filtered through gauze, and only sperm-rich fractions were collected. Within 30 minutes after collection, semen volume, pH (using pH paper, MERCK, Darmstadt, Germany), subjective sperm motility by phase contrast microscope (Olympus CX31, New York, USA) at 100x and 400x magnification, were recorded. A semen sample of 1 ml was collected to Eppendorf for future analysis at the semen laboratory, including analysis for concentration by using Neubauer hemocytometer (Improved neubauer's chamber, Germany), sperm viability by living cell nucleic acid stain, SYBR-14 (Fertilight®; Sperm Viability Kit, Molecular Probes, Europe BV, The Netherland), sperm acrosome integrity with FITC-PNA staining and morphology with William's staining. Only ejaculate with motility of ≥ 70% and ≥ 80% morphologically normal was used for cryopreservation. The remaining fresh semen was diluted with (1:1 v/v) Modena™ extender (extender I)

and transported by cell incubator (Micom control system 20Q, Continental plastic CORP, Wisconsin, USA) at 15°C to the semen laboratory.

Semen freezing process and supernatant collection: All semen samples were frozen by using a control rate freezer (Icecube 14s, Sylab, Purkersdorf, Australia). After collection and evaluation, the semen was diluted with (1:1 v/v) Modena™ extender (extender I) (Swine Genetics international, Ltd). Diluted semen was convey to 50 ml centrifuge tubes, cooled at 15°C for 120 min and later centrifuged at 800xg 15°C for 10 min (Hettich Rotanta 460R, Tuttlingen, Germany). The supernatant was collected, centrifuged at 800xg 15°C for 10 min and kept at -20°C for further using as post-thawing solution and semen extender, and the sperm pellet was re-suspended (about 1-2:1) with lactose-egg yolk (LEY) extender (80 ml of 11% lactose solution and 20 ml egg yolk, extender II) to a concentration of $1.5x10^9$ sperm/ml. To prepare Extender II (plus semen) supplement with *L*-cysteine (Fluka Chemie GmbH, Sigma-Aldrich, Switzerland), stock solution of *L*-cysteine was prepared 10 mM of concentration. (stock solution contained 605.8 mg/5 ml distilled water) Each ml of extender II (plus semen) was mixed with 0.6058 mg (10 µl from stock solution) and was supplemented in Group B and C.

The diluted semen was cooled to 5°C for 90 min. Semen were mixed with extender III (89.5% Lactose-Egg-Yolk extender with 9% glycerol and 1.5% Equex-STM® (Novo chemical sale, INC, USA)). The final concentration of semen sample to be frozen was approximately $1.0x10^9$ spermatozoa/ml and contained 3% glycerol. The processed semen was loaded into 0.5 ml straws (Bio-Vet, Z.I. Le Berdoulet, France) and sealed with plasticine. All the straws were transferred into the chamber of a programmable freezer (Icecube 14s, Sylab, Purkersdorf, Austria) set to +5°C. The two steps of cooling and freezing rates were 3°C/min from +5 to -5°C, and thereafter 50°C/min from -5 to -140°C. Then, straws were immediately plunged into liquid nitrogen (-196°C) for storage and further artificial insemination.

Semen thawing process: Random of thawing of straws was carried out in warm water using thermos, at 50°C for 12 sec (Kaeoket et al., 2008). After thawing the thaw samples were diluted (1:4) with supernatant in Group B and diluted with Modena™ extender in Group C in a test tube and kept in water bath at 37°C for 10 min, before evaluation and further artificial insemination. The subjective progressive motility was evaluated and only frozen-thawed motility of ≥ 30% was used for artificial insemination.

Animals and general management: After weaning, 15 multiparous sows with a lactation length between 21-24 days and an average parity number of 4.07±1.91 (means±SD) were used in this study and kept in individual crates with an open-house system of a commercial herd. Ahead of this study, the sows had shown normal reproductive characteristics (e.g., average total number of piglets born = 10.4±2.9; number of piglets born alive = 10.1±2.8). The average WOI was 3.6±0.5 days. The sows were fed twice a day with a commercial diet formulated for weaning sows. Water was provided *ad libitum*.

Oestrous detection: Oestrous detection was carried out by examination of the vulva for reddening and swelling as well as by control of the standing reflex in the presence of a boar (Kaeoket et al., 2002). The oestrous detection was performed daily (Kaeoket et al., 2005; Kaeoket et al., 2009).

Artificial insemination

Altogether 15 sows were allocated into one of the following experimental groups:

Groups A (control, n=5): Sows were inseminated with a dose of $4x10^9$ spermatozoa (fresh semen) in 60 ml of Modena™ extender. All inseminations were performed by using an intrauterine catheter, depending on their WOI (day of weaning = day 0) as follows:

- If WOI of 3-4 days, then inseminated at 24 h and 36 h after standing oestrous,
- if WOI of 5-6 days, then inseminated at 12 h and 24 h after standing oestrous,
- if WOI of more than 7 days, then inseminated at 0 h and 12 h after standing oestrous

Group B (n=5): Sows were inseminated with frozen semen twice (i.e., depending on their WOI) with a dose of frozen semen, containing $2x10^9$ spermatozoa in 60 ml of supernatant (50% v/v of seminal plasma plus Modena™ extender).

Group C (n=5): Sows were inseminated with frozen semen twice (i.e., depending on their WOI) with a dose of frozen semen, containing $2x10^9$ spermatozoa in 60 ml of Modena™ extender (Swine Genetics International, Ltd., Iowa, USA).

All the inseminations in groups B and C were also performed in the presence of a boar, by using an IUI catheter.

Data collection: All the sows were subjected to pregnancy diagnosis (trans abdominal) twice on Days 20-22 at first time and on 31-44 day after insemination by using real time ultrasound (50STringa, sector probe with 5 MHz, ESAOTE Pie Medical, The Netherlands) (Kaeoket et al., 2009). The farrowing rate and following parameters were recorded: total number of piglets born, number of piglets born alive, number of stillbirths and number of mummies.

Statistical analysis: Data were analysed by using Group *T*-test (SPSS/PC+statistic package version 14 for Windows Inc, Chicaco. IL, USA) and expressed as mean values±standard deviations (SD.). The pregnancy rates (PR) and farrowing rates (FR) are presented as percentages and were analyzed using the Kruskal-Wallis Test (non-parametric).The total number of piglet born and number of piglet born alive were analyzed by using the Group *T*-test. When the GLM revealed a significant effect, the mean values were compared by Group *T*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Fresh semen analysis: The progressive motility, viability (alive spermatozoa) and acrosome integrity (intact acrosome) of fresh semen are more than 80%, 80.8±3.3% and 75.0±28.7%, respectively.

Post thawing semen analysis

Progressive motility: There was higher percentage of post-thaw sperm motility in group B (frozen semen diluted with supernatant; 50%) than group C (frozen semen diluted with Modena™ extender; 40%)

Viability of spermatozoa (alive and non-alive spermatozoa, percentage): The higher percentage of live spermatozoa appeared to be in group B (live spermatozoa = 39.0±7.8) than group C (live spermatozoa = 37.2±8.4)

Acrosome integrity in live spermatozoa (intact and non-intact spermatozoa, percentage): The higher percentage of intact acrosome appeared to be in the group B (intact acrosome 69.7±26.3) than group C (intact acrosome 60.7±19.0)

Clinical observation and fertility: The average WOI was 3.6±0.5 days (range 3-5). For fresh semen (group A), the pregnancy rates and farrowing rates were 100% (Table 1). For frozen semen; the pregnancy rates and farrowing rates in group B were higher than group C (100% versus 60%: 60% versus 0%, respectively) (Table 1). However, two sows in group B were aborted with 6 embryos at 24 days and 9 embryos at 34 days after insemination. No sow farrowed in group C. For farrowing data, total number of pig born and number of piglet born alive are presented in Table 1.

Table 1 Total number of piglet born, number of piglet born alive, pregnancy and farrowing rates (Means±S.D.) follow artificial insemination in different groups (Group A: Fresh semen; Group B: Frozen semen+Supernatant; Group C: Frozen semen+Modena™)

Parameters	Group A	Group B	Group C	P-value
Pregnancy rate (%)	100 (n=5)	100 (n=5)	60 (n=5)	0.37
Farrowing rate (%)	80 (n=4)*	60 (n=3)**	0 (n=5)	0.37
Total number of piglet born	7.8±3.9 (n=4)	6.0±2.7 (n=3)	-	0.53
Piglet born alive	7.0±3.9 (n=4)	6.0±2.7 (n=3)	-	0.72

*One sow died during experiment., **Two sows aborted at 24 days with 6 embryos and 34 days with 9 embryo after insemination

Discussion

In the present study, supplementation of *L*-cysteine (10 mM) in the freezing extender II yield a satisfied motility (40%), viability (37.16%) and acrosome integrity (60.67%) which is in agreement with a recently study reported by Kaeoket et al.(2010). The reason might be that *L*-cysteine is a precursor of intracellular cysteine and glutathione (Bilodeau et al., 2001). It has also been reported that *L*-cysteine had ability to pass into the cell rapidly and is transformed to taurine, which combine to a fatty acid in membrane and then transformed to acyl-taurine, subsequently result in improve surfactants property and osmoregulation of sperm membrane (Weir and Robaire, 2006).

In the present study, there is an evident of a higher percentage of post-thaw spermatozoa parameters (motility, viability and acrosome integrity) diluted with supernatant (50% seminal plasma v/v plus Modena™) compare with semen diluted with Modena™. The possible mechanism might be that seminal plasma is a complex mixture of hormones (e.g. estrogen and prostaglandins), electrolytes and protein, which in turn initiating sperm motility, particularly; these seminal plasma proteins coat the sperm and act to stabilize the acrosome (Maxwell et al., 1998). It has been suggested that an improved efficacy of a greater seminal plasma inclusion rate on the ability to establish a pregnancy and maintain a greater number of fetuses to term may be a consequence of repair of sperm cryo-injury, or possibly its antioxidant activity limiting sperm damage, consequently resulting in a larger viable sperm population. Maxwell et al. (1999) demonstrated in frozen-thawed ram sperm that a considerably higher motility when re-suspended in thawing solution containing 20% seminal plasma compared with thawing solution without seminal plasma. In addition adding of 50% seminal plasma to the medium post-thaw increased percentage of live boar sperm and percentage of boar sperm motility (Garcia et al., 2010) and stallion sperm motility (Alghamdi et al., 2005). This is in agreement with the present results in that the seminal plasma is constituent in thawing solution (i.e., supernatant), 50% v/v. Moreover, it has been postulated by Kirkwood et al. (2008) that seminal plasma may prevented or reversed capacitation in frozen-thawed boar sperm, and in ram sperm, seminal plasma may also repaired membrane damage associated with cold shock (Barrios et al., 2000).

In the present study, seminal plasma existed in thawing solution, not only be active directly on the sperm, but may also modulated sow uterine functions. Seminal plasma in a dose of semen may

improve fertility by influencing the time of ovulation, by enhancing sperm transport via stimulation of uterine contractions, and by providing immune modulation in the uterus (Claus, 1990; Waberski, 1996; Engelhardt et al., 1997; Rozeboom et al., 1999). Additionally, estrogen that is secreted along with seminal plasma in an ejaculate have been suggested to be partially involved in improving sperm transport to the uterotubal junction (UTJ) (Claus, 1989; Rath et al., 1989; Weitze et al., 1990). The result of the present study is in agreement with Rozeboom et al. (2000, fresh semen) and Larsson et al. (1976, frozen semen) in that pregnancy and farrowing rates were higher in female pigs that were inseminated with a dose of semen diluted with seminal plasma compared with normal semen extender. Furthermore, it has been shown that thawing frozen semen in solution, containing 10% seminal plasma, has a positive effect on in vivo fertilization competence in poor freezability boars (Okazaki et al., 2009). However, in the field trial, Kirkwood et al. (2008) reported that supplementation of seminal plasma (30% v/v) in a dose of semen prior to insemination will be necessary to exert the positive effect on sow reproductive performances.

It is well documented that numbers of spermatozoa at the site of fertilization and also time of insemination are of important factors in order to achieve a good fertilization rate and to sustain normal embryonic development (Kaeoket et al., 2002, 2005). Thus, the number of spermatozoa in a dose of frozen semen may have an effect on little size of sows. The little size of 6 piglets per sows (inseminated with frozen semen) in this study is comparable with the sows inseminated with fresh semen. However, it is considered lower than other studies. In the present study, the average post-thaw sperm motility is 50%, suggesting that an average number of $1.0x10^9$ live spermatozoa for an insemination dose of $2.0x10^9$ spermatozoa were deposited twice in the uterine body of the experimental sows. In the studies of IUI using frozen-thawed boar semen, using a dose of $5x10^9$ spermatozoa, yielded a litter sizes of 10.7 piglets (Erikson et al., 2002) and 12.5 piglets (Garcia et al., 2009). These data and our present results indicate that sperm number in a dose of insemination is one of the important factors for sow fertility. However, further study is needed in order to clarify the optimal dose of frozen semen for intrauterine insemination.

It has been reported by Kemp et al. (1996) and Soede et al. (1997) that sows with a short WOI (3-4 days) on average have long oestrous duration, which is associated with a longer time from onset of oestrous to ovulation. On the other hands, sows with a WOI of 5-6 days or longer, had a shorter time from onset of oestrous to ovulation, and should be inseminated sooner after the onset of oestrous to ensure that the first insemination occurs before ovulation, varying between 64-70% of the duration of oestrous (Soede et al., 1997). However, owing to a short life span of frozen thawed spermatozoa, FT-boar spermatozoa require an insemination-to-ovulation interval not longer than 4-6 hrs, result in a peri-ovulatory insemination is a pre-condition to attain the highest fertility outcome. Spontaneous ovulation in sows most often occurs at two-thirds of oestrous duration. Estimation of the duration of oestrous of female pigs, with regards to the weaning-to-oestrous interval, is useful when setting up a suitable insemination programme (Roca et al., 2006). In order to improve the fertility outcome (i.e., farrowing rates and little sizes) with frozen semen, it is crucial to inseminate the female pigs as close to ovulation as possible. However, sows were considered to ovulate approximately 38-40 hrs after the onset of their estrus (Kemp et al., 1996). Soede et al. (1995) indicated that the optimal insemination-to-ovulation interval, for fresh semen, is from 24 to 0 hr before ovulation. However, because the other factors of sows such as parity and breed, and also the factors of semen such as quality and number of sperm cells (Waberski et al., 1994), storage time of semen, preservation method and type of semen extender used may shorten the optimal period for insemination relative to ovulation, therefore the optimal period of fertilization may be 12 hrs before ovulation. This is in agreement with our study in that sows were inseminated twice at 16 hrs and 4 hrs before ovulation (calculated from an average ovulation time of 38-40 hrs). Interestingly, Weitze et al. (1990) showed that insemination with seminal plasma at the onset of estrus may advance ovulation by up to 14 hrs (approximately 59% of oestrous duration) consequently we may have to correct the insemination time. In the present study, most sows have a WOI of 3-4 days. Therefore, only the second insemination at 36 hrs after standing oestrous is considered to be the optimal insemination time (Steverink, 1999; Kaeoket et al., 2009). However, further study in a larger population of sows is needed in order to study the timing of insemination under field condition.

In conclusion, using supernatant (50% v/v of Seminal plasma plus ModenaTM extender) as post-thawing solution and semen extender improve sow fertility, and the correlation of WOI-oestrus duration-ovulation time is a good tool for AI in a field conditions.

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