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BOAR SEMEN CRYOPRESERVATION: STUDIES ON THE INFLUENCE OF THE SEMEN DONORS, FREEZING PROTOCOLS, INSEMINATION TECHNIQUES AND *IN VIVO* FERTILITY

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A Thesis submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
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Thesis Title	BOAR SEMEN CRYOPRESERVATION: STUDIES ON THE INFLUENCE OF THE SEMEN DONORS, FREEZING PROTOCOLS, INSEMINATION TECHNIQUES AND *IN VIVO* FERTILITY
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คคนางค์ บูรณะอำนวย : น้ำเชื้อสุกรแช่แข็ง: อิทธิพลของพ่อสุกร วิธีการแช่แข็ง วิธีการผสมเทียมและความสมบูรณ์พันธุ์ของแม่สุกรหลังการผสมเทียมด้วยน้ำเชื้อแช่แข็ง. (BOAR SEMEN CRYOPRESERVATION: STUDIES ON THE INFLUENCE OF THE SEMEN DONORS, FREEZING PROTOCOLS, INSEMINATION TECHNIQUES AND *IN VIVO* FERTILITY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.น.สพ.ดร. มงคล เตชะกำพุ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ.ดร. เผดิจ ธรรมรักษ์, Prof. Heriberto Rodriguez-Martinez, Prof. Detlef Rath, 89 หน้า

การทดลองที่ 1 และ 2 ศึกษาปัจจัยที่มีผลต่อคุณภาพน้ำเชื้อสุกรแช่แข็ง รีดน้ำเชื้อจากสุกรพันธุ์แลนด์เรซ ยอร์กเชียร์ และดูรอค พันธุ์ละ 5 ตัวๆ ละ 3 ครั้ง แบ่งน้ำเชื้อเป็น 2 ส่วนเพื่อแช่แข็งในสารละลายที่เติมและไม่เติมสารอีเควิกซ์ เอสทีเอ็ม เพส บรรจุน้ำเชื้อในหลอดขนาด 0.25 และ 0.5 มล. ผลพบว่าการเติมสารอีเควิกซ์ในสารละลายก่อนการแช่แข็งมีผลเพิ่มคุณภาพน้ำเชื้อหลังการละลายอย่างมีนัยสำคัญ ($P<0.05$)น้ำเชื้อแช่แข็งที่บรรจุในหลอดขนาด 0.5 มล. มีคุณภาพดีกว่าในหลอดขนาด 0.25 มล. อย่างมีนัยสำคัญทางสถิติ และพบว่าความแตกต่างของพันธุ์และตัวพ่อสุกรมีผลต่อคุณภาพน้ำเชื้อแช่แข็ง โดยอสุจิของสุกรพันธุ์ยอร์กเชียร์มีแนวโน้มที่จะไวต่อกระบวนการแช่แข็งและละลายมากกว่าอีกสองพันธุ์ จากผลข้างต้นจึงเลือกวิธีการแช่แข็งน้ำเชื้อสุกรในสารละลายแช่แข็งที่เติมสารอีเควิกซ์และบรรจุน้ำเชื้อในหลอดขนาด 0.5 มล.

การทดลองที่ 3 และ 4 ศึกษาอัตราการปฏิสนธิที่ 2 วัน (การทดลองที่ 3) และอัตราการคลอดและขนาดครอก(การทดลองที่ 4) ของแม่สุกรหลังได้รับการผสมเทียมด้วยน้ำเชื้อแช่แข็งแบบสอดท่อเข้าตัวมดลูก (ใช้อสุจิทั้งหมด 2 พันล้านตัวต่อครั้ง) และแบบสอดท่อเข้าปีกมดลูก (ใช้อสุจิทั้งหมด 1 พันล้านตัวต่อครั้ง) และใช้การอัลตร้าซาวน์ผ่านทางทวารหนักเพื่อตรวจหาเวลาตกไข่ของสุกร *การทดลองที่ 3* แบ่งสุกรหลังหย่านมออกเป็น 2 กลุ่ม เพื่อผสมเทียมด้วยวิธี 1) สอดท่อเข้าตัวมดลูก (6 ตัว) และ 2) สอดท่อเข้าปีกมดลูก (6 ตัว) โดยผสมครั้งแรกหลังจากเริ่มพบการเป็นสัด 24 ชม. และผสมซ้ำทุกๆ 12 ชม. จนตกไข่ พบว่าอัตราการปฏิสนธิที่เกิดขึ้นในท่อนำไข่ข้างซ้ายและขวาของแม่สุกรแต่ละตัวทั้งในกลุ่มที่ 1 และ 2 มีค่าไม่แตกต่างกัน แต่เมื่อเปรียบเทียบผลระหว่างกลุ่มพบว่าอัตราการปฏิสนธิในกลุ่มที่ 1 (66%) สูงกว่ากลุ่มที่ 2 (31%) อย่างมีนัยสำคัญ ($P<0.05$) *การทดลองที่ 4* แบ่งแม่สุกรหลังหย่านมเป็น 2 กลุ่มๆ ละ 9 ตัวเพื่อผสมเทียมด้วยวิธี 1) สอดท่อเข้าตัวมดลูก และ 2) สอดท่อเข้าปีกมดลูก สุกรแต่ละตัวได้รับการผสมเทียม 2 ครั้งที่ 24 และ 36 ชม. หลังเริ่มพบการเป็นสัด ผลพบว่าไม่มีความแตกต่างกันของอัตราการคลอดและขนาดครอกระหว่างแม่สุกรทั้ง 2 กลุ่ม อย่างไรก็ดีขนาดครอกของกลุ่มที่ 1 มีแนวโน้มมากกว่ากลุ่มที่ 2 (10.5±2.9 และ 7.7±3.0 ตัวต่อครอก, $P>0.05$) จากผลข้างต้นจึงเลือกวิธีการผสมเทียมแบบสอดท่อเข้าตัวมดลูกมาใช้กับน้ำเชื้อสุกรแช่แข็งในการทดลองต่อไป

การทดลองที่ 5 ศึกษาประสิทธิภาพของฮอร์โมน 2 ชนิด ได้แก่ เอช ซี จี และจี เอ็น อาร์ เอช (บูเซอรีลิน) ในการควบคุมการตกไข่ของแม่สุกร: แบ่งสุกรหลังหย่านมเป็น 3 กลุ่ม 1) เป็นสัดตามธรรมชาติ (10 ตัว) 2) เหนี่ยวนำการตกไข่ด้วยฮอร์โมน เอช ซี จี (750 หน่วยสากลต่อตัว) (10 ตัว) และ 3) เหนี่ยวนำการตกไข่ด้วยฮอร์โมน จี เอ็น อาร์ เอช (10 มก. ต่อตัว) (13 ตัว) ตรวจการตกไข่ด้วยเครื่องอัลตร้าซาวน์แบบตรวจผ่านทางทวารหนัก ผลพบว่าฮอร์โมน เอช ซี จี มีแนวโน้มควบคุมการตกไข่ได้ดีกว่าฮอร์โมน จี เอ็น อาร์ เอช เนื่องจากไม่พบว่ามีแม่สุกรตัวใดในกลุ่มนี้เกิดถุงน้ำบนรังไข่ภายหลังได้รับฮอร์โมน และระยะเริ่มเป็นสัดถึงตกไข่ของแม่สุกรกลุ่มนี้มีความแปรปรวนน้อยที่สุด

การทดลองที่ 6 และ 7 ศึกษาความสมบูรณ์พันธุ์ของสุกรที่เลี้ยงในฟาร์มหลังได้รับการผสมเทียมแบบสอดท่อเข้าตัวมดลูกด้วยน้ำเชื้อแช่แข็ง: แบ่งแม่สุกรหลังหย่านมเป็น 2 กลุ่ม 1) สุกรตกไข่ธรรมชาติผสมครั้งแรกที่ 24 ชม. หลังพบการเป็นสัดและผสมซ้ำทุก 12 ชม. จนตกไข่ และ 2) สุกรที่ได้รับการเหนี่ยวนำการตกไข่ด้วยฮอร์โมน เอช ซี จี ผสมเทียม 1 ครั้ง ที่ 41 ชม. หลังพบการเป็นสัด (การทดลองที่ 6) และผสมที่ 36 และ 42 และ/หรือ 48 ชม. หลังพบการเป็นสัดจนกระทั่งตกไข่ (การทดลองที่ 7) ผลการทดลองไม่พบความแตกต่างกันของอัตราเข้าคลอดและขนาดครอกของแม่สุกรทั้ง 2 กลุ่ม ในทั้ง 2 การทดลอง ($P>0.05$) แต่ในการทดลองที่ 6 พบว่าสุกรที่ได้รับการเหนี่ยวนำการตกไข่และผสมเทียมเพียง 1 ครั้งมีแนวโน้มของอัตราการคลอดและขนาดครอกต่ำกว่าสุกรที่ตกไข่ธรรมชาติซึ่งได้รับการผสมหลายครั้ง และ ในการทดลองที่ 7 พบว่าจำนวนครั้งของการผสมเทียมสุกรในกลุ่มที่ 1 มีแนวโน้มมากกว่ากลุ่มที่ 2 (+0.5 ครั้งต่อตัว)

จากผลการทดลองทั้งหมดแสดงให้เห็นถึงความสำเร็จเบื้องต้นของการผลิตน้ำเชื้อสุกรแช่แข็งในประเทศไทย โดยการผสมเทียมแบบสอดท่อเข้าตัวมดลูกเป็นวิธีการที่เหมาะสมสำหรับน้ำเชื้อสุกรแช่แข็ง ซึ่งหากปฏิบัติในแม่สุกรที่ได้รับการเหนี่ยวนำการตกไข่ก่อนการผสมเทียมจะสามารถลดจำนวนครั้งของการผสมลงได้โดยไม่กระทบต่อความสมบูรณ์พันธุ์ของแม่สุกร

ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์
สาขาวิชา วิทยาการสืบพันธุ์สัตว์
ปีการศึกษา 2552

ลายมือชื่อนิสิต.....คคนางค์ บูรณะอำนวย.....
ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS: PIG, FROZEN SEMEN, INSEMINATION, FERTILITY

KAKANANG BURANAAMNUAY: BOAR SEMEN CRYOPRESERVATION: STUDIES ON THE INFLUENCE OF THE SEMEN DONORS, FREEZING PROTOCOLS, INSEMINATION TECHNIQUES AND *IN VIVO* FERTILITY. THESIS ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., Doctoratde 3^{e} cycle, THESIS CO-ADVISOR: ASSOC. PROF. PADET TUMMARUK, D.V.M., Ph.D, PROF. HERIBERTO RODRIGUEZ-MARTINEZ, D.V.M., Ph.D, PROF. DETLEF RATH, D.V.M., Ph.D, 89 pp.

EXP I and EXP II were conducted to evaluate factors affecting FT boar sperm quality. Three ejaculates from each of 15 purebred boars (5L, 5Y and 5D) were frozen using the lactose-egg yolk extender and 9% glycerol with or without 1.5% Equex STM Paste. The processed semen was loaded in 0.25- and 0.5-ml straws and frozen in a styrofoam box. The FT sperm quality was improved when adding Equex STM Paste into the freezing media ($P<0.05$) and was higher when loading into a 0.5-ml straw compared with a 0.25-ml straw ($P<0.05$). The breed of boar and the individual boars within the same breed significantly influenced most of the FT sperm quality and sperm of the Y boars seemed to be most sensitive to freezing-thawing process. Therefore, in the following experiments, boar semen were cryopreserved in the freezing extender containing Equex STM Paste and packaged in a 0.5-ml straw.

EXP III and EXP IV were conducted to evaluate fertilization rate at 2 days (EXP III) and farrowing rate (FR) and number of total piglets born per litter (TB) (EXP IV) of sows after IUI (using $2x10^9$ total sperm per dose) and DIUI (using $1x10^9$ total sperm per dose) with FT boar semen. Ovulation was determined using real-time B-mode ultrasonography. In EXP III, weaned sows were divided into 2 groups, IUI (n=6) and DIUI (n=6). The sows were inseminated at 24 h after the detection of estrus and repeated every 12 h until ovulation. At 48 h after ovulation, numbers of fertilized and unfertilized oocytes flushed from the oviducts were counted. The results revealed that fertilization rates between the left and right oviducts did not differ significantly in both groups. However, the rate of fertilization was superior for IUI compared to DIUI (66% vs 31%, $P<0.05$). In EXP IV, weaned sows were inseminated twice at 24 and 36 h using IUI (n=9) or DIUI (n=9). There were no differences in both FR and TB between IUI and DIUI. Nonetheless, TB of the IUI group tended to be higher than the DIUI group (10.5±2.9 vs 7.7±3.0 piglets per litter, $P>0.05$). Based on the results of EXP III and IV, the IUI procedure was therefore used in further studies.

EXP V Effectiveness of exogenous hormones, hCG and GnRH (buserelin), in controlling ovulation in sows was investigated. Weaned sows were randomly allocated to 3 groups: 1) the sows (n=10) were allowed to ovulate spontaneously; 2) the sows (n=10) were induced ovulation with 750 IU hCG; and 3) the sows (n=13) were controlled ovulation with 10 μg GnRH. The hormones were given at the standing estrus. Ovulation was determined using transrectal ultrasonography. The results indicated the efficacy of both hormones in controlling ovulation in sows. However, hCG seemed to be more effective than GnRH since, after administration, no incidence of ovarian cysts and less variation in the estrus-to-ovulation interval (EOI) among treated sows were observed.

EXP VI and EXP VII Fertility of sows after IUI with FT semen studied under farm conditions was evaluated. Weaned sows were assigned to 2 groups for IUI with low numbers of FT spermatozoa. Group I, spontaneously ovulating sows were inseminated at 24 h after estrus detection and repeated every 12 h until ovulation. Group II, hCG induced ovulating sows were inseminated once at 41 h after estrus detection (EXP VI) and were inseminated at 36 and 42 and/or 48 h after estrus detection, until ovulation (EXP VII). It was shown that no significant differences were found for the fertility between group I and II in both experiments ($P>0.05$). However, in EXP VI, both FR and TB in the induced ovulating sows which were inseminated once tended to be lower than the spontaneously ovulating sows inseminated several times; and in EXP VII, number of insemination in group I was higher than group II (+0.5 time/estrus).

The results of all trials indicated that semen cryopreservation and artificial insemination with FT boar semen could be successfully performed in Thailand. IUI seemed to be an appropriate insemination technique for FT boar semen and the number of insemination required for achieving acceptable fertility could be decreased when IUI was conducted in sows with induced ovulation.

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Field of study: Theriogenology Advisor's signature M. Techakumphu
Academic year: 2009 Co-advisor's signature Padet Tummaruk

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFLP	amplified restriction fragment length polymorphism
AI	artificial insemination
BA	number of piglets born alive per litter
BTS	Beltsville Thawing Solution
°C	Celsius
CL	corpora lutea
cm	centimeter
conc.	concentration
CPAs	cryoprotective agents
D	Duroc
DF	degree of freedom
DIUI	deep intra-uterine insemination
DMSO	dimethyl sulfoxide
eCG	equine chorionic gonadotrophin
EOI	estrus to ovulation interval
Exp.	experiment
FR	farrowing rate
FSH	follicle stimulating hormone
FT	frozen-thawed
GLM	general linear model
GnRH	gonadotrophin releasing hormone
h	hour
hCG	human chorionic gonadotrophin
i.m.	intramuscular
IU	international unit
IUI	intra-uterine insemination
kg	kilogram
L	Landrace
LEY	lactose-egg yolk

LH	luteinizing hormone
LN_2	liquid nitrogen
LS	litter size
LSD	least significant difference
LSM	least-square means
m	meter
mg	milligram
MHz	megahertz
min	minute
ml	milliliter
mm	millimeter
mOsm/kg	milli-osmole per kilogram
NAR	normal apical ridge acrosome
NR	non-return rate
ns	not significant
PI	propidium iodide
pLH	porcine luteinizing hormone
r	correlation coefficient
ROS	reactive oxygen species
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	second
SEM	standard error of means
sHOST	short hypo-osmotic swelling test
TB	number of total piglets born per litter
UTJ	utereotubal junction
wk	week
WOI	weaning to estrus interval
Y	Yorkshire
µg	microgram
µl	microliter

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The procedures for freezing boar semen have been studied and developed continuously during the past 60 years (Polge, 1956; Crabo and Einarsson, 1971; Crabo et al., 1972; Pursel and Johnson, 1975; Bamba and Cran, 1985; Almlid and Johnson, 1988; Fiser and Fairfull, 1990; Almlid and Hofmo, 1996; Eriksson and Rodriguez-Martinez, 2000a, b; Wongtawan et al., 2006; Kaeoket et al., 2008). The advantages for development of frozen semen include the preservation of the good genetic resource, the distribution of superior genetic boars, and the improvement of the transportation of spermatozoa across countries (Johnson et al., 2000). However, the utilization of frozen-thawed (FT) semen prepared for artificial insemination (AI) at present is estimated to be less than 1% of all insemination world wide (Wagner and Thibier, 2000). The most important reason is a lower fertilizing capacity of FT semen, when used for conventional AI compared to fresh semen. Conventional AI in pigs with a high number (5 to $6x10^9$ per dose) of FT spermatozoa often results in a decrease of 20 to 30% in farrowing rates (FR) and 2 to 3 in number of total piglets born per litter (TB) compared to extended fresh semen (Johnson et al., 2000). Poor sperm quality frequently found in FT boar semen is partly due to a high sensitivity of the boar spermatozoa to rapid cooling to a few degrees above 0 °C, the so-called “cold shock”, which the spermatozoa have to traverse during cryopreservation process. This is evidenced by the loss of viable spermatozoa and by more capacitation-like changes in the viable sperm (Watson, 2000). These changes result in a shorter survival time of the FT sperm in the female genital tract in comparison to its fresh and liquid-preserved counterparts (Pursel et al., 1978b; Watson, 1995).

A couple of the factors related to the susceptibility to cryodamage of boar spermatozoa and hence sperm quality after cryopreservation is the breed of boar and individual variation among boars (Johnson et al., 1981; Park and Yi, 2002; Thurston et al., 2002; Roca et al., 2006). Thurston et al. (2002) studied the effect of breed in 129 boars, 3 breeds: Landrace (L), Yorkshire (Y) and Duroc (D) by assessments of sperm motility and

sperm membrane integrity after freezing-thawing. It was found that spermatozoa of the L breed had the highest resistance to cryoinjury, while the Y breed yielded the maximal variation of sperm freezability among boars. On the other hand, Johnson et al. (1981) found that in comparison to L boars, a higher percentage of post-thaw sperm motility was found in Y boars. FT sperm quality in the Y boars was also better than that in the D boars under investigation of Park and Yi (2002). Because unanimous conclusion is not drawn yet and no such study has been performed under tropical climate, the study on this topic requires additional investigation.

The freezing package is one factor that affects FT sperm qualities. Theoretically, packages with a larger surface-to-volume ratio, e.g. pellets (Schorner, 1974), 0.25- and 0.5-ml straws (Larsson et al., 1976; Baron, 1986) allow more homogeneous freezing and thawing throughout the sample. Thus, FT spermatozoa using small packages are less damaged than those in the large packages, e.g. 5-ml straws (Eriksson, 2000). However, the small container contains a low number of spermatozoa, i.e. 250 to $500x10^6$ total spermatozoa per straw. These are not enough for a single dose of conventional AI in pig. Recently, a new large flat plastic container (the FlatPack®) has been developed (Eriksson and Rodriguez-Martinez, 2000b). This slightly improves the FT semen qualities and also *in vivo* fertility. However, using a large number of spermatozoa per dose, i.e. 3 to $6x10^9$ per dose restricts the number of insemination doses from each ejaculate (approximately 20 to 25 doses per ejaculate). This point should be concerned particularly when semen is collected from the high genetic boars. Therefore, new insemination procedures (intra-uterine insemination, IUI and deep intra-uterine insemination, DIUI) have been utilized. These techniques allow the use of a fewer number of spermatozoa per insemination dose, i.e. 150 to $2000x10^6$ per dose, without affecting *in vivo* fertility (Martinez et al., 2001; 2002). Hence, small freezing packages (e.g., 0.25-, 0.5-ml straws) that could load a low number of sperm become a suitable container for being used with these insemination methods. Nothling and Shuttleworth (2005) studied the effect of two straw sizes, i.e. 0.25- and 0.5-ml straws on FT sperm quality in dog and found that at 60 min post-thaw, 0.5-ml straws yielded 5.7% more progressively motile sperm and 6.5% fewer abnormal acrosomes than 0.25 straws. It was indicated that 0.5-ml straws are more favorable for FT dog semen.

Nonetheless, there have been no comparisons of these small containers for cryopreserved boar semen.

In order to improve FT boar sperm qualities, various substances have been added to semen during cropreservation. One of these substances includes Sodium Dodecyl Sulphate (SDS), which is known as Equex STM Paste (Nova Chemical Sales, Scituate In.C., MA, USA). This substrate can maintain sperm quality by enhancing protective action of egg yolk rather than affecting directly on the sperm plasma membrane (Pursel et al., 1978a; Strzezek et al., 1984). Pursel et al. (1978a) found that boar sperm incubated with 0.1% SDS in the extender devoid of egg yolk had a marked deleterious effect on acrosome morphology and motility of sperm. Since previous studies of SDS efficacy used relatively large freezing package, the study on effect of SDS on FT semen loaded in small containers seems to be necessary.

Owing to the weakness of FT spermatozoa as well as a large number of spermatozoa required per dose to achieve high fertility results, the cervical AI is likely to be inappropriate for FT boar semen. The deep insemination procedures for depositing sperm cells into the uterine body (IUI) or in the proximal 1/3 of one uterine horn (DIUI) have thus been utilized (Martinez et al., 2001; Watson et al., 2001; Sumransap et al., 2007; Tummaruk et al., 2007). Using IUI with fresh semen, an approximately 3-fold reduction in the number of spermatozoa ($1x10^9$ vs $3x10^9$ spermatozoa per dose) has been used without any significant adverse effects on FR and litter sizes (LS) in comparison to cervical AI with $3x10^9$ spermatozoa in 80 to100 ml (i.e., 91.1% and 86.9% FR, and 12.5 and 12.1 LS after cervical AI and IUI, respectively) (Watson and Behan, 2002). Presently, no fertility data are available using this insemination technique with FT boar spermatozoa. Using DIUI procedure, sperm dose can be deposited caudally 8 to 55 cm from the uterotubal junction (UTJ) (Martinez et al., 2002) and a 20-fold reduction in the number of fresh spermatozoa inseminated could be used without decreasing fertility when insemination were conducted in induced ovulating weaned sows (Martinez et al., 2001; 2002). On the other hand, DIUI with the same dose in the spontaneously ovulating sows yielded similar fertility but lower LS, despite no significant differences, compared with those obtained after conventional AI with $3x10^9$ sperm per dose (Day et al., 2003). Martinez et al. (2006) found that these problems could be overcome by increasing the number of spermatozoa used in each DIUI

dose to $600x10^6$. Using DIUI in hormonally treated weaned sows, the number of FT spermatozoa can be decreased from the 5 to $6x10^9$ used in conventional AI to $1x10^9$ without altering reproductive performance (Roca et al., 2003). However, the fertility of naturally estrous sows after DIUI with a low number ($1x10^9$) of FT spermatozoa (70% FR and 9.25 LS) were lower than those received following DIUI with $150x10^6$ of fresh spermatozoa (84% and 9.9). Insemination outside of the optimal insemination-ovulation period was a reason for the lower fertility of FT spermatozoa (Roca et al., 2003).

In tropical countries, cryopreservation of boar semen is nowadays performed in a limited scale and it has yet to be conducted in Thailand particularly for the commercial purpose. Concerning this point and obtained benefit in the future, the thesis topic is established.

1.2 Literature Review

At present, frozen boar semen is used on a very limited scale owing mainly to its lower fertility results compared with fresh semen (Johnson et al., 2000; Wagner and Thibier, 2000). Therefore, use of frozen boar semen is mainly confined to the introduction and/or improvement of superior genetics in pigs into the nucleus herds of the pig industry. It is of considerable importance to appreciate the future role that frozen boar semen would play such as in breeding programs with progeny testing, a proper timing of insemination times with semen available at the time of need and the use of semen in areas that are geographically far apart from AI centres.

The present review focuses on: 1) factors affecting the success of boar semen cryopreservation in the aspects of the semen donors, the composition of freezing extenders and freezing packages, 2) control of ovulation in sows by hormonal treatments for development of a fixed-time insemination protocol, as well as 3) *in vivo* fertility of FT boar semen.

1.2.1 Factors affecting the success of boar semen cryopreservation

Boar semen differs in several respects from the semen of other domestic animals. It is produced in large volume (200 to 250 ml) and is extremely sensitive to cold shock (Polge, 1956). The success of freezing boar semen depends on both internal and external factors. Internal factors include the inherent characteristics of spermatozoa and the existing

differences among boars and ejaculates, while external factors are composed of the composition of the extenders, freezing packages, and the method of freezing and thawing of the semen, for example (Johnson et al., 2000).

1.2.1.1 The semen donors

Variation between individuals in the extent to which their spermatozoa are damaged by freezing-thawing has been reports in many species including pig (Johnson et al., 1981; Park and Yi, 2002; Thurston et al., 2002; Holt et al., 2005). For instance, Thurston et al. (1999) assigned individual boars into good, average and poor freezability groups on the basis of their post-thaw sperm viability using a system of multivariate pattern analysis, and suggested that cryosurvival of the spermatozoa was not necessarily related to the observed quality of the semen sample. In addition to inter-animal variation, intra-animal variation such as difference between ejaculate fractions has also been described as a source of difference in boar sperm freezability (Pena et al., 2003b, 2006). Pena et al. (2003b) found that spermatozoa present in the first 10 ml of the sperm-rich fraction (portion I) better sustain cooling and freezing-thawing compared to those present in the rest of the ejaculate (portion II). These differences were manifested by motility patterns, the maintenance of membrane integrity and capacitation-like changes of spermatozoa after thawing. However, variation between ejaculate fractions is dependent of individual boars, with some boars differing in the ability of the two ejaculate portions to sustain cryopreservation, while in other boars such differences were not detected (Pena et al., 2006). The mechanisms underlying differences in cryosensitivity between different individuals and different ejaculate portions have yet to be elucidated, but there is some evidence for physiological differences between spermatozoa from individual boars. Harrison et al. (1996) demonstrated that the stimulatory effects of bicarbonate on the process of capacitation differ among individual boars. Also, the existence of differences in seminal plasma composition and sperm morphology has been hypothesized as a possible explanation for the distinct ability of different boars and different ejaculate portions to sustain cryopreservation (Zhu et al., 2000; Thurston et al., 2001). In general, boar sperm heads present in portion I were significantly shorter and wider than those present in portion II, detected by using computerized morphometric analysis (ASMA) (Pena et al., 2006). It has been hypothesized that such differences could be genetic in origin. Thurston et al. (2002)

using amplified restriction fragment length polymorphism (AFLP) technology to analyze genome of 22 Y boars indicated that 16 candidate genetic markers linked to genes controlling sperm freezability and these genomes varied among individual boars. Consequently, they may be useful for the prediction of both post-thaw semen quality and fertility of individual boars.

1.2.1.2 The composition of freezing extenders

A number of substances have been added to boar semen during cryopreservation in order to improve FT sperm quality. It has been investigated that egg yolk added to boar semen could protect sperm acrosomes during cold shock and hence reduce cryodamage of FT boar spermatozoa (Pursel et al., 1973; Paquignon, 1985). Protection has been claimed to be due to both phospholipids and the low density lipoprotein fraction in egg yolk (Gebauer et al., 1970; Foulkes, 1977). The mechanism of action is unclear but could be mediated by either a less intense cellular dehydration or by stabilization of the spermatozoa plasma membrane (Watson, 1975; Courtens and Paquignon, 1985).

Cryoprotective agents (CPAs) have been divided into those that penetrate the cell and those which remain extracellular. Glycerol considered as penetrating agents and other non-penetrating agents such as various sugars have been evaluated for cryoprotective effect in boar spermatozoa (Wilmut and Polge, 1977a, b). Glycerol in low concentrations (3 to 4%) has been utilized in various techniques of spermatozoa cryopreservation (Westendorf et al., 1975; Pursel and Johnson, 1975; Paquignon and Courot, 1976; Larsson et al., 1977). At these concentrations, glycerol gives maximum post-thaw viability and also *in vitro* fertilizing capacity of spermatozoa (Almlid and Johnson, 1988). Graham and Crabo (1972) demonstrated that both post-thaw motility and acrosome integrity of boar spermatozoa would be decreased when glycerol concentration reached 5%. Glycerol and other penetrating agents could improve FT sperm survival by penetrating spermatozoa and reduce the shrinkage of the cells developed during cooling (Mazur and Rigopoulos, 1983). They could also lower the freezing point of extra-cellular fluid via action of non-penetrating CPAs (Bwanga, 1991). Therefore, the damage of spermatozoa from the formation of intracellular ice occurred during freezing is reduced.

The success of the boar sperm cryopreservation was dramatically increased when the detergent SDS (later known as Equex STM Paste) was included in the cryopreservation protocol (Graham et al., 1971; Pursel et al., 1978a; Fraser and Strzezek, 2007). The addition of SDS to semen extenders decreases freeze-thaw damage to sperm in several species, including boar (Graham et al., 1971; Westendorf et al., 1975; Eriksson, 2000; Pena et al., 2003a; Axner et al., 2004). Pursel et al. (1978a) stated that the use of 0.5% Orvus Es Paste, a commercial preparation of SDS, in the BF5 extender significantly enhanced the preservation of fertilizing capacity concomitant with an increase in post-thaw percentages of acrosomal normal apical ridge (NAR) and motility of boar sperm. The beneficial effect of SDS on the sperm membrane is not fully understood, but it has been suggested that its protective effect is mediated through a change in the extending medium, by solubilization of the protective lipids in the egg yolk contained in the extenders. This effect enhanced the cold shock resistance of sperm (Arriola and Foote, 1987; Penfold and Moore, 1993).

1.2.1.3 Freezing packages

Boar spermatozoa have been frozen in many forms of packages. Pellet, a form of freezing bull semen on dry ice, was adapted to freeze boar semen and first reported by Pursel and Johnson in 1975. Boar spermatozoa have also been frozen in 5-ml maxi- (Westendorf et al., 1975), 0.5-ml medium- (Fazano, 1986; Baron, 1986) and 0.25-ml mini-straws (Larsson et al., 1976), as well as different types of 5-ml flat plastic bags (Bwanga et al., 1991; Eriksson and Rodriguez-Martinez, 2000a). All package forms have their own advantages and drawbacks. The 5-ml maxi-straw contains one insemination dose but has a relatively small surface-to-volume ratio, which constrains optimal freezing and thawing throughout the sample (Weitze et al., 1987). The plastic bags allow even more homogeneous freezing and thawing and also contain a whole insemination dose, but they are not suited for storage in standard liquid nitrogen containers, and therefore are not in commercial use (Eriksson, 2000). Pellets and the small straws (0.25- and 0.5-ml straws) have a cryobiologically suitable shape with a large surface-to-volume ratio; thus theoretically, FT spermatozoa in pellets and small straws are less damaged than those in maxi-straws (Bwanga et al., 1990; Berger and Fischerleitner, 1992). However, with pellets, there are difficulty in the identification of the doses and a risk of cross-contamination during storage, and the thawing procedure is rather complicated as well (Eriksson, 2000).

Also, the small packages could contain relatively few spermatozoa such as 250 to 500 $x10^6$ sperm per straw, which are not enough for a single dose of conventional AI in pigs. Eriksson and Rodriguez- Martinez (2000b) developed a new flat plastic container (the FlatPack®) for freezing boar semen. This package could contain a complete insemination dose, allows a quick and uniform freezing and thawing due to its large surface-to-volume ratio, and fits into any conventional liquid nitrogen container. Nonetheless, insemination with large numbers of spermatozoa, such as 5 to $6x10^9$ sperm per dose, reduces the number of AI doses per ejaculate. Achieving successful AI with fewer sperm is more important if using boars of superior genetic merit.

Nowadays, small freezing packages are generally used in conjunction with the new deep transcervical AI procedures (i.e., IUI and DIUI). Using these insemination techniques, semen could be deposited directly into different parts of the uterus (Martinez et al., 2001; Watson et al., 2001); therefore, the number of thawed spermatozoa required for achieving acceptable fertility is diminished. Roca et al. (2003) indicated that the DIUI technique potentially allows the number of FT boar spermatozoa to be as few as $1x10^9$ without adversely affecting *in vivo* fertility. Since small packages are appropriate for use with deep insemination procedures, further studies on the effect of straw size (0.25- vs 0.5-ml) on FT boar sperm quality are likely to be needed in order to find the most suitable freezing container.

1.2.2 Control of ovulation in sows by hormonal treatments for development of a fixed-time insemination protocol

In pigs, ovulation time can be precisely monitored by non-invasive methods such as the determination of luteinizing hormone (LH), estrogen or progesterone (Soede et al., 1994). However, such methods are of no practical value for AI application. Non-invasive transcutaneous ultrasonography (Weitze et al., 1989; Waberski et al., 1994) and transrectal ultrasonography (Soede et al., 1992; Mburu et al., 1995) of the ovaries have been used to monitor the occurrence of ovulation. On average, ovulation occurs when 2/3 of the standing estrus has passed, which means for most sows 35 to 45 h after onset of estrus (Soede and Kemp, 1997). However, a large variation in the duration of estrus among individuals exists,

and occurrence of ovulation has been reported to occur between 10 to 85 h (Waberski et al., 1994), 10 to 58 h (Soede et al., 1995) or 35 to 43 h (Mburu et al., 1995) after estrus onset. This renders difficulty to determine the optimal time of AI in pig.

Timing of insemination in relation to ovulation is one of the key roles for successful fertilization (Soede et al., 1995). This is even more important when FT boar spermatozoa are used. The reason is that the lifespan of the FT spermatozoa in the female reproductive tract is as short as 4 to 8 h (Einarsson and Viring, 1973; Pursel et al., 1978b; Holt, 2000). Repeated insemination every 4 to 8 h might be needed for FT boar semen to ensure that the insemination occurs close to the time of ovulation. However, such multiple insemination regimens are unpractical for routine use.

Consequently, the control of ovulation using exogenous hormones in gilts and sows has been established to allow a more precise timing of insemination relative to ovulation (Guthrie, 1977; Gooneratne et al., 1989). Roca et al. (2003) controlled estrus and ovulation in sows by using 1,250 IU equine chorionic gonadotrophin (eCG) at 24 h after weaning and 750 IU human chorionic gonadotrophin (hCG) at 72 h later, and the ovulation took place at about 40 h after the hCG application. Gilts at 8.5 months of age received 800 IU eCG at 24 h after the 15 days feeding of progesterone (20 mg/day) and were injected with 75 μg gonadotrophin releasing hormone (GnRH) analog at 80 h thereafter. The mean time from GnRH administration to ovulation onset was 36.4 h (Brussow et al., 1996). In addition, estrus to ovulation interval (EOI) of approximately 38 h was investigated in sows which were controlled ovulation using porcine luteinizing hormone (pLH) (Candini et al., 1999, 2001; Cassar et al., 2005). The exogenous eCG can induce estrus by mimicking action of the reproductive hormone named “follicle stimulating hormone” (FSH) which is secreted from the anterior pituitary gland and results in follicle growth, increased levels of estradiol, and estrus. The hCG component mimics the endogenous hormone LH, also released from an anterior portion of the pituitary gland, that leads to ovulation (Coffey et al., 1997).

1.2.3 Fertility after transcervial deep AI of FT boar semen

1.2.3.1 Conventional AI in pigs

Conventional AI in domestic pigs is practiced with doses of approximately $3x10^9$ spermatozoa extended to a volume of 80 to 100 ml (Stratmen and Self, 1960). Semen doses

are stored at temperatures ranging 16 to 20°C, usually for up to 3 days in simple extenders, but longer when using other extenders (Dube et al., 2004; Vyt et al., 2004). The semen is deposited into the posterior region of the cervix by using a disposable, often an intra-cervical, catheter whose tip stimulates the corkscrew shape of the boar penis and engages with the posterior folds of the cervix as it occurs during natural mating. In general, the AI process starts 12 h after detection of standing estrus and it is repeated every 12 to 18 h until standing estrus is no longer shown. When proper detection of estrus is performed, the FR and LS are comparable with those achieved by natural mating, reaching over 90% of FR and mean LS of 14 piglets (Nissen et al., 1997).

1.2.3.2 Use of FT semen in porcine AI

Contrary to what occurs in cattle, where FT semen is routinely used for AI (Curry, 2000), cryopreserved boar semen is used in less than 1% of the AIs performed around the world (Johnson et al., 2000; Wagner and Thibier, 2000). The reasons behind this restricted use of FT boar semen are the low survivability of spermatozoa after the freezing-thawing process and the shorter lifespan of the surviving spermatozoa. These result in lower FR and small LS compared with AI using semen preserved in liquid form (Johnson et al., 2000). Furthermore, owing to the restricted lifespan of the FT boar spermatozoa, excessive sperm numbers are used, often 5 to 6 $x10^9$ spermatozoa per dose. Moreover, at least two AIs are usually performed per estrus in order to reach acceptable fertility rates in the field (Johnson, 1985; Eriksson et al., 2002). Altogether, few doses can be obtained from a single ejaculate and too many spermatozoa are used to ensure fertilization. A decrease in the number of spermatozoa per dose is therefore required to improve the use of ejaculates, so that the production will be cheaper and the use of genetically superior sires more effective.

1.2.3.3 Transcervical deep AI

Although few spermatozoa are required for fertilization within the oviduct, this reduced number is the product of a sequential and very effective reduction along the process of sperm transport in the female reproductive tract (i.e., 25 to 40% of inseminated spermatozoa are lost with the blackflow and 50% of the rest of the spermatozoa are ingested by leukocytes in the uterus; Matthijs et al., 2003). The problem to be overcome during AI is to get an adequate number of spermatozoa to the UTJ that could ensure the

establishment of the functional sperm reservoir with enough viable, potentially-fertile spermatozoa to ensure maximal fertilization. One strategy proposed to accomplish this is to decrease the number of spermatozoa per AI-dose, by depositing the semen directly in the uterus, and get sufficient spermatozoa into the UTJ. Such deep AI with reduced sperm numbers is a relatively new reproductive practice that has attracted the attention of the swine industry. Such a method could also be advantageous for the spreading of AI with FT semen.

There are basically two non-surgical procedures for depositing spermatozoa into the pig uterus. These include semen deposition either in the uterine body (Watson et al., 2001; Watson and Behan, 2002) or into the uterine horn (Martinez et al., 2001; 2002).

1.2.3.3.1 Intra-uterine insemination (IUI)

A non-traumatic transcervical catheter that allows an easy penetration of the cervix and deposition of semen in the uterine body of the sow has been designed. Briefly, a conventional catheter (outer catheter) is placed toward and locked into the cervix. An inner tube (around 4 mm outer diameter) is passed through the outer catheter, along the cervical lumen, to reach the uterine body or the posterior part of one of the uterine horns (about 200 mm beyond the tip of the outer catheter). The IUI catheter can be used with minimal training and it does not seriously delay the process of insemination, although it can only be safely used in sows (Watson and Behan, 2002). Under commercial conditions, use of the IUI catheter with extended fresh semen can reduce sperm numbers to $1x10^9$ spermatozoa per insemination dose and results in a comparable effect on both FR and LS (89% FR and 12 LS) compared with 91% FR and 12.5 LS after conventional AI with $3x10^9$ spermatozoa (Watson and Behan, 2002). However, in the field trials carried out by Rozeboom et al. (2004) and Roberts and Bilkei (2005), FR were similar between IUI with $1x10^9$ spermatozoa and conventional AI with $3x10^9$ spermatozoa, but IUI sows had significantly less piglets born per litter (1.5 to 2 smaller LS). The reasons for the loss in LS have not been clarified. Rozeboom et al. (2004) suggested that several factors such as aged spermatozoa, improper semen handling or insemination-ovulation interval can cause decreases in reproductive performances when low numbers of spermatozoa are used, and in order to obtain consistently high fertility results, a slightly higher number of spermatozoa

should be considered. Presently, whereas IUI with fresh semen has been reported, fertility results after IUI with FT semen are not available yet.

1.2.3.3.2 Deep intra-uterine insemination (DIUI)

Non-surgical DIUI has been performed in non-sedated pigs using a flexible fiber optic endoscope (1.35 m length, 3.3 mm outer diameter) inserted via the vagina and cervix to reach the upper segment of one uterine horn (Martinez et al., 2001). The procedure required 3 to 5 min in 90% of the females. After this DIUI, only 1% of the sows showed signs of uterine infection. However, the endoscope is a highly expensive instrument and unpractical for routine use. A flexible catheter was therefore developed on the basis of the propulsion force and flexibility of the fibroendoscope (Martinez et al., 2002). The method allows deposition of low sperm doses of either fresh or FT spermatozoa. Moreover, the technology can be successfully used to produce piglets with sex-sorted spermatozoa (Vazquez et al., 2003), or for embryo transfer (Martinez et al., 2004).

Using fresh semen, FR and LS were not statistically different between DIUI with $150x10^6$ spermatozoa per dose and conventional AI with $3x10^9$ spermatozoa, ranging from 83 to 87% FR and 9.2 to 10.4 LS (Vazquez et al., 2001). Nonetheless, LS was always lowest in the DIUI sows. Similarly, although no differences in FR were found (83% and 90% for DIUI and conventional AI, respectively), DIUI sows had less LS (10.5 and 12.9, respectively) (Day et al., 2003). The low LS achieved in the DIUI sows inseminated with $150x10^6$ spermatozoa probably resulted from the high incidence of unilateral or incomplete bilateral fertilization, and could be overcome by increasing the number of inseminated spermatozoa to $600x10^6$ sperm per dose (Martinez et al., 2006). On the other hand, when a single DIUI with $150x10^6$ spermatozoa was performed in hormonally induced ovulating sows, both FR and LS of DIUI sows (83% and 9.7) were not different from those of conventional AI sows (83% and 10) (Martinez et al., 2002). When FT semen ($1x10^9$ spermatozoa per dose) was used for DIUI, promising results were obtained. With hormonally induced ovulation and a single DIUI, the FR was 77.5% and LS was 9.3, while with spontaneous ovulation and two DIUIs, the FR was 70% and LS was 9.3. The lower fertility obtained in the latter group resulted from the suboptimal insemination-ovulation period (Raca et al., 2003). Bolarin et al. (2006) working with spontaneously ovulating sows

(n=407) obtained FR of over 80% and about 10 piglets born per litter when two DIUIs, at 6 h interval, with only $1x10^9$ FT sperm per dose were conducted at the peri-ovulatory period. It has been suggested that DIUI should be carried out ≤ 8 h before spontaneous ovulation when FT spermatozoa are used (Wongtawan et al., 2006).

1.3 Objectives

1. To produce cryopreserved boar semen in Thailand. The specific objectives were to investigate effects of straw volume, Equex STM Paste added to a freezing extender and of the individual differences on boar sperm quality after cryopreservation.

2. To evaluate fertility results (fertilization rate, FR and LS) of FT boar semen after DIUI compared with IUI in multiparous sows

3. To study feasibility of the use of FT boar semen for transcervical deep AI in a commercial pig farm. The specific objectives were to investigate the timing of ovulation in sows treated with hormones for development of a fixed-time insemination protocol and to evaluate fertility results (FR and LS) of FT boar semen after transcervical deep AI in spontaneous and induced ovulating sows.

CHAPTER II

FACTORS INFLUENCING FT BOAR SPERM QUALITY

2.1 Experiment 1: Effects of straw volume and Equex STM Paste on boar sperm quality after cryopreservation

2.1.1 Abstract

The present experiments were designed to study the effect of adding the detergent Equex STM Paste to freezing extender, and of straw volume (0.25-ml versus 0.5-ml), on boar sperm quality after cryopreservation. Three ejaculates from each of four purebred boars (3 Landrace and 1 Yorkshire) were collected and frozen with a lactose-egg yolk extender containing glycerol with or without 1.5% Equex STM Paste. The extended semen was loaded into either 0.25- or 0.5-ml straws. The straws were placed in liquid nitrogen (LN_2) vapor approximately 3 cm above the level of LN_2 for 20 min and then were plunged into LN_2. Thawing was achieved in warm water at 50°C for 12 sec and then was incubated in a 38°C water-bath for 30 min before evaluating sperm quality. Results showed that the sperm motility, viability and acrosomal normal apical ridge (NAR) were improved (P<0.001) when Equex STM Paste was added to the freezing extender. There was no difference (P=0.48) in sperm motility between 0.25- and 0.5-ml straws when Equex STM Paste was added. The percentages of viable and of NAR sperm in 0.5-ml straws were higher than those in 0.25-ml straws (P=0.02, P=0.0003, respectively). The percentages of membrane intact sperm evaluated using the short hypo-osmotic swelling test (sHOST) were not affected by straw volume or the adding of Equex STM Paste (P>0.05). The results of these investigations suggested that Equex STM Paste exerts a beneficial effect on the quality of cryopreserved boar semen and this cryopreservation protocol was favorable for a 0.5-ml straw.

2.1.2 Introduction

The production of frozen-thawed (FT) semen in pig was first reported more than 50 years ago (Polge, 1956). The advantages of FT boar semen included the preservation of the good genetic resource, which can be used for a longer period than the herd life of the boar, and the transportation of valuable genetic material over a long distance, e.g. export. However, low fertility is a common problem following insemination of sows with frozen semen compared to fresh semen (e.g., Almlid and Hofmo, 1996). This resulted from a dramatic reduction in the sperm viability post-thaw due to high sensitivity of boar sperm to cold shock.

The quality of the sperm after cryopreservation is affected by a number of factors, including the type of semen package (Bwanga et al., 1991). In general, packages with a larger surface-to-volume ratio, such as pellets, 0.25- and 0.5-ml straws, allow a more homogeneous freezing and thawing temperature throughout the sample. Thus, FT sperm frozen in small packages are less damaged than those frozen in larger packages such as 5-ml straws (Eriksson, 2000). However, small packages contain relatively few sperm, usually approximately 250 to 500 $x10^6$ total sperm per straw. These are not enough for a single dose of conventional artificial insemination (AI) in pigs. Recently, a new large flat plastic container (the FlatPack®) was developed (Eriksson and Rodriguez-Martinez, 2000b), which can slightly improve quality and *in vivo* fertility of the FT boar sperm. However, insemination with large numbers of sperm per dose, such as 5000 to 6000 $x10^6$, reduces the number of AI doses per ejaculate. Achieving successful AI with fewer sperm is more important if using boars of superior genetic merit or sex-sorted sperm. Recently, the deep insemination techniques have been developed for swine. Using these techniques, semen could be deposited into the uterine body (intra-uterine insemination, IUI; Watson et al., 2001) or into the proximal third of the uterine horn (deep intra-uterine insemination, DIUI; Martinez et al., 2001). The DIUI technique potentially allows the number of FT boar sperm per insemination to be as few as 1000 $x10^6$ per dose without adversely affecting *in vivo* fertility (Roca et al., 2003). Normally, 250 and 500 $x10^6$ boar sperm can be loaded into 0.25- and 0.5-ml straws, respectively, making these packages suitable for use with DIUI. In dog, the 0.25 and 0.5 straws are commonly used as the freezing package of sperm and

Nothling and Shuttleworth (2005) found that at 60-min post-thaw, the 0.5-ml straws yielded 5.7% more progressively motile sperm and 6.5% fewer abnormal acrosomes than 0.25 straws, indicating that 0.5-ml straws are more favorable for FT dog semen. Nonetheless, we are not aware of any comparisons of these small containers for cryopreservation of boar sperm.

The success of the boar sperm cryopreservation was dramatically increased when the detergent sodium dodecyl sulphate (SDS; later known as Equex STM Paste) was included in the cryopreservation protocol (Graham et al., 1971; Pursel et al., 1978a; Fraser and Strzezek, 2007). The addition of SDS to semen extenders decreases freeze-thaw damage to sperm in several species, including boar (Graham et al., 1971; Westendorf et al., 1975; Eriksson, 2000; Pena et al., 2003; Axner et al., 2004). Pursel et al. (1978a) stated that the use of 0.5% Orvus Es Paste, a commercial preparation of SDS, in the BF5 extender significantly enhanced the preservation of fertilizing capacity concomitant with an increase in post-thaw percentages of acrosomal normal apical ridge (NAR) and motility of boar sperm. The beneficial effect of SDS on the sperm membrane is not fully understood, but it has been suggested that its protective effect is mediated through a change in the extending medium, by solubilization of the protective lipids in the egg yolk contained in the extenders. This effect enhanced the cold shock resistance of sperm (Arriola and Foote, 1987; Penfold and Moore, 1993). Since previous studies of SDS efficacy used relatively large FT semen packages, the present study was conducted to evaluate the effects of straw volume (0.25-ml and 0.5-ml) used for packaging frozen semen on boar semen quality after cryopreservation, and to investigate the effect of Equex STM Paste (Nova Chemical Sales Inc., Scituate, MA, USA) added to a freezing extender, on boar sperm quality post-thaw.

2.1.3 Materials and methods

2.1.3.1 Sperm freezing and thawing protocols

The sperm-rich fractions of three ejaculates from each of four purebred boars (3 Landrace and 1 Yorkshire) aged between 1 and 3 years old were used. The boars were of proven fertility and held in 2 commercial herds in Nakorn Pathom province, Thailand. The

ejaculates were collected with a minimum of a one-week interval using the gloved-hand technique. The ejaculates were kept in an insulated thermos flask at approximately 38°C during transport to the laboratory within 40 min after collection. Fresh semen that had a minimum of 70% sperm motility was used for further processing. The semen was processed according to Westendorf et al. (1975) and Gadea et al. (2004) with some modifications. Shortly after collection, the semen was diluted with isothermal Beltsville Thawing Solution (BTS; Minitüb, Abfüll-und Labortechnik GmbH & Co. KG, Germany) extender at a ratio of 1:1 (v/v). Diluted semen was placed at 15°C for 2 h and later centrifuged at 800x g for 10 min. The supernatant was discarded and the pellet resuspended (1:1) with lactose-egg yolk (LEY) extender (80 ml of 11% lactose solution and 20 ml egg yolk). After further cooling to 5°C over a 90-min period, two parts of semen were mixed with one part of extender III (LEY extender and 9% glycerol with or without 1.5% Equex STM Paste).

In experiment A, effect of straw volume on boar semen quality after cryopreservation was studied. The processed semen (with 1.5% Equex STM Paste) was loaded into 0.25- and 0.5-ml straws (Bio-Vet, Z.I. Le Berdoulet, France). In experiment B, effect of Equex STM Paste on FT boar semen quality was determined. The extended semen with and without Equex STM Paste was loaded into 0.5-ml straws. The final concentration of sperm frozen was approximately $1x10^9$ sperm per ml with 3% glycerol. The straws were sealed with PVC powder before placing in liquid nitrogen (LN_2) vapor at 3 cm above the level of LN_2 for 20 min and then plunged into LN_2.

Because storage of frozen boar semen in LN_2 for 30-min and 1-wk yielded similar post-thaw sperm quality ($P>0.05$; data not shown), the straws were thawed at approximately 30 min after freezing. Thawing was achieved by immersing the straws in water at 50°C for 12 sec (Selles et al., 2003). Immediately after thawing, the semen was diluted (1:4) with an extender consisting of 95% BTS and 5% LEY extender. The addition of LEY extender prevented the sperm from sticking to the glassware used during motility analysis. The extended thawed semen was incubated in a 38°C water-bath for 30 min before evaluating the post-thaw sperm quality.

2.1.3.2 Evaluation of semen quality

Sperm concentration was assessed by direct cell count using Bürker haemocytometer (Boeco, Germany) (Ax et al., 2000). Visual motility of both fresh and FT sperm was evaluated at 38°C under a light microscope with 400x magnification. The motility was assessed by the same technician, throughout the experiments.

The percentages of viable sperm and NAR were determined separately by eosin-nigrosin staining as described by Dott and Foster (1972), it having a similar ability to delineate intact and damaged acrosomes to examination on a wet smear by differential interference-contrast microscopy (Dott and Foster, 1972; Chenoweth et al., 1984; Bamba, 1988). Evaluation was undertaken using a bright-field microscope at 1000x magnification with 200 sperm being examined for each smear.

The integrity of sperm plasma membranes was assessed using the short hypo-osmotic swelling test (sHOST) described by Perez-Llano et al. (2001), with some modifications. Briefly, aliquots of each semen sample (100 µl) were incubated at 38°C for 10 min, with 1000 µl of either hypo-osmotic (75 mOsm/kg) or iso-osmotic (300 mOsm/kg) solutions. The solutions were prepared with fructose and Na-citrate in distilled water and final osmolality was measured by freezing point depression. Following the 10-min incubation, 200 µl of the semen-hypo-osmotic solution was fixed in 1000 µl of hypo-osmotic solution plus 5% formaldehyde (Merck, Germany) for later evaluation. Sperm coiling was assessed by placing 20 µl of well-mixed sample on a warm slide, which was covered with a cover slip before being observed under a light microscope (1000 x) and 200 sperm per slide were counted. In order to determine the percentage of sperm with intact membranes, the proportion of coiled tail sperm from a control sample (300 mOsm/kg) was subtracted from the result of hypo-osmotic condition.

2.1.3.3 Statistical analysis

The statistical analyses were performed using SAS (SAS version 9.0, Cary, N.C., USA). Descriptive statistics was used to describe semen quality before and after thawing. Data on the motility, viability, NAR and sHOST positive sperm after thawing were analyzed using the General Linear Model (GLM) procedure. The first statistical model (Exp.A) included the effects of individual boars (1 to 4), repeated ejaculation (1 to 3) and straw volume (0.25- and 0.5-ml). The second statistical model (Exp.B) included the

effects of individual boars (1 to 4), repeated ejaculation (1 to 3) and Equex STM Paste (0 and 1). Least-square means were obtained from each class of the factors and were compared by using least significant difference test (LSD). Differences with $P<0.05$ were regarded as statistical significance.

2.1.4 Results

In the present study, repeated ejaculation had no significant effect on FT semen quality, while individual boars significantly affected all parameters studied (Table 1). However, all boars displayed similar trends across treatments (data not shown) and so only the effects of straw volume and Equex STM Paste on boar sperm quality after cryopreservation were considered and presented in Table 2.

The motility, viability and NAR of FT sperm were significantly improved ($P<0.001$) when Equex STM Paste was added into the freezing media. There was no significant difference in FT sperm motility between 0.25- and 0.5-ml straws (Table 2). The percentages of viable and NAR sperm in 0.5-ml straws were significantly higher than those in 0.25-ml straws ($P=0.02$, $P=0.003$, respectively). Post-thaw sperm response to sHOST were not significantly affected by either the volume of straws or the addition of Equex STM Paste ($P>0.05$).

Table 1 Significance levels of factors affecting sperm quality after frozen-thawed

Factors[1]	DF[2]	Motility	Viability	NAR	sHOST
Boar	3	*	*	*	*
Ejaculate	2	ns	ns	ns	ns
Straw volume[3]	1	ns	*	*	ns
Equex STM Paste[4]	1	*	*	*	ns

*= $P\leq0.05$; ns = not significant [1]All factors were included in the same statistical model, [2]DF= degree of freedom, [3]Statistical model 1 (Exp.A), [4]Statistical model 2 (Exp.B)

Table 2 Fresh and post-thaw sperm quality (means±SD) of the 4 boars (3 ejaculates per boar)

		Exp.A		Exp.B	
Sperm quality	Fresh	0.25 ml	0.5 ml	0.5 ml (+Equex)	0.5 ml (-Equex)
Motility (%)	74.2± 3.6	23.8± 8.8^{a}	25.8± 10.8^{a}	25.8± 10.8^{A}	6.3± 5.3^{B}
Viability (%)	75.5± 5.8	24± 10.1^{b}	31.4± 10.5^{a}	31.4± 10.5^{A}	7.8± 6.1^{B}
NAR (%)	85.4± 5.1	15.8± 5.5^{b}	28.5± 12.4^{a}	28.5± 12.4^{A}	3.8± 5.3^{B}
sHOST (%)	57.5± 21.3	10.5± 7.7^{a}	11.5± 9^{a}	11.5± 9^{A}	7.8± 4.2^{A}

Value with different letters (a, b or A, B) indicate significant difference within rows in each experiment (P<0.05)

2.1.5 Discussion

Although there was no difference in post-thaw sperm quality between ejaculates of the same boar, significant differences among boars were observed (Table 1). It was a result of individual differences in the composition of sperm plasma membrane (e.g., cholesterol/ phospholipids ratio, phospholipids/protein ratio) and the distribution of membrane components which influence semen freezability (Holt, 2000; Zeng et al., 2001). However, in this study, the individual differences were disregarded because all of the four boars showed similar trends of FT sperm quality across treatments.

Due to the smaller surface-to-volume ratio of 0.5-ml straws compared to 0.25-ml straws, theoretically, FT sperm quality packaged in 0.5-ml straws should not be better than that in 0.25-ml straws when using the same freezing and thawing protocols. Nothling and Shuttleworth (2005) studied the effect of straw size on post-thaw quality of dog sperm, found that during the freezing process a change in temperature of extended semen in 0.25- and 0.5-ml straws while being suspended at 3.5 cm above LN_2 vapor in a styrofoam box was very similar. The temperature inside both straws tended to stabilize between -130 and -140°C from 10-min post-lowering the straws into the box. Therefore, in the present study, we hypothesized that boar sperm frozen in 0.25- and 0.5-ml straws using the same

cryopreservation protocol (i.e., placed 3 cm above LN_2 for 20 min) should not differ in their post-thaw quality. The results showed that there was no significant difference in FT sperm motility or response to sHOST between 0.25- and 0.5-ml straws. On the other hand, the proportions of viable and NAR acrosomes of FT sperm in 0.5-ml straws were higher than those in 0.25-ml straws (Table 2). The reason for this is not known but we suggest it does not involve the freezing and thawing protocols, since they are the same as used by other authors (Gadea et al., 2004; Pena et al., 2004; Pelaez et al., 2006). Interestingly, a favorable result of 0.5-ml compared to 0.25-ml straws has also been observed in dog semen (Nothling and Shuttleworth, 2005). Despite being frozen and thawed using their own optimal protocols, the quality (i.e., motility, acrosome integrity, longevity and GOT activity) of FT dog sperm packaged in 0.5-ml containers was still better than that in 0.25-ml (Nizanski and Dubiel, 2003). To confirm similar conclusions with boar sperm, it might be necessary to conduct additional studies to increase the number of boars tested, to change the type of freezing extender and to change type and/or concentration of cryoprotective agents.

In experiment B, FT sperm quality parameters, except sHOST, were significantly improved when Equex STM Paste was added into a freezing extender. This result is in agreement with previous studies indicating that the addition of SDS to a freezing extender enhanced boar sperm quality (e.g., membrane integrity, motility, and normal acrosomes) after cryopreservation (Graham et al., 1971; Pursel et al., 1978a; Fraser and Strzezek, 2007). Similar findings have also been observed with the sperm of other species including bull (Arriola and Foote, 1987), dog (Pena and Linde-Forsberg, 2000; Tsutsui et al., 2000; Pena et al., 2003a), cat (Axner et al., 2004), mouse (Penfold and Moore, 1993), ram (Akourki et al., 2004) and goat (Mohamed et al., 2004). The mechanism for the action of SDS in protecting sperm from cryo-damage has not been fully elucidated. It was reported that the protective effect of SDS is an indirect effect, by dissolving or dispersing protective components in the egg yolk of the freezing extender, which increases the protective effect of the egg yolk on the sperm membrane (Pursel et al., 1978a; Strzezek et al., 1984). In the present study, Equex STM Paste had no significant effect on the percentages of sHOST positive sperm after being frozen and thawed. This might be due to the Equex STM Paste having an ability to protect membrane structural integrity but not ion exchange capacity,

and hence osmotic regulation, of the sperm membrane. This suggestion remains to be verified.

Although beneficial effects of SDS on sperm are well documented, negative effects have also been observed. For instance, boar sperm were severely damaged (i.e., the decrease in the percentages of NAR acrosomes and motile sperm) when incubated for 1 h at 25°C in an extender containing as little as 0.1% SDS but lacking egg yolk (Pursel et al., 1978a), or frozen in an extender with an excessive amount of SDS, which varied under different conditions (Graham and Crabo, 1972; Pursel et al., 1978a). Moreover, Pena and Linde-Forsberg (2000) reported that a prolonged exposure of dog sperm to SDS or to SDS-treated egg yolk lipoproteins exerted a direct negative effect, by excessively increasing sperm cell membrane permeability and fluidity possibly through the solubilization and disruption of the lipid and lipoprotein constituents of the sperm cell membrane (Koefoed-Johnsen and Mann, 1954; Helenius and Simons, 1975).

Equex STM Paste included in the semen extender can enhance general characteristics (e.g., motility, NAR acrosomes) of FT boar sperm packaged in 0.5-ml straws, as shown in results of the present study (experiment B). Nevertheless, success in fertilization of sperm frozen in presence of Equex STM Paste might not be superior to that of sperm frozen in the absence of the detergent, since Equex STM Paste may interact with oocytes, sperm and/or the oviductal environment at the time of fertilization which affects fertilization outcome. To examine this suggestion, assessment of *in vitro* and/or *in vivo* fertility of FT boar sperm packaged in 0.5-ml straws cryopreserved with or without Equex STM Paste needs to be determined.

In summary, the results of present study suggested that Equex STM Paste exerts a beneficial effect on the quality of cryopreserved boar semen and this cryopreservation protocol was favorable for a 0.5-ml straw.

2.2 Experiment 2: The production of boar semen cryopreservation in Thailand: post-thaw semen quality and variation among ejaculates

2.2.1 Abstract

The present study aimed to produce cryopreserved boar semen in Thailand. Fresh and post-thaw semen quality and variation among ejaculates were investigated. Forty-five ejaculation of semen from 15 boars were collected. The semen was diluted with an isothermal Beltsville Thawing Solution (BTS) extender, held at 15 °C for 2 h and centrifuged. The semen precipitant was re-suspended with lactose-egg yolk (LEY) extender. The diluted semen was cooled down to 5°C within 90 min. Two parts of semen were mixed with one part of LEY extender containing 9% glycerol and 1.5% Equex STM Paste. The extended semen samples were loaded into 0.5-ml straws. The straws were placed on liquid nitrogen (LN_2) vapor for 20 min, then plunged into LN_2 and frozen. Thawing was conducted by immersing the straws in water at 50 °C for 12 sec. Sperm concentration, sperm motility, sperm viability, percentage of normal apical ridge (NAR) acrosomes, sperm plasma membrane function (sHOST) and sperm plasma membrane integrity (SYBR) were evaluated. On average, the sperm concentration post-thaw was 811 $x10^6$ spermatozoa per ml. The motility, the viability, the NAR, the sHOST and the SYBR of boar spermatozoa post-thaw were 28%, 36%, 26%, 19% and 31%, respectively. The motility of frozen-thawed (FT) spermatozoa significantly correlated with the sperm viability (P<0.001), the NAR (P<0.02), the sHOST (P<0.001) and the membrane integrity (SYBR) (P<0.001). The higher concentration of FT semen resulted in the lower membrane integrity (r=-0.3, P=0.04). Post-thaw sperm concentration was significantly correlated with the volume of fresh semen (r=-0.30, P=0.04). The breed of boar and the individual boars within the same breed significantly influenced most of the post-thaw sperm parameters.

2.2.2 Introduction

Artificial insemination (AI) in pig is nowadays widely used in the pig industry all over the world. Conventionally, the AI was performed using fresh semen with 2 to $5x10^9$

motile spermatozoa in 80 to100 ml of volume. AI in sows using frozen-thawed (FT) semen has been developed long time ago in many countries in Europe and USA (Larsson and Einarsson, 1976). However, the success of AI using FT semen has just markedly progressed during a recent year (Eriksson, 2000). The main reasons may include the development of insemination technique and the advance of knowledge concerning time of ovulation.

The advantages for development of FT semen in pig include the preservation of the good genetic resource that can be done for a longer period than the herd life of the boar, the distribution of superior genetic boar that is much quicker than fresh semen and also the improvement of the transportation of spermatozoa across countries (Polge, 1956; Almlid and Hofmo, 1996; Johnson, 1998). However, boar spermatozoa after freeze-thaw are weak, and have a lower fertility rate than fresh semen (Eriksson and Rodriguez-Martinez, 2000a). The reason is due partly to the lipid structure within the plasma membrane of the boar spermatozoa that is very sensitive to the change in temperature (Johnson et al., 2000).

FT boar sperm quality is influenced by many factors such as freezing and thawing protocols, composition of diluents used in the processes, susceptibility to cryo-injury of the spermatozoa, etc. (Johnson et al., 2000; Suzuki et al., 2005). The susceptibility to cryo-damage of the boar spermatozoa, so called ‘sperm freezability’, is related to the differences of individual boars and the breed of boar (Larsson and Einarsson 1976; Thurston et al., 2002; Holt et al., 2005).

In tropical countries, cryopreservation of boar semen is nowadays performed in a limited scale and it has yet to be conducted in Thailand particularly for the commercial purpose. The present study therefore aimed to produce cryopreserved boar semen in Thailand. Fresh and post-thaw semen quality and variation among ejaculates were investigated.

2.2.3 Materials and methods

2.2.3.1 Animals and semen

Fifteen purebred boars (5 Landrace; L, 5 Yorkshire; Y and 5 Duroc; D) from commercial herds in Nakorn Pathom province, Thailand were used in the experiment. The

boars aged between 1 to 3 years old and were being used for routine AI in the herds. The boars were fed approximately 3 kg per day with a corn-soybean-fishmeal base feed containing 15 to 16% crude protein. Water was provided *ad libitum*. Three ejaculates from each boar were collected at one week interval using the gloved-hand technique. The fresh semen used must contain a minimum of 70% sperm motility. The collected semen was sent to the laboratory within 40 min after collection.

2.2.3.2 Semen cryopreservation

After collection, the semen was diluted with an isothermal Beltsville Thawing Solution (BTS, Minitüb, Abfüll-und Labortechnik GmbH & Co. KG, Germany) extender with a ratio of 1:1 (v/v). Diluted semen was held at 15 °C for 2 h and centrifuged at 800xg for 10 min. The supernatant was discarded and the semen precipitant was re-suspended (1:1) with lactose-egg yolk (LEY) extender (80 ml of 11% lactose solution and 20 ml egg yolk). The diluted semen was cooled down to 5°C within 90 min. Two parts of semen were mixed with one part of extender III consisting of LEY extender with 9% glycerol and 1.5% Equex STM Paste (Nova Chemical Sales, Scituate Inc., MA, USA) (modified after Westendorf et al., 1975 and Gadea et al., 2004). Thereafter, the processed semen was loaded into 0.5-ml straws (Bio-Vet, Z.I. Le Berdoulet, France). The straws were sealed with PVC powder before placing in liquid nitrogen (LN_2) vapor approximately 3 cm above the level of the LN_2 for 20 min and then were plunged into LN_2.

2.2.3.3 Thawing process

Thawing was achieved, approximately one day after storing in LN_2, by immersing the straws in water at 50 °C for 12 sec (Selles et al., 2003). After thawing, the semen was diluted (1:4) with an extender consisting of 95% BTS and 5% LEY extender. The addition of LEY extender was performed to avoid the spermatozoa sticking to the glassware during the sperm motility analysis. The extended thawed semen was incubated in a 38 °C water-bath for 30 min before evaluating the semen quality after thawing.

2.2.3.4 Evaluation of semen quality

Sperm concentration and motility

Sperm concentration was assessed by direct cell count using Bürker haemocytometer (Boeco, Germany) (Ax et al., 2000). Sperm concentration was expressed as spermatozoa $x10^6$ per ml. Subjective motility of both fresh and post-thaw spermatozoa

was evaluated at 38 °C under a light microscope with 400x magnification. The sperm motility examinations for all semen samples were assessed by one person.

Sperm viability and normal apical ridge acrosomes

The percentages of sperm viability and normal apical ridge (NAR) acrosomes were determined separately by eosin-nigrosin staining (Dott and Foster, 1972). The semen sample (50 µl) was well mixed with a drop of eosin-nigrosin dyes (Fluka Chemie GmbH, Sigma-Aldrich, Switzerland), and the mixture (10 µl) was smeared and dried on a glass slide. Evaluation was undertaken by counting 200 spermatozoa with 1000x magnification. Spermatozoa with an unstained head were regarded as the live spermatozoa. The spermatozoa with a crescent shaped apical ridge were regarded as the NAR spermatozoa.

Functional integrity of sperm plasma membrane

Functional integrity of sperm plasma membrane was assessed using the short hypo-osmotic swelling test (sHOST) (Perez-Llano et al., 2001). Spermatozoa were incubated at 38 °C for 10 min, with 75 mOsm/kg a hypo-osmotic solution consisting of 0.368% (w/v) Na-citrate and 0.675% (w/v) fructose (Merck, Germany) in distilled water. Following this incubation time, 200 µl of the semen-hypo-osmotic solution was fixed in 1000 µl of a hypo-osmotic solution plus 5% formaldehyde (Merck, Germany), for later evaluation. Sperm coiling was assessed by placing 20 µl of well-mixed sample on a warm slide, which was covered with a coverslip before being observed under a light microscope (1000x). Two hundred spermatozoa per slide were counted. In order to determine the percentage of spermatozoa with functional membrane intact, the proportion of coiled tail spermatozoa from a control sample (300 mOsm/kg) was subtracted from the result of hypo-osmotic condition.

Sperm plasma membrane integrity

The integrity of sperm plasma membrane was evaluated with SYBR-14/propidium iodide (PI) (Fertilight®; Sperm Viability Kit, Molecular Probes Europe BV, The Netherlands). For staining procedure, 50 µl aliquots of semen (approximately $50x10^6$ spermatozoa per ml) were mixed with 2.7 µl of the user solution of SYBR-14 and 10 µl of PI. The user solution was SYBR-14 diluted (1:100) in dimethyl sulfoxide (DMSO), fractionated and frozen in eppendorfs. After incubation at 37 °C for 20 min, 2x100

spermatozoa were assessed (1000x) under a fluorescence microscope. The nuclei of spermatozoa with intact plasma membrane stained green with SYBR-14 while those with damaged membranes stained red with PI. The results were expressed as the percentage of spermatozoa with intact membranes (SYBR).

2.2.3.5 Statistical analysis

The statistical analyses were performed using SAS (SAS version 9.0, Cary, N.C., USA). Descriptive statistics was used to describe semen quality before and after thawing. Shapiro-Wilk test was used to examine the normal distribution of data. The semen qualities before and after thawing were compared for each boar using paired *t*-test. Pearson's correlation was used to evaluate the correlation among all sperm parameters that were measured including the sperm motility, the sperm viability, the NAR, the sHOST and the SYBR. Data on the motility, the viability, the NAR, the sHOST positive spermatozoa and the SYBR after thawing were analyzed using the General Linear Model (GLM) procedure of the SAS. The statistical model used included the effects of the breed of boar (D, L, Y), repeated ejaculation (1 to 3) and the effect of the individual boar nested within breed. Least-square means were obtained from each class of the factors and were compared by using least significant difference test (LSD). The differences with $P<0.05$ were regarded as statistical significance.

2.2.4 Results

Semen quality before and after freeze-thaw

The semen quality including the sperm motility, the sperm viability, the NAR, the sHOST and the SYBR, of fresh and FT semen are presented in Table 3. On average, the sperm concentration of pre-diluted fresh semen was $530x10^6$ spermatozoa per ml and the sperm concentration of FT semen was $811x10^6$ spermatozoa per ml, varied between $325x10^6$ and $1620x10^6$ spermatozoa per ml. The motility of FT spermatozoa was 28%, the FT sperm viability was 36%, and the proportion of FT spermatozoa with intact membrane (SYBR) was 31% (Table 3). All of the sperm parameters significantly decreased after being frozen and thawed. For instance, the sperm motility decreased by 44%, the sperm viability decreased by 38%, and the NAR deceased by 60% ($P<0.001$). It was found

that the quality of FT semen varied dramatically such as sperm motility varied from 5 to 45%, sperm viability varied from 13 to 60% and SYBR varied from 13 to 53% (Table 3).

Correlations among post-thaw sperm parameters

Most of the sperm parameters measured after freeze and thaw were significantly correlated (Table 4). The motility of FT spermatozoa was significantly correlated with the sperm viability (r=0.74; *P*<0.001), the NAR (r=0.33; *P*=0.03), the sHOST (r= 0.58; *P* <0.001) and the membrane integrity (r=0.67; *P* <0.001) (Table 4). Additionally, it was found that the higher concentration of the FT semen resulted in a significantly lower percentage of spermatozoa with intact membranes (r=-0.3, *P*=0.04). Post-thaw sperm concentration was significantly correlated with the volume of fresh semen (r=-0.30, *P*=0.04) (data not shown).

Factors influencing post-thaw semen quality

It was revealed that the breed of boar and the individual boars within the same breed significantly influenced most of the FT sperm parameters (Table 5 and Fig. 1). For instance, the post-thaw sperm viability in D and L boars was significantly higher than Y boars (*P*<0.05). The motility and the NAR of FT spermatozoa were lowest in Y boars. The membrane integrity (SYBR) of FT spermatozoa in the L boars was significantly higher than the Y boars (*P*<0.05; Fig 1). Repeated ejaculation significantly influenced the percentage of FT spermatozoa with intact membranes (*P*=0.01; Table 5). On average, the last ejaculate had the highest percentage of spermatozoa with intact membranes (29, 29 and 35% for ejaculation 1, 2 and 3, respectively; data not shown). However, repeated ejaculation within boar did not influence other post-thaw sperm parameters (*P*>0.1).

2.2.5 Discussion

The present study demonstrated that cryopreservation of boar semen could be successfully performed in Thailand. However, a considerably high variation of post-thaw semen quality was observed. In general, successful boar semen cyopresevation depends on a number of factors, which could be classified as internal factors and external factors. The internal factors include the inherent characteristic of spermatozoa, and differences between

Table 3 Descriptive statistics for sperm parameters of fresh (n=45) and frozen-thawed semen (n=45) from 15 boars

Sperm parameters	Fresh semen		frozen-thawed semen	
	mean	range	mean	range
Conc. ($x10^6$sperm/ml) [1]	530	175-2125	811	325-1620
Sperm motility (%)	72	70-80	28	5-45
Sperm viability (%)	74	57-87	36	13-60
NAR (%)[2]	86	67-98	26	8-55
sHOST (%)[3]	54	21-89	19	3-45
SYBR (%)[4]	NA[5]	NA	31	13-53

[1]Conc.= concentration, [2]NAR= normal apical ridge, [3]sHOST = functional integrity of sperm plasma membrane, [4]SYBR = percentage of spermatozoa with intact membranes, [5]NA = not available

Table 4 Pearson's correlation coefficient (r) and significance level (*P*-value) among sperm parameters measured after frozen-thawed

	Viability (%)	NAR (%)	sHOST (%)	SYBR (%)[3]
Sperm motility (%)	0.74***	0.33*	0.58***	0.67***
Sperm viability (%)		0.50***	0.72***	0.54***
NAR (%)[1]			0.18 ns	0.11 ns
sHOST (%)[2]				0.49***

* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, ns = $P > 0.05$, [1]NAR= normal apical ridge, [2]sHOST = functional integrity of sperm plasma membrane, [3]SYBR = percentage of spermatozoa with intact membranes

Table 5 Significance levels of factors affecting post-thaw semen quality

Factors [1]	DF[2]	Motility (%)	Viability (%)	NAR (%)	sHOST (%)	SYBR (%)
Breed	2	0.14[3]	0.01	0.11	0.87	0.05
Ejaculate	2	0.23	0.22	0.46	0.39	0.01
Boar [4]	12	0.04	0.04	0.03	0.002	0.05

[1]All factors were included in the same statistical model, [2]DF= degree of freedom, [3]*P*-value were calculated base on type III sum of square, [4]Effect of individual boars was nested within breed

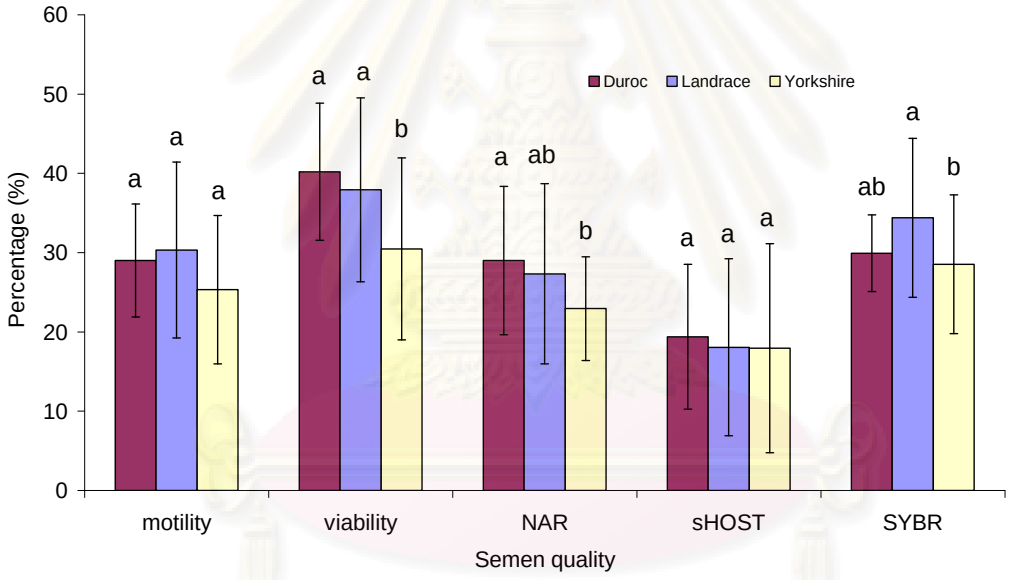


Fig. 1 Semen quality after freeze-thaw in Duroc, Landrace and Yorkshire boars, different letters within each sperm parameter differ significantly ($P<0.05$) (NAR= normal apical ridge, sHOST= functional integrity of sperm plasma membrane, SYBR = percentage of spermatozoa with intact membranes). Error bars represent the standard deviation.

boar and ejaculate. The external factors include the composition of diluents, type and concentration of cryoprotective agents, rates of dilution and of cooling, equilibration and method of freezing and thawing of semen (Johnson et al., 2000). The present study demonstrated a significant impact of internal factors, i.e. the breed of boar and individual boars, on the post-thaw semen quality. The individual boars influenced the sperm susceptibility to cryoinjury as shown that the post-thaw sperm quality differed significantly among boars within the same breed. This was in agreement with previous findings by Larsson and Einarsson (1976) and Holt et al. (2005). Watson (1996) suggested that cold shock resistance of spermatozoa may relate to the lipid composition within the membrane bilayer of the sperm plasma membrane. Generally, boar spermatozoa which have a high sensitivity to cold shock contain a low percentage of phosphatidylcholine and a high percentage of phosphatidylethanolamine and sphingomyelin in their plasma membrane (Johnsson et al., 2000). The proportion of cholesterol in the sperm plasma membrane also influences the thermotrophic behavior of spermatozoa. In boar spermatozoa, cholesterol/phospholipids ratio is low as well as the cholesterol is distributed asymmetrically and is presented in the outer than the inner layer of the membrane. These render the inner layer of the membrane is vulnerable to cold shock. The difference in physiological characteristic of the spermatozoa from each boar may contribute to the individual variation. Further studies concerning the fat composition of the boar spermatozoa are of interest.

In the present study, L boars seemed to have the most variations in many of the FT sperm parameters. This finding was in contrast to the results of Thurston et al. (2002) who found that the Y breed was more evenly split of sperm freezability than the others. The reason for this is not known. Additional studies with a higher number of boars within each breed need to be performed. It has been demonstrated that there is a correlation between some genetic markers and the differences in the sensitivity of boar spermatozoa to cryoinjury (Thurston et al., 2002; Holt et al., 2005). Holt et al. (2005) conducted an experiment with more than 100 boars and used the genetic analyses to test that the susceptibility of boar spermatozoa to cryo-injury is a consistent feature of an individual and found that those individuals differed in susceptibility. The genomic differences between individual boars correlated with freezability and post-thaw quality of their spermatozoa.

Thurston et al. (2002) analyzed genome of 22 boars by amplified restriction fragment length polymorphism and found that 16 candidate molecular markers linked to genes controlling semen freezability. These genomes varied among individual boars, and may lead to an improvement in the predictability of both post-thaw semen quality and fertility of individual males.

The number of spermatozoa loaded in each semen package also influences the FT sperm quality. In this study, higher concentration of FT semen resulted in the lower percentage of membrane intact spermatozoa. In our view, it was probably because, at too high concentration, spermatozoa in the centre of straw were frozen and thawed with inappropriate rates, compared with those at the periphery, which resulted in the sperm damage. However, interestingly, other sperm parameters were not affected by the difference of frozen-thawed sperm concentration. The reason for this is still being elucidated. Effect of sperm concentration during freezing-thawing on FT sperm quality has been reported earlier. Graham and Crabo (1972) stated that a low concentration ($250x10^6$ spermatozoa per ml) raised the level of motile spermatozoa, whereas a high concentration ($1000x10^6$ spermatozoa per ml) lowered it. Westendorf et al. (1975) recommended a concentration of $900x10^6$ while Paquignon and Courot (1976) found a concentration of 400 to $800x10^6$ spermatozoa per ml was most suitable. Nowadays concentrations of between 450 and $1000x10^6$ spermatozoa per ml are commonly used.

In conclusions, boar semen cryopreservation could be successfully performed in Thailand with the averages of $811x10^6$ per ml FT sperm concentration, 28% the sperm motility and of 36% the sperm viability. However, post-thaw sperm concentration and semen quality varied considerably. The higher concentration of FT semen resulted in the lower membrane integrity. The breed of boar and the individual boars within the same breed significantly influenced most of the post-thaw sperm parameters.

CHAPTER III

INSEMINATION TECHNIQUES AND *IN VIVO* FERTILITY OF FT BOAR SEMEN

3.1 Experiment 3 and 4: Fertility in spontaneously ovulating sows after intra-uterine and deep intra-uterine insemination with frozen-thawed boar semen

3.1.1 Abstract

Fertilization rate, farrowing rate (FR), number of total piglets born per litter (TB) and number of live born piglets per litter (BA) obtained following intrauterine insemination (IUI) with $2x10^9$ frozen-thawed (FT) spermatozoa and deep intrauterine insemination (DIUI) with $1x10^9$ FT spermatozoa were studied in spontaneously ovulating weaned sows. Estrus detection was performed at 12 h interval (6:00 and 18:00 h) and transrectal ultrasonography was used to determine the time of ovulation. Experiment 3 evaluated fertilization rate after IUI or DIUI. The estrous sows were inseminated at 12 h interval until ovulation, beginning at 24 h after the detection of estrus, using IUI (n=6) or DIUI (n=6). At 45.1±7.2 h after ovulation, the recovery rate of oocytes and embryos flushed from the oviducts of the slaughtered sows was 83.8%. IUI yielded better fertilization rate than DIUI (66.0 vs 31.0%; $P<0.001$). The pregnancy rate was 83.3% (5/6 sows) and 33.3% (2/6 sows) for IUI and DIUI, respectively ($P=0.24$). The percentage of sows with unilateral fertilization was higher in the DIUI (60%, 3/5) compared with the IUI group (16.7%, 1/6; $P=0.24$). The number of embryos recovered was 13.5±6.7 and 6.6±8.2 embryos per sow in IUI and DIUI groups, respectively ($P=0.16$). Experiment 4 studied FR, TB and BA after IUI or DIUI. Each of the eighteen sows was inseminated twice, at 24 and 36 h after the detection of standing estrus, by IUI (n=9) or DIUI (n=9). No significant differences were found in any of fertility parameters evaluated between IUI and DIUI groups (89 vs 67% non-return rate, 67 vs 67% FR, 10.5±2.9 vs 7.7±3.0 piglets for TB, and 9.5±3.0 vs 7.5±3.0 piglets for BA, respectively; $P>0.05$). However, TB and BA achieved in the IUI group tended to be higher compared to the DIUI group (+2.8 TB and +2.0 BA, $P>0.05$). The

results of both experiments indicated that inseminations with 1 to $2x10^9$ FT boar spermatozoa per dose using IUI or DIUI technique in naturally ovulating sows could result in either unilateral or bilateral fertilization as well as acceptable fertility rates. However, incidence of unilateral fertilization, number of unfertilized oocytes per sow and smaller litter sizes were more pronounce in the DIUI than the IUI group, indicating that DIUI with $1x10^9$ FT spermatozoa per dose seemed not to be sufficient to obtain good fertility results.

3.1.2 Introduction

Conventional artificial insemination (AI) in pigs with a high number (5 to 6 $x10^9$ per dose) of frozen-thawed (FT) spermatozoa often results in a decrease of 20 to 30% in farrowing rates (FR) and 2 to 3 in number of total piglets born per litter (TB) compared to extended fresh semen (Almlid and Hofmo, 1996; Johnson et al., 2000). Under field conditions, the use of FT boar spermatozoa for AI in commercial swine herds is limited (Wagner and Thibier, 2000). In an attempt to obtain satisfactory fertility results using low numbers of sperm per dose, non-surgical deep insemination procedures to deposit semen into the uterine body (intra-uterine insemination; IUI) or into the uterine horn (deep intra-uterine insemination; DIUI) have been developed (Martinez et al., 2001; Watson et al., 2001). Using DIUI, sperm are deposited at about the proximal third of the uterine horn close to the uterotubal junction (Martinez et al., 2002). It has been shown that DIUI with a 20-fold reduction in the number of extended fresh spermatozoa has been conducted without decreasing fertility (Martinez et al., 2001; 2002). However, Day et al. (2003) found that DIUI in spontaneously ovulating sow yields a lower litter size compared to conventional AI. Martinez et al. (2006) suggested that the reduction of litter size might be overcome by increasing the number of spermatozoa for DIUI. Using DIUI in induced ovulating weaned sows, $1x10^9$ FT spermatozoa per dose can be used without altering the reproductive performance (Roca et al., 2003). Nonetheless, DIUI in spontaneously ovulating sows with $1x10^9$ of FT spermatozoa resulted in 70% FR and 9.3 TB, which was lower than DIUI with $150x10^6$ extended fresh spermatozoa (84% FR and 9.9 TB). Using FT semen, insemination outside of the optimal insemination-ovulation period (4 to 6 h before ovulation) significantly decreased FR and TB (Roca et al., 2003). In addition to DIUI, IUI is another

insemination technique that has been developed to reduce the number of spermatozoa per insemination dose (Watson and Behan, 2002). It has been demonstrated that an approximately 3-fold reduction in the number of spermatozoa for extended fresh semen can be used with IUI without affecting FR and TB, compared to conventional AI. To our knowledge, fertility in sows inseminated with FT semen using IUI, in comparison to DIUI has not been evaluated. The present study was therefore performed to investigate fertility results such as fertilization rate, FR and TB after IUI or DIUI with reduced numbers of FT boar spermatozoa in spontaneously ovulating weaned sows.

3.1.3 Materials and methods

3.1.3.1 Animals

The inseminations reported in this study were conducted at the farm animal hospital, Faculty of Veterinary Science, Chulalongkorn University, Nakorn Pathom (experiment 3) and at the Animal Breeding Extension Division, Regional Development Office, Royal Thai Supreme Command Head Quarters, Chachoengsao, Thailand (experiment 4). The animal welfare and treatment were approved by the faculty's ethical committee (Animal Use Protocol No. 0731055). Animals from the herds were randomly assigned to the study. The weaned sows used in the study were Landrace (L) x Yorkshire (Y) crossbreeds of parities 2 to 8 and weaning-to-estrus intervals of $\leq$ 7 days. Sows were allocated in individual pens adjacent to an adult boar and fed with 2 kg of a corn-soybean-fish based commercial feed with 15% crude protein, twice a day. Water was provided *ad libitum* via water nipple.

3.1.3.2 Semen collection and cryopreservation

The sperm-rich fraction of ejaculates was collected once a week from each of 4 L and 3 Y boars, aged between 1 and 3 years, of proven fertility by the gloved-hand method. Semen was kept in an insulated thermos flask during transport to the laboratory accomplished within 40 min after collection. Only ejaculates with $\geq$70% motile sperm, as estimated subjectively using a light microscope at 400x magnification, were cryopreserved (Buranaamnuay, 2009). The semen was processed and frozen according to an adaptation of the Westendorf method (Westendorf et al., 1975; Gadea et al., 2004). Briefly, sperm-rich fractions were extended (1:1, v/v) in Beltsville Thawing Solution (BTS; Minitüb, Abfüll-

und Labortechnik GmbH & Co. KG, Germany) and cooled down to15°C for 2 h. After centrifugation at 800g for 10 min, the pellets were diluted in lactose-egg yolk (LEY) extender (80 ml of 11% lactose solution and 20 ml egg yolk) to a concentration of $1.5x10^9$ cells per ml. After cooling to 5°C over a 90-min period, the diluted spermatozoa were re-suspended with extender III (LEY extender, 9% glycerol and 1.5% Equex STM Paste; Nova Chemical Sales Inc., Scituate, MA, USA) to a final concentration of $1x10^9$ sperm per ml. The processed spermatozoa were packed into 0.5 PVC-french straws (Bio-Vet, Z.I. Le Berdoulet, France) and frozen by placing in liquid nitrogen (LN_2) vapor approximately 3 cm above the level of LN_2 for 20 min. The frozen doses were plunged into LN_2 (-196^oC) until thawing.

3.1.3.3 Thawing procedure and evaluation of the post-thaw sperm quality

Thawing was achieved by immersing the straws in 50°C water for 12 sec. Immediately after thawing, the semen was diluted (1:4) with an extender consisting of 95% BTS and 5% LEY extender. The addition of LEY extender prevents the sperm from sticking to the glassware used during motility analysis. The extended thawed semen was incubated in a 38°C water-bath for 30 min and the post-thaw sperm quality was evaluated. The subjective sperm motility was assessed using a bright-field microscope (400x) (Buranaamnuay, 2009). Sperm concentration was evaluated using Bürker haemocytometer (Boeco, Germany) (Ax et al., 2000) and viability of spermatozoa was determined by eosin-nigrosin staining (Dott and Foster, 1972). The FT semen used for insemination must have sperm motility of ≥ 35%. Before insemination, the qualifying FT semen (≥ 35% motility) was diluted with 20 ml and 10 ml of BTS extender for IUI and DIUI, respectively. The diluted semen was incubated at 38°C for 10 min and checked for the post-thaw motility before insemination.

3.1.3.4 Detection of estrus and ovulation

Estrus detection was performed twice daily (06:00 and 18:00 h), starting from the day after weaning, by allowing the females to have direct contact with a mature boar and back pressure test. Sows that exhibited a standing reflex were considered as estrous sows. The occurrence of ovulation was investigated every 4 h (experiment 3) or 12 h (experiment 4) after detection of standing estrus, by using real-time B-mode ultrasonography (Aloka, SSD 500, Japan) (Soede et al., 1992; Mburu et al., 1995). Briefly, after removal of feces

from the rectum, a 5 MHz multiple angle transducer was carefully introduced into the rectum. The appearance of follicles on the ovaries was observed. The ovulation time was defined as 2 h (experiment 3) or 6 h (experiment 4) before the first time when no preovulatory follicles (≥ 6 mm diameter) were visible.

3.1.3.5 Intra-uterine and deep intra-uterine insemination

The inseminations were performed in the gestation crates. Briefly, for IUI group, the IUI device (Deep goldenpig™ catheter; IMV Technologies, L'Aigle, France) was inserted through the vagina into the cervix. The inner tube extended 200 mm beyond the tip of the outer catheter lying in the uterine body or the posterior uterine horn (Figure 2a). Then, $2x10^9$ spermatozoa diluted in 20 ml BTS were inseminated and 2 ml of BTS extender were flushed before removing the device. For DIUI group, the standard Goldenpig® catheter was introduced through the vagina into the cervix. Thereafter, the DIUI device (length 180 cm, out diameter 4 mm, and working inner channel of 1.8 mm; Firflex®, Magapor, Ejea de los Caballeros, Spain) was inserted through the catheter, handled through the cervix and propelled forward along one uterine horn (Figure 2b). A syringe containing a predetermined number of spermaotzoa ($1x10^9$) in 10 ml of BTS extender was then connected to the device, and the extended FT semen slowly infused into the uterus. The BTS extender (2 ml) was flushed to force remaining spermatozoa out of the device.

3.1.3.6 Recovery of embryos

The reproductive organs, i.e. the uteri, the uterine horns, the oviducts, and the ovaries were collected from the slaughtered sows in experiment 3. The number of corpora lutea (CL) was counted on both ovaries. Each oviduct was separated from the uterus and later flushed with 20 ml TCM 199 Hepes at 38°C in order to collect the embryos and oocytes. Recovery rate was determined as the percentage of embryos and oocytes recovered based on the number of CL counted. Examination of the retrieved embryos and oocytes was performed under a stereomicroscope (40x). An oocyte was classified as fertilized when ≥ 2 cells per embryo were observed. Fertilization rate was determined on the basis of the total number of recovered embryos and oocytes. Bilateral fertilization was deemed to be established when at least one embryo could be retrieved from both oviducts and unilateral fertilization was regarded when only oocytes were collected from an oviduct. Pregnancy

rate was calculated as the ratio of sows with ≥ 4 embryos to the total number of sows inseminated (Martinez et al., 2006).

3.1.3.7 Management of sows after insemination

The sows in experiment 4 were checked for estrus symptoms twice a day (AM/PM) from about 17 days after insemination onwards using a mature boar. The sows were kept in individual crate from insemination until 15 wk of gestation before moving to the farrowing barn at about one week before expected farrowing time. The sows could access to water nipple freely. Feed was provided twice a day. At about 24 days after insemination, a 24-day non-return rate (NR) was recorded. At farrowing, the number of sows that farrowed, TB and number of live born piglets per litter (BA) were evaluated.

3.1.3.8 Experimental design

In experiment 3, the study on fertilization rate and number of embryos after IUI and DIUI with FT boar semen, weaned sows were inseminated with FT spermatozoa obtained from a proven Y boar using either IUI (n=6) or DIUI (n=7). Inseminations were conducted at 24 h after the onset of estrus and repeated every 12 h until ovulation took place. Approximately 48 h following ovulation, the inseminated sows were brought to an abattoir and the reproductive organs were obtained for embryo collection.

In experiment 4, the study on FR, TB and BA after IUI and DIUI with FT boar semen, weaned sows were given double inseminations using either IUI (n=9) or DIUI (n=9) at 24 and 36 h after the onset of estrus. In each sow, double inseminations were performed using FT semen of the same boar; and FT semen prepared from each boar (4L and 3Y boars) was used in both groups to minimize effect of individual boar variation. Success of inseminations was evaluated and recorded as a 24-day NR. All pregnant sows were allowed to carry litter to term for assessment of FR, TB and BA.

3.1.3.9 Statistical analysis

The statistical analyses were performed using the General Linear Model (GLM) procedure of SAS version 9.0 (SAS Inst. Cary, NC, USA). Descriptive statistics including means, standard deviations (SD) and range of the data were calculated. Insemination techniques (IUI and DIUI) were included as a fixed effect. Numbers of CL, embryos and oocytes were analyzed. Least-square means were obtained and compared between groups using Student's *t*-test. Number of CL between the left and right ovaries were compared

using paired *t*-test. Recovery rate and fertilization rate were compared between IUI and DIUI groups using chi-square test. Pregnancy rate, 24-day NR and FR were analyzed by Fisher's exact test. TB and BA were analyzed by Student's *t*-test. Differences were considered to be significant at $P<0.05$.

3.1.4 Results

Experiment 3

On average, FT semen containing motile spermatozoa of 35% (35.0±0) was used. One inseminated sows in the DIUI group was excluded from the analyses because < 20% recovery rate was revealed, owing to the late slaughter. Therefore, the results in this study were based on 12 sows.

Of the 12 sows, the weaning to estrus interval was 2.9±0.5 days (range 2 to 4 days). Ovulation took place at 49.6±10.9 h (range 35.0 to 72.0 h) after the onset of estrus. The sows were slaughtered at 45.1±7.2 h after ovulation. Interval from last insemination to ovulation in the IUI group was 1.4±1.5 h (range 0 to 3 h) and in the DIUI group was 4.7±4.8 h (range 0 to 12 h; $P=0.16$). The proportion of sows with an insemination to ovulation interval ≥ 6 h was 0/6 and 2/6 sows in the IUI and DIUI groups, respectively. In both groups, the number of CL on the left and right ovaries did not differ significantly (10.5±2.9 vs 12.1±5.8; $P=0.49$).

The fertilization results are presented in Table 6. The overall recovery rate of oocytes and embryos in relation to the number of CL was 83.3% and did not differ significantly between groups (85.3% in IUI and 82.0% in DIUI; $P=0.46$). There were no significant differences in fertilization rate between the left and right sides of the oviducts within either group ($P>0.05$). The proportion of fertilized oocytes after IUI (81/122, 66.4%) was higher than DIUI (31/105, 31.4%; $P< 0.001$). Of the six females in each group, fertilization was found in all IUI sows (6/6, 100%) and in 5 DIUI sows (5/6, 83.3%; $P=1.0$). Among sows in which embryo(s) were found, fertilization took place in both sides of the oviducts in 5 sows in the IUI group (5/6, 83.3%) and 2 sows in the DIUI group (2/5, 40%; $P=0.24$). The number of recovered embryos after IUI (13.5±6.7 embryos per sow) was higher than after DIUI (6.6±8.2 embryos per sow; $P=0.16$). Unfertilized oocyte numbers

did not differ significantly between insemination groups but tended to be higher in the DIUI (12.0±8.9 oocytes per sow) compared to the IUI group (6.8±7.3 oocytes per sow; *P*=0.30). The stages of embryos retrieved were between 2 and 8 cells (Figure 3).

Table 6 Weaning to estrus interval, numbers of insemination, corpora lutea, sows with embryo(s) and of sows with unilateral fertilization and recovery, fertilization and pregnancy rates after intra-uterine (IUI) and deep intra-uterine insemination (DIUI) with frozen-thawed boar semen in spontaneously ovulating multiparous sows

Parameters	Insemination procedure	
	IUI (n=6)	DIUI (n=6)
Weaning-estrus interval (days) (mean±SD)	2.8±0.3	3.0±0.7
No. of insemination	3	2-4
No. of corpora lutea (mean±SD)	23.8±6.2	21.3±4.0
No. of oocytes and embryos recovered (%)	122/143 (85.3)	105/128 (82.0)
No. of embryos (%)	81/122 (66.4)[a]	33/105 (31.4)[b]
left oviducts	39/55 (70.9)	12/45 (26.7)
right oviducts	42/67 (62.7)	21/60 (35.0)
No. of sows with ≥ 1 embryo(s) (%)	6/6 (100.0)	5/6 (83.3)
No. of sows with ≥ 4 embryos (%)	5/6 (83.3)	2/6 (33.3)
No. of sows with unilateral fertilization (%)	1/6 (16.7)	3/5 (60)

Values within rows with different superscripts are significantly different (*P*<0.05).

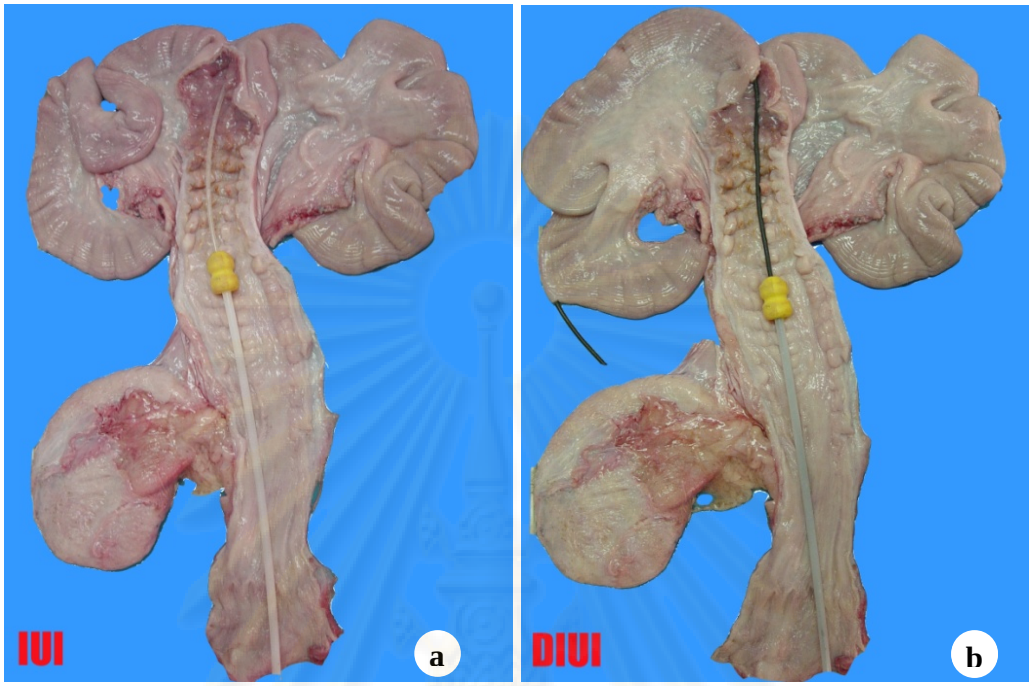
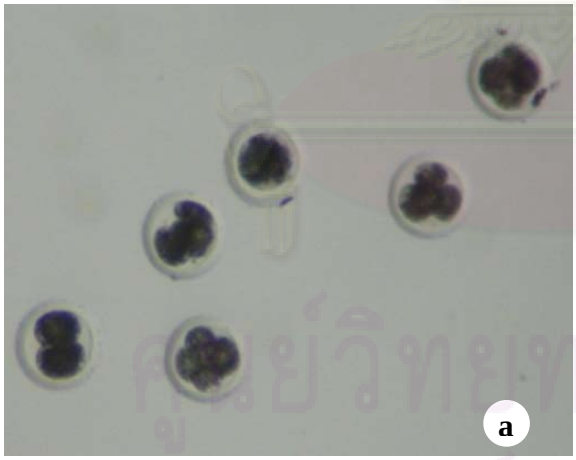


Fig. 2 Sperm deposit site in different procedures: (a) intra-uterine insemination (IUI) and (b) deep intra-uterine insemination (DIUI)



b

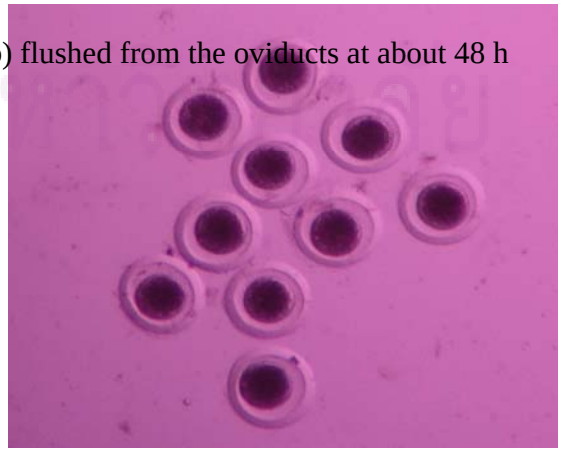


Fig. 3 Embryos (a) and unfertilized oocytes (b) flushed from the oviducts at about 48 h after ovulation

Experiment 4

Eleven ejaculates (6 ejaculates from the 4 L boars and 5 ejaculates from the 3 Y boars) were used for insemination. On average, the percentages of motile spermatozoa used for IUI and DIUI were 41.7±5.6% (range 35 to 50%) and 38.9±3.3% (range 35 to 45%), respectively (P=0.22). The FT sperm viability used for IUI and DIUI was 50.6±4.8% (range 43 to 59%) and 48.6±5.0% (range 43 to 55%), respectively (P=0.46). The interval from the detection of estrus to ovulation was 28.7±10.3 h (range 6 to 42 h) and 32.2±6.3 h (range 19 to 42 h) in the IUI and DIUI groups, respectively (P=0.39). The interval between insemination and ovulation was 6.0±5.0 h (range 0 to 18 h) and 3.6±2.4 h (range 0 to 6 h) in the IUI and DIUI groups, respectively (P=0.20).

The reproductive performances of all inseminated sows are presented in Table 7. The 24-day NR, FR, TB and BA were not significantly different between the IUI and DIUI groups (P>0.05). However, the sows inseminated using DIUI with $1x10^9$ FT spermatozoa had a 2.8 TB (P=0.13) and 2.0 BA (P=0.27) lower than IUI with $2x10^9$ FT spermatozoa.

3.1.5 Discussion

The results in experiment 3 demonstrated that fertilization occurred in both sides of the sow oviducts after IUI or DIUI. This indicates that a low number of FT spermatozoa inseminated using these procedures can be transported via the genital tract and then successfully fertilize the oocytes. This finding is in agreement with previous studies in which extended fresh semen was used for IUI (Sumransap et al., 2007) and DIUI (Martinez et al., 2002, 2006; Tummaruk et al., 2007). Martinez et al. (2002) stated that spermatozoa deposited in only one uterine horn by DIUI can migrate to the contralateral oviduct and fertilize the oocytes via both transuterine and transperitoneal migrations. Nevertheless, DIUI with 150 $x10^6$ chilled sperm diluted in 10 ml extender per dose in spontaneously ovulating sows resulted in unilateral and partial fertilizations in some sows (Martinez et al., 2006; Tummaruk et al., 2007). This probably resulted from too low spermatozoa number and/or too low semen volume used. Martinez et al. (2006) found that no incidence of unilateral fertilization was observed in naturally ovulating sows after DIUI with $600x10^6$ spermatozoa in 20 ml of extender. All of these suggest that when DIUI is performed, the

number of spermatozoa and/or volume of the doses have to be high enough to establish an adequate sperm population in the oviductal sperm reservoirs so that oocytes can be fertilized in both oviducts.

Table 7 Non-return rate, farrowing rate, number of total piglets born per litter and number of piglets born alive per litter after intra-uterine insemination (IUI) and deep intra-uterine insemination (DIUI) with frozen-thawed boar semen

	Insemination procedure	
	IUI	DIUI
No. of sows	9	9
Parity number (mean±SD)	5.0±1.9	4.8±1.9
Weaning to estrus interval (days) (mean±SD)	4.9±0.9	5.1±1.5
Sows inseminated within 6 h before/after ovulation (%)	8/9 (89)	9/9 (100)
Non-return rate at 24 days (%)	8/9 (89)	6/9 (67)
Sows return-to-estrus after 24 days (%)	2/8 (25)	0 (0)
Farrowing rate (%)	6/9 (67)	6/9 (67)
Number of total piglets born per litter (mean±SD)	10.5±2.9	7.7±3.0
Number of piglets born alive per litter (mean±SD)	9.5±3.0	7.5±3.0

In the third experiment, low fertilization rate, unilateral fertilization and low number of embryos per sow were observed in the DIUI group. These might be a consequence of a longer interval from last insemination to ovulation of the DIUI compared with the IUI group (P>0.05). It has been demonstrated that FT boar spermatozoa remain viable in the female reproductive tract for only 6 h after insemination, due to the capacitation-like change of the sperm plasma membranes induced by freeze-thaw process (Waberski et al., 1994; Watson, 2000; Wongtawan et al., 2006). In this study, last insemination to ovulation interval of more than 6 h was observed in 2 out of 6 sows in the DIUI group. These two sows had no fertilization (one sow) and unilateral fertilization (one sow). Our results

confirm earlier findings that the risk of no fertilization or even unilateral fertilization appeared to be maximal when insemination, regardless of the AI method used, was carried out far from optimal timing with respect to the moment of ovulation (Hunter, 1967; Soede et al., 2000). Using extended fresh spermatozoa, optimal percentage of embryos is reached when insemination is performed in the interval of 0 to 24 h before ovulation, and fertilization rates are reduced before and after this interval (Waberski et al., 1994; Soede et al., 1995). However, the optimal insemination-ovulation interval may not be the only one factor for successful fertilization since high percentages of unilateral fertilization have been found when a single conventional AI were carried out at 0 to 8 h before ovulation in gilts during spontaneous estrus (Waberski et al., 1994). The above suggestion is also affirmed by the current observations that unilateral fertilization was still observed in two sows in the DIUI group, although the interval between last insemination and ovulation of these two sows was no longer than 3 h.

Since the timing of insemination relative to ovulation is essential for attaining optimal fertility, an inappropriate time of insemination results in low FR, TB and BA (Johnson et al., 2000). In experiment 4, a sow that did not get pregnant ovulated at about 6 h after detection of estrus, meanwhile insemination was begun at 24 h (i.e., the interval between insemination and ovulation was 18 h). This might be one reason for the unsuccessful fertilization in this sow. In pigs, it is well documented that ovulation takes place at approximately 70% of the estrus duration (Belstra et al., 2001). However, the duration of estrus varies among animals, making it difficult to predict the exact time of ovulation and the best time of insemination. At a 12 h interval of ovulation detection, 18 sows used in this study ovulated at a range of 6 to 42 h after the detection of standing estrus. Hence, double inseminations with FT spermatozoa at 24 and 36 h were somewhat too early for the late-ovulating sows and too late for the early-ovulating sows. To minimize this problem, the control of ovulation using different types of hormones, i.e. human chorionic gonadotrophin (hCG), gonadotrophin releasing hormone (GnRH) or porcine luteinizing hormone (pLH), has been recommended (Roca et al., 2003). Earlier studies have demonstrated that ovulation took place at 36 to 40 h after the application of these hormones in sows (Brussow et al., 1996; Candini et al., 1999; 2001; Roca et al., 2003). It has been demonstrated that the fertility of the hormonally-treated sows after a single DIUI with FT

spermatozoa (77.5% FR and 9.31 TB) were not significantly different from extended fresh semen (75.7% FR and 9.6 TB) (Roca et al., 2003).

Besides insemination to ovulation interval, Martinez et al. (2006) demonstrated that the number of spermatozoa, the semen volume and the insemination procedure also play major roles in successful fertilization. In experiment 3, despite optimal insemination-ovulation interval the percentage of sows with unilateral fertilization was higher in the DIUI sows (60%) compared with the IUI sows (16.7%). It could be suggested that DIUI using a total of $1x10^9$ FT spermatozoa (~$350x10^6$ motile spermatozoa) may not be sufficient for fertilization in both oviducts of the spontaneously ovulating sows. It has been well documented that several factors are necessary for the formation of the functional sperm reservoir in the female oviducts including selective binding of spermatozoa to the oviductal epithelium (Fazeli et al., 1999). The sperm population that enters the oviductal sperm reservoirs, which is partly related to the achievement in fertilization, depends on the number of spermatozoa inseminated (Rodriguez-Martinez et al., 2005). Nonetheless, spermatozoa that are able to attach to the oviductal epithelial cells in the reservoir must be viable and uncapacitated cells (Mburu et al., 1996, 1997; Tienthai et al., 2004). In general, about 40 to 50% of the sperm population does not survive following the freeze-thaw process even with optimized protocols (Garcia-Herreros et al., 2008) and some of the rest spermatozoa which are still alive lose their fertilizing capacity, manifested through the capacitation-like appearance of the sperm plasma membrane (Watson, 1995). Therefore, a low number of FT spermatozoa ($1x10^9$) and thus low numbers of intact spermatozoa after freeze-thaw used for each DIUI dose in this study probably were not enough to stimulate the formation of the functional oviductal sperm reservoirs in some inseminated sows or, in case of sufficiency, the number of intact spermatozoa colonized in the reservoirs might be too low to fertilize all ovulated oocytes. Earlier research demonstrated that a significantly low number of spermatozoa were recovered from the oviductal reservoir(s) at 24 h after DIUI with a reduced number of extended fresh spermatozoa, when compared to IUI and conventional AI (Tummaruk and Tienthai, 2008).

The results of previous studies revealed that decreases in the number of embryos and the number of total piglets born were observed in sows inseminated with the DIUI procedure using a reduced number of fresh spermatozoa (Vazquez et al., 2001; Day et al.,

2003; Martinez et al., 2006) and FT spermatozoa (Roca et al., 2003). Similarly, in the present experiments, the numbers of embryos per sow and piglets born per litter in the DIUI group was lower than the IUI group ($P>0.05$). These suggested that the lower number of embryos per sow and the smaller litter sizes obtained after DIUI with a relatively low number of spermatozoa might be related to the presence of unilateral fertilization. Moreover, the proportion of sows with at least 4 embryos (termed "pregnancy rate") tended to be higher after IUI (83.3%) compared to DIUI (33.3%). It could be speculated that a higher farrowing rate might be obtained after IUI with $2x10^9$ FT spermatozoa. The results in the fourth experiment showed that two sows in the IUI group exhibited standing estrus after 24 days post-insemination resulting in an approximately 20% decrease of FR, which finally was not greater than that of the DIUI group (see Table 7). It could be implied that, in these two sows, fertilization occurred but the embryos were dead during 3 to 4 weeks of gestation, and consequently resulted in return-to-estrus at irregular intervals (26 to 28 days after insemination). This finding may not relate to insemination technique, since it has been demonstrated that the use of FT spermatozoa for insemination regardless of methods used frequently resulted in high early embryonic mortality or low embryonic development due to damages of chromatin structure of the post-thaw boar spermatozoa (Evenson et al., 1980; Sailer et al., 1996; Hammadeh et al., 1999). Nonetheless, to our knowledge, this study is the first report on IUI with a reduced number of FT spermatozoa in which the fertility obtained was acceptable and tended to be greater than results achieved after DIUI. This indicated that IUI could also be an optimal insemination method for FT semen under field conditions.

In conclusion, the present study demonstrated that using IUI or DIUI with relatively low numbers of FT spermatozoa in spontaneously ovulating sows resulted in either unilateral or bilateral fertilization as well as reasonable FR, TB and BA. The advantages of IUI and DIUI include the reduction in the number of spermatozoa required per dose and hence an increase in the overall production of valuable boars. However, the higher fertilization and pregnancy rates as well as higher numbers of embryos and piglets born per litter were obtained after IUI compared with DIUI, suggesting that it is more possible to use IUI with a minimum of $2x10^9$ total FT boar spermatozoa in order to produce acceptable fertility in spontaneously ovulating sows under field conditions.

CHAPTER IV

FERTILITY AND FECUNDITY AFTER DEEP AI WITH FT BOAR SEMEN UNDER FIELD CONDITIONS

4.1 Experiment 5: Control of ovulation in sows using human chorionic gonadotrophin (hCG) compared with gonadotrophin releasing hormone (GnRH)

4.1.1 Abstract

The present study aimed to investigate the effects of human chorionic gonadotrophin (hCG) and gonadotrophin releasing hormone (GnRH) on the time of ovulation in sows. Thirty-three purebred Landrace (L) and Yorkshire (Y) multiparous sows were used. Estrus detection was performed every 6 h after weaning. Estrous sows were randomly allocated to three groups, i.e. spontaneous ovulation (n=10), hCG induced ovulation (n=10) and GnRH induced ovulation (n=13). The hormones were given to the hCG and GnRH induced ovulating sows at the detection of estrus. Ovulation was determined every 6 h by transrectal ultrasonography. All of the sows in the spontaneous ovulation and hCG groups ovulated within 5 days after estrus onset. Three out of 13 (23%) sows in the GnRH group had cystic ovaries and had not ovulated. The intervals from onset of estrus to ovulation were not significantly different among the spontaneous ovulation (46.0±19.2 h), hCG induced ovulation (43.2±5.5 h) and GnRH induced ovulation (40.5±10.3 h) (P>0.05). The percentages of sows ovulated within 45 h after estrus onset in the hCG and GnRH groups were equal (70%, 7/10 sows in each group) and seemed to be higher in comparison to the spontaneous ovulation group (40%, 4/10 sows) (P>0.05). In conclusion, the present results indicate the efficacy of both hCG and GnRH to induce ovulation, in the predictable times, in spontaneously estrous sows.

4.1.2 Introduction

In the pig, it is well documented that ovulation takes place at about 2/3 of the

duration of estrus (Weitze et al., 1994; Soede and Kemp, 1997). However, large variation in the estrus duration among individual exists. This makes difficulty to determine the optimal time for artificial insemination (AI) in pig. Time of insemination relative to the moment of ovulation plays an important role in fertilization results especially when frozen-thawed (FT) boar semen was used (Soede et al., 1995). Suboptimal time of insemination, before and after ovulation, accounts for low fertilization and pregnancy rates and smaller litter sizes (Waberski et al., 1994; Soede et al., 1995; Nissen et al., 1997). In our previous study, deep intra-uterine insemination with FT boar semen at 12 to 9 h before ovulation as investigated by transrectal ultrasonography resulted in failure of fertilization or fertilization in only one side of the uterine horn. It has been demonstrated that FT boar spermatozoa maintain their viability and are capable of fertilizing oocytes for approximately 4 to 8 h after insemination (Einarsson and Viring, 1973; Pursel et al., 1978; Holt, 2000). Therefore, insemination every 4 h might be necessary to ensure successful fertilization but it is difficult for industry implementation.

Exogenous hormones such as human chorionic gonadotrophin (hCG), gonadotrophin releasing hormone (GnRH) and its analogs and porcine luteinizing hormone (pLH) have been used in the female pig to induce/synchronize ovulation (Hunter, 1972; Brussow et al., 1996; Wahner and Huhn, 1996; Duanyair and Srikandakumar, 1998; Candini et al., 1999, 2001; Baer and Bilkei, 2004). These hormones may be used alone or along with equine chorionic gonadotrophin (eCG) in order to induce both estrus and ovulation with the aim of fixed-time inseminations without detection of estrus (Brussow et al., 1996; Kauffold et al., 2007). It has been found that fertility in post-weaned sows after insemination at fixed-time did not differ from sows that were inseminated based on estrus (Knox et al., 2003).

The reproductive performance of sows is influenced by numerous factors such as genetics, management, season, outdoor environment (Vesseur et al., 1994; Kemp and Soede, 1996). Therefore, the moment of ovulation in response to hormonal treatment might also differ between sows being in different climates. Also, since control of ovulation in the pig using hormonal treatment has never been studied in the tropical climate, the present study was conducted to evaluate the effect of hCG and GnRH on ovulation time in multiparous weaned sows.

4.1.3 Materials and methods

4.1.3.1 Animals

The present study was performed in a commercial swine farm in the middle part of Thailand between May and October 2007. Thirty-three purebred Landrace (L, n=14) and Yorkshire (Y, n=19) multiparous sows with parities of 6 to 8 and weaning to estrus interval (WOI) of ≤ 7 days were used. The sows were weaned at 22.2±1.2 days (range 20 to 24). After weaning, the sows were housed in groups of between 3 and 5 sows per group with space allowance of 1.5 to 2.0 m^2 per sow. The sows received a corn-soybean-fish based commercial sow diet containing 15% crude protein (Betagro 306®, Betagro-Agro group Co., Ltd., Thailand) twice daily. Water was provided up to *ad libitum* via water nipple. The sows were randomly assigned to three groups according to ear tag, i.e. spontaneous ovulation (4Y and 6L), hCG induced ovulation (hCG; 5Y and 5L) and GnRH induced ovulation (GnRH; 5Y and 8L).

4.1.3.2 Detection of estrus

The sows were examined for signs of pro-estrus twice a day after weaning. The pro-estrous sows were thereafter checked for onset of standing estrus by back pressure test in the presence of a mature boar every 6 h (2:00, 8:00, 14:00 and 20:00 h). The time of estrus onset was decided to be 3 h before the first time the sows showed standing response.

4.1.3.3 Detection of ovulation

Transrectal ultrasonography (Aloka, SSD 500, Japan) was performed using a 5 MHz linear array probe in order to diagnose ovulation (Soede et al., 1992). The presence of ovarian follicles was observed after estrus detection. The follicular development was investigated every 6 h until ovulation. Ovulation time was defined as 3 h after the last time when follicles of ≥ 6 mm diameter were visible (Mburu et al., 1995).

4.1.3.4 Induction of ovulation

Once the estrus was observed, 750 IU hCG (Chorulon®, Intervet International B.V., Boxmeer, The Netherlands) and 10 μg GnRH-agonist (buserelin; Receptal®, Intervet International B.V., Boxmeer, The Netherlands) were i.m. injected into the sows in hCG and GnRH groups, respectively so as to induce ovulation. The sows in spontaneous ovulation group were given no treatment.

4.1.3.5 Statistical analysis

The statistical analyses were performed using SAS version 9.0 (SAS Inst. Cary, NC, USA). Descriptive statistics including means, standard deviations (SD) and range of the data were calculated. Interval from onset of estrus to ovulation (EOI) was analyzed using general linear model (GLM) procedures of SAS. The statistical model included group (spontaneous ovulation, hCG, GnRH), breed (L, Y) and interaction between group and breed. Least-square means (LSM) were compared using analysis of varience (ANOVA). The percentage of sows ovulated within 45 h after the onset of estrus was analyzed and compared between groups using Fisher's exact test. Differences with $P<0.05$ were considered significant.

4.1.4 Results

Three of 13 (23%) sows in the GnRH group were considered to have ovarian cysts because follicles with a diameter of ≥ 15 mm existed on their ovaries for longer than 5 days after estrus onset (Martinat-Botte et al., 1996; Castagna et al., 2004); and therefore, these sows were excluded from the analyses.

No breed effect was registered in the present study. Descriptive statistics on the reproductive data are shown in Table 8. On average, WOI was 3.8±0.9 days and did not differ significantly among the groups ($P>0.05$). Due to late ovulation in 4 spontaneously ovulating sows (96 to 102 h), analyses of the average EOI were only based on the data of 6 sows. EOI in the induced ovulation groups were not significantly different from the spontaneous ovulation group ($P=1.0$) and among induced ovulation groups, EOI in the hCG did not differ significantly from that in the GnRH group ($P=1.0$, Table 8). In the spontaneous ovulation group, Y sows had EOI of 48.0±24.0 h and L sows had EOI of 42.0±8.5 h ($P=1.0$). In the hCG group, 44.4±6.8 and 42.0±4.2 h in EOI were investigated in the Y and L sows, respectively ($P=1.0$). EOI of 52.0±3.5 h in Y sows and 35.6±7.8 h in L sows were observed in the GnRH group ($P=0.3$). The percentage of sows ovulated within 45 h after the onset of estrus was 40% (4/10 sows) in spontaneous ovulation, 70% (7/10 sows) in hCG induced ovulation and 70% (7/10 sows) in GnRH induced ovulation ($P>0.05$).

Table 8 Intervals from weaning to the onset of estrus (WOI) and from onset of estrus to ovulation (EOI) of spontaneous ovulation, hCG and GnRH induced ovulation groups (d = days, h = hours)

	Spontaneous ovulation	hCG	GnRH
No. of sows	10	10	10
Parity (range)	6-8	6-8	6-8
WOI (d)	3.8±0.6	4.1±0.7	3.4±1.1
EOI (h)	46.0±19.2 *	43.2±5.5	40.5±10.3
Sows with EOI≤ 45 h (%)	4/10 (40)	7/10 (70)	7/10 (70)

Values are mean±SD, * Calculated from data of 6 sows

4.1.5 Discussion

In the present study, the EOI of spontaneously ovulating sows was not significantly different from those of the induced ovulation; nonetheless, a high variation of EOI (19.2 h SD) was observed in this group. Furthermore, variation will be even higher (30.3 h) if data of the 4 late ovulating sows are included in the analyses. Such a high variation makes it difficult to determine an optimal time of insemination with respect to ovulation in spontaneously ovulating sows.

The mean ovulation times of the sows induced ovulation with hCG (40.2 h after hCG injection) or with GnRH (37.5 h after GnRH injection) in the present study were consistent with previous findings which studied in induced estrous gilts (Brussow et al., 1996; Duanyai and Srikandakumar, 1998) and sows (Roca et al., 2003; Kauffold et al., 2007). Interestingly, when multiparous sows were given with GnRH-agonist-gel via intravagina at 96 h after weaning, the time of ovulation from administration of hormone were extended to 51 h (Baer and Bilkei, 2004). The longer interval from intravaginally hormonal treatment to ovulation, compared with intramuscular injection, might be due to differences in the time of hormonal application with respect to the estrous cycle and/or in

the route of GnRH administration which affects absorption rate and finally LH release (Martinez et al., 2003). However, these indicate that the use of GnRH-agonists in any preparation in gilts and sows is able to induce a preovulatory LH surge and ovulation at the certain times.

In addition to induction of ovulation, GnRH can be used for treatment of ovarian cysts in sows (Bollwahn, 1974; Ogasa et al., 1983; Itoh, 1999; Cech and Dolezel, 2007). Nonetheless, interestingly, ovarian cysts were investigated in 23% (3/13) of sows treated with GnRH. The reason for explaining this observation has not been elucidated but in our viewpoint, it might or might not be associated with the effect of hormone. Palasz et al. 1989, Martinez et al. 2003 and Brussow et al. 2007 stated that qualities and quantities of the GnRH product used for ovulation induction affect LH release and ovulation. In our present study, 10 μg GnRH-agonist (buserelin; Receptal®) used were probably able to rapidly increase plasma LH but maximal release of LH might not be high enough to induce ovulation in three treated sows. Therefore, to verify this suggestion, further studies on determination of the effective doses of buserelin required for inducing LH release with subsequent ovulation in pigs are of interest. Contrarily, ovarian cysts observed in 23% of sows in the GnRH group could also deem to be an accidental incidence and were not the consequence of the hormone, since ovarian cysts could affect 2.4 to 40% animals in any swine herd (Vandeplassche et al., 1971; Kudlac, 1980; Schmidt et al., 1995; Castagna et al., 2004). Hence, 9.1% (3/33 sows) of cyst incidence investigated in the present study seem to be common.

After hormonal treatments, the percentages of sows ovulated within 45 h were improved (70%, 7/10 sows) in comparison to spontaneously ovulating sows (40%, 4/10 sows). This indicates that both hCG and GnRH can be used effectively to control ovulation in multiparous weaned sows. It has been demonstrated that synchronization of ovulation with hormones followed by one to two times AI at the fixed-time using fresh or FT boar semen resulted in the high level of farrowing rate and litter size in large numbers of gilts and sows (Iwamoto et al., 1978; Brussow et al., 1996; Martinez et al., 2001, 2002; Roca et al., 2003). However, in this study, the EOI of 30% of sows treated with hormone, either with hCG or GnRH, was still longer than 45 h suggesting that ovulation still showed individual variations in spite of controlling ovulation with hormones. This was similar to

what was already demonstrated, that an exact induction of ovulation cannot be achieved with either substance (Brussow et al., 1990a, b; Waberski et al., 1998; Kauffold et al., 2007).

Although similar efficiencies between hCG and GnRH in controlling ovulation were observed, GnRH has some more advantages over hCG. For instance, GnRH, a peptide hormone, is more suitable for the intended purpose owing to its short-lived effects and can be used repeatedly in each animal without stimulation of antibody production (Brussow et al., 1996). Consequently, it seems to be more possible to incorporate the induction of ovulation regimen by using GnRH into field conditions.

The results of the present study showed that hCG and GnRH have an efficiency in controlling ovulation in naturally estrous weaned L and Y sows, indicated through less variations of EOI and high numbers of sows ovulated at the predicted times (within 45 h after estrus onset) in comparison to those in the spontaneously ovulating sows.

4.2 Experiment 6 and 7: Intra-uterine insemination with low numbers of frozen-thawed boar semen in spontaneous and induced ovulating sows under field conditions

4.2.1 Abstract

The objective of the present study was to evaluate non-return rate (NR), farrowing rate (FR), and number of total pigs born per litter (TB) of weaned sows after intra-uterine insemination (IUI) using low numbers of frozen-thawed (FT) spermatozoa. Semen from 6 boars was cryopreserved individually in a 0.5-ml straw, at a concentration of $1x10^9$ spermatozoa per ml. A total of 60 multiparous sows with weaning-to-estrus interval of ≤ 7 days were included. The sows were detected for standing estrus twice daily and were randomly assigned to two groups: I) spontaneous ovulation and II) induced ovulation, the sows were given 750 IU human chorionic gonadotrophin (hCG) i.m. immediately at estrus detection. Ovulation was determined every 12 h using transrectal ultrasonography. Experiment 6 used FT semen containing $2x10^9$ total spermatozoa per dose. In group I, the sows (n=10) were inseminated at 24 h after the detection of estrus and repeated every 12 h until ovulation. In group II, the sows (n=10) were inseminated once at 41 h after hCG injection. The results showed that the interval from standing estrus to ovulation (EOI) was 39.8±8.0 and 41.5±4.7 h in group I and II, respectively (*P*=0.58). The interval between last insemination and ovulation was 3.8±1.8 and 3.9±2.5 h in group I and II, respectively (*P*=0.96). No significant differences were found in any fertility parameters evaluated between the groups (60 vs 20% NR, 40 vs 20% FR, and 9.3±2.2 vs 7.5±3.5 TB in group I and II, respectively; *P*>0.05). In experiment 7, FT semen containing $1x10^9$ motile spermatozoa per dose was inseminated at 24 h after estrus detection and repeated every 12 h until ovulation in group I (n=20) and at 36, 42 and/or 48 h after hCG treatment in group II (n=20). EOI differed significantly between group I (40.2±5.5 h) and group II (35.6±4.5 h; *P*=0.01). The number of IUI per sow was 2.9±0.6 times in group I and was 2.4±0.5 times in group II. There were no significant differences (*P*>0.05) in the NR (80 vs 85%), FR (60 vs 65%) and the TB (8.0±2.8 vs 9.4±3.7 piglets per litter) between the groups. In conclusion, fertility of induced ovulating sows after a single dose of IUI with low numbers of FT

spermatozoa tended to have the inferior results compared to that of spontaneously ovulating sows after multiple doses of IUI. Multiple doses of IUI with a low number of FT boar spermatozoa provided a fairly good NR, and reasonable FR and TB both in spontaneous and induced ovulating sows.

4.2.2 Introduction

The procedure for artificial insemination (AI) in pigs using frozen-thawed (FT) semen involves the deposition of a high number of FT spermatozoa (5 to 6 $x10^9$ cells) in a large volume of diluent (80 to 100 ml) into the cervix. Despite inseminating with this high sperm number, low fertility levels are usually obtained (about 20 to 30% conception (CR) and farrowing rates (FR) and 2 to 3 total piglets born per litter (TB) lower than those achieved by fresh semen) (Almlid and Hofmo, 1996; Johnson et al., 2000). Therefore, the use of FT semen in commercial AI swine herds is still limited (Wagner and Thibier, 2000).

Recently, a procedure for non-surgical intra-uterine insemination (IUI) in sows, which allows the transcervical deposition of semen into the uterine body, has been developed (Watson and Behan, 2002; Sumransap et al., 2007). The fertility rates achieved by IUI with fresh semen using $1x10^9$ spermatozoa per dose were comparable to those of conventional AI with $3x10^9$ spermatozoa per dose (Watson and Behan, 2002). Furthermore, it has been demonstrated that the number of spermatozoa in the sperm reservoir at about 24 h after insemination was not significantly different between IUI and conventional AI (Wiesak et al., 1990). However, no fertility data are available using IUI with FT boar semen.

Owing to high variation of the ovulation time among individual females and a short survival time of FT boar spermatozoa in the female reproductive tract (Pursel et al., 1978; Weitze et al., 1994; Soede et al., 1995), the induction of ovulation before insemination with FT semen seems to be necessary for maximizing *in vivo* fertility (Konig et al., 1977; Huhn and Konig, 1989). The control of estrus and ovulation with exogenous hormones in gilts and sows has been demonstrated (Wiesak et al., 1990; Brussow et al., 1996; Roca et al., 2003; Baer and Bilkei, 2004). Roca et al. (2003) using human chorionic gonadotrophin (hCG) for controlling ovulation reported that 77.5% FR and 9.31 TB were obtained in

hormonally treated sow after a single deep AI using a low number of FT spermatozoa. The fertility results were not significantly different from those received following insemination with extended fresh semen (75.7% FR and 9.6 TB).

The present study was conducted to evaluate the efficacy of hCG for the control of ovulation in spontaneously estrous weaned sows and to investigate fertility of spontaneous and hCG induced ovulating sows after IUI with low doses of FT boar semen.

4.2.3 Materials and methods

4.2.3.1 Animals

The experiments were conducted in commercial pig farms located in the middle part of Thailand from March to December 2007. The study included a total of 60 purebred sows (30 Landrace; L and 30 Yorkshire; Y) with parity of 2 to 6 and weaning to estrus interval (WOI) of $\leq$ 7 days. After 3 weeks in lactation length, the weaned sows were allocated individually to pens in the mating/gestation units, fed with a commercial diet (Betagro 306®, Betagro-Agro group Co., Ltd., Thailand) according to the nutritional requirements for weaned sows, and were given *ad libitum* access to water via water nipple.

4.2.3.2 Detection of estrus and ovulation

The sows were carefully detected for standing estrus twice daily (8:00 and 16:00 h) after weaning using a back pressure test with a present of a mature boar. Standing estrous sows were then randomly equally distributed to one of two groups, i.e. spontaneous ovulation (group I) and induced ovulation (group II). Induction of ovulation in sows was performed immediately after estrus detection by a single i.m. injection of 750 IU hCG (Chorulon®, Intervet International B.V., Boxmeer, The Netherlands). The occurrence ovulation was investigated every 12 h, beginning 24 h after estrus detection, by using transrectal real-time ultrasonography (Agroscan, L 5/7.5 064, Adelaide, Australia) as described by Soede et al. (1992). Briefly, after manual removal of feces from the rectum and addition of lubricant, a 5 MHz wide band rectal linear probe covered with a plastic sheath containing ultrasound gel was carefully introduced into the rectum. The appearance of preovulatory follicles (6 to 8 mm diameter) on the ovaries was observed. Time of

ovulation was defined as 6 h before the first time when most preovulatory follicles were not found.

4.2.3.3 Semen collection and cryopreservation

The sperm-rich fractions from six mature (2 to 4 years of age) purebred boars (3L and 3Y) were used. The boars were of proven fertility and held in the same herd as the sows in experiment 6. The ejaculates were collected twice a week using the gloved-hand technique, as an ordinary program. Only ejaculates with ≥ 70% motile sperm, as estimated subjectively under a light microscope at 400x magnification, were selected for cryopreservation. The ejaculates were processed and frozen using the straw freezing procedure (Gadea et al., 2004; Buranaamnuay et al., 2009). Briefly, ejaculates were extended (1:1, v/v) in Beltsville Thawing Solution (BTS; Minitüb, Abfüll-und Labortechnik GmbH & Co. KG, Germany) and cooled down to 15°C for 2 h. After centrifugation at 800xg for 10 min, the supernatant was removed by aspiration and the pellets were re-extended with lactose-egg yolk (LEY) extender (80 ml of 11% lactose solution and 20 ml egg yolk) to a concentration of $1.5x10^9$ cells per ml. After cooling to 5°C over a 90-min period, diluted sperm were re-suspended with extender III (LEY extender, 9% glycerol and 1.5% Equex STM Paste; Nova Chemical Sales Inc., Scituate, MA, USA) to a final concentration of $1x10^9$ sperm per ml. The processed sperm were packed into 0.5 ml PVC-french straws (Bio-Vet, Z.I. Le Berdoulet, France) and frozen by placing in liquid nitrogen (LN_2) vapor approximately 3 cm above the level of LN_2 for 20 min. The frozen doses were plunged into LN_2 until thawing. Thawing was achieved by immersing the straws in 50°C of water for 12 sec. Immediately after thawing, the semen was diluted (1:4) with an extender consisting of 95% BTS and 5% LEY extender. The addition of LEY extender prevents the sperm from sticking to the glassware used during motility analysis. The extended thawed semen was incubated in a 38°C water-bath for 30 min before evaluation of sperm motility. FT semen containing ≥ 30% motile spermatozoa was used to insemination.

4.2.3.4 Insemination

FT semen of L boars was inseminated to Y sows and vice versa in order to produce LY crossbred offspring. To minimize effect of individual boar variation, each batch of FT

semen was divided into two portions and used to inseminate sows in both groups by IUI procedure. The IUI device (Deep goldenpig^{TM} catheter; IMV Technologies, L'Aigle, France) was used by inserting through the vagina into the cervix. The inner tube was gently pushed through the outer catheter and situated presumably in the body of the uterus. A syringe containing a predetermined number of FT spermatozoa in 20 ml of BTS was then connected to the device. After insemination, 5 ml of BTS extender were flushed to force remaining sperm out of the device. In experiment 6, all sows studied (n=20) were intra-uterine inseminated with FT semen containing a total of $2x10^9$ spermatozoa per dose. The spontaneously ovulating sows (5L and 5Y) were inseminated 24 h after the detection of estrus and re-inseminated every 12 h until ovulation took place. The induced ovulating sows (5L and 5Y) were fixed-time inseminated once at approximately 41 h following hCG treatment. In experiment 7, $1x10^9$ motile spermatozoa were included in each IUI dose. The sows in group I (10L and 10Y) were inseminated 24 h after detection of estrus and repeated every 12 h until ovulation. The sows in group II (10L and 10Y) were fixed-time inseminated 36 and 42 h after hCG administration. In some cases, the third insemination, at 48 h, was also conducted if at least one preovulatory follicle(s) still remain on the ovaries.

4.2.3.5 Fertility parameters

A 24-day non-return rate (NR), farrowing rate (FR), the number of total piglets born per litter (TB) and the number of piglets born alive per litter (BA) were evaluated.

4.2.3.6 Statistical analysis

The statistical analyses were carried out using SAS (SAS version 9.0, Cary, NC, USA.). The reproductive data and the sperm parameters were presented as mean ± standard deviation (SD). The detection of estrus to ovulation interval (EOI), interval between insemination and ovulation (IOI), TB and BA were analyzed using a general linear model. The statistical model included the effects of group (spontaneous vs induced ovulation), sow breed (L vs Y) and interaction between group and sow breed. NR and FR between groups were compared using Fisher's exact test (experiment 6) or chi-square test (experiment 7). The post-thaw sperm motility, TB and BA between the groups were compared using Student's *t*-test. The differences with $P<0.05$ were considered statistically significant.

4.2.4 Results

Experiment 6

On average, the WOI was 4.4±0.8 days (range 3 to 6) and 3.6±1.4 days (range 1 to 6) in group I and II, respectively (P=0.13). The EOI was 39.8±8.0 h (range 28 to 52) and 41.5±4.7 h (range 32 to 44) in group I and II, respectively (P=0.58).

On average, FT semen used for IUI in both groups had the sperm motility of 38.5% (range 35 to 45%). The number of insemination per sow was 2.2±0.4 times (range 2 to 3) and 1.0 time in group I and II, respectively. The interval between last insemination and ovulation (IOI) was 3.8±1.8 h (range 0 to 7.5) and 3.9±2.5 h (range 2 to 9) in group I and II, respectively (P=0.96). The number of sows with IOI ≤ 6 h was 9 sows and 8 sows in group I and II, respectively. Of the sows having IOI ≤ 6 h, 4 sows in group I and 6 sows in group II did not conceive. All of the sows farrowing had an IOI of ≤ 6 h.

The fertility results including NR, FR, TB and BA of the sows in group I and II are presented in Table 9. No differences were found in any fertility parameters measured between the groups (P=0.17, P=0.63, P=0.48 and P=0.52 for NR, FR, TB and BA, respectively).

TB after IUI with FT semen varied from 6 to 11 piglets per litter in group I and from 5 to 10 piglets per litter in group II. Totally, 37 piglets were born from four sows in group I and 15 piglets were born from two sows in group II.

Experiment 7

Overall, the sperm motility of FT semen was 35.3±5.7 % (range 30 to 45 %) and 36.5±4.0 % (range 30 to 40 %) used in L and Y sows, respectively (P=0.43). Sow breed had no influence on all parameters assessed (P>0.05). No significant correlations were found between the sperm motility and IOI and all of fertility parameters (P>0.05).

There was a significant difference in the EOI between spontaneous and induced ovulation groups (P=0.01). On average, the spontaneously ovulating sows ovulated at 40.2±5.5 h (range 24.5 to 51) after the estrus detection and the induced ovulating sows ovulated at 35.6±4.5 h (range 28 to 43) after the detection of estrus. The number of insemination conducted in spontaneous ovulation group was 2 to 4 times and in induced

Table 9 Estrus to ovulation interval (EOI), non-return rate at 24 days after IUI (NR), farrowing rate (FR), number of total piglets born per litter (TB) and number of piglets born alive per litter (BA) after IUI with frozen-thawed boar semen in spontaneously ovulating sows (group I) and induced ovulating sows (group II); h = hours

Parameters	Group	
	I (spontaneous ovulation)	II (hCG induced ovulation)
No. of sows	10	10
EOI (h)	39.8±8.0	41.5±4.7
Sows conceived at 24 days (%)	6/10 (60)	2/10 (20)
Sows farrowing (%)	4/10 (40)	2/10 (20)
Total piglets born per litter	9.3±2.2	7.5±3.5
Piglets born alive per litter	8.8±3.2	6.5±4.9

Values are mean±SD

ovulation group was 2 to 3 times. Of the 20 spontaneously ovulating sows, 4 sows were inseminated twice, 14 sows were inseminated three times and 2 sows were inseminated four times. For induced ovulation sows, 13 sows were inseminated twice and 7 sows were inseminated three times.

Reproductive performances of the spontaneous and induced ovulating sows are presented in Table 10. In the present study, irregular return to estrus and abortion of 25% (4/16) and 23.5% (4/17) were found in spontaneous and induced ovulation groups, respectively. The overall fertility of spontaneously ovulating weaned sows did not differ from the sows with hCG induced ovulation ($P>0.05$).

4.2.5 Discussion

The interval from hCG treatment to ovulation observed in experiment 6 (41.5 h) was consistent with previous reports; on the other hand, in experiment 7, the value

Table 10 Non-return, abortion and farrowing rates and litter sizes obtained after intra-uterine insemination with frozen-thawed semen in spontaneous (Spon. ovulation) and hCG induced ovulating sows

Parameters	Spon. ovulation	Induced ovulation
No. of sows	20	20
Sows non-return to estrus (%)	16/20 (80)	17/20 (85)
Sows returned to estrus at irregular interval (%)	4/16 (25)	1/17 (5.9)
Sows aborted (%)	0 (0)	3/17 (17.6)
Sows farrowing (%)	12/20 (60)	13/20 (65)
Total piglets born per litter (mean±SD)	8.0±2.8	9.4±3.7
Piglets born alive per litter (mean±SD)	7.8±2.8	8.7±3.7

investigated (35.6 h) was shorter than that of previous studies (Wiesak et al., 1990; Roca et al., 2003). It might be partly resulted from difference in the individual animal response. Martinat-Botte et al. (1985) stated that the responsiveness of sows to exogenous gonadotrophins is different and may be also dependent on farm-specific factors such as genotype and nutrition. The results of earlier studies which were in agreement with above suggestions revealed that ovulation still showed individual variations even though the control of ovulation with hormones have been performed, indicating that an exact induction of ovulation cannot be absolutely achieved with any substance (Brussow et al., 1990a, b; Waberski et al., 1998; Kauffold et al., 2007). However, variations of EOI within group were lower in the induced ovulating sows compared to the spontaneously ovulating sows both in experiment 6 and 7. These suggested that the application of hCG at the estrus detection in weaned sows could control/synchronize the time of ovulation and thus reduces the number of inseminations required per estrus, as shown in experiment 7 (0.5 time per sow less in the induced ovulation group). The mechanism of action of exogenous hCG in controlling ovulation is by mimicking preovulatory luteinizing hormone (LH) peak, which generally occurs 27 to 48 h before ovulation (Mburu et al., 1995; Brussow et al., 1996).

Nevertheless, induction of ovulation using hCG may not be an appropriate method for the routine use since hCG, a glycoprotein hormone, causes antibody production which reduces its efficacy when used several times (Brussow et al., 1996). Furthermore, it has been reported that hCG also causes early embryonic loss in Meishan gilts (Hunter and Picton, 1995).

After insemination, low fertility of the sows in both spontaneous and induced ovulation groups was observed in experiment 6. This problem might be related to the insufficient numbers of spermatozoa per insemination dose ($2x10^9$ total spermatozoa) used in both groups. Also, it could be speculated that the number of inseminations conducted in the induced ovulation group (1 time per sow) might be inadequate. In the present experiment, about $800x10^6$ motile spermatozoa per dose (i.e., ~ 40% post-thaw motility) were inseminated to each sow, and 4 of 9 sows in group I and 6 of 8 sows in group II that were inseminated at the optimal time in relation to ovulation (IOI) $\leq$ 6 h did not conceive. These clearly indicated that $800x10^6$ motile FT spermatozoa are not an adequate number of functional spermatozoa to colonize in the reservoirs. It has been demonstrated that low numbers of spermatozoa per insemination dose result in the low number of functional spermatozoa that colonize in the oviductal sperm reservoirs and hence decrease fertilization rate and litter size (Watson, 2000; Flowers, 2001). Previous studies have shown that one insemination with low numbers of spermatozoa in gilts resulted in fewer embryos, more unfertilized oocytes and lower fertilization rate compared to two inseminations with high numbers of spermatozoa even though precise time relative to ovulation has been practiced (Bracken et al., 2003). It has also been shown that a fixed-time two-insemination scheme provided higher pregnancy rates than a single fixed-time insemination, following control of ovulation with hormones (Heidler et al., 1977; Iwamoto et al., 1978); and two inseminations versus one during estrous period could increase litter size by one piglet (Conrad and Henze, 1988). Therefore, we suggested that the number of FT spermatozoa per dose as well as the number of inseminations during standing estrus should be increased in order to improve fertility results.

Interestingly, the induced ovulating sows in experiment 6 had smaller litter sizes (about 2 piglets per litter) than the spontaneously ovulating sows, although the results were not significant. The exact reasons behind this were not known, but it was probably the

consequence of a single IUI with relatively low numbers of FT spermatozoa as performed in this study and/or the effect of hCG. It has been reported that the injection of hCG at the onset of estrus in order to induce ovulation resulted in an increase of progesterone at 48 h post estrus and shorten the interval from onset of estrus to luteinizing hormone (LH) surge, which was not good for the embryonic survival and fetal development in Meishan gilts (Hunter and Picton, 1995). On the other hand, Kauffold et al. (2007) found no significant differences in litter sizes between LY crossbred sows induced ovulation with hCG and gonadotrophin releasing hormone (GnRH). It could therefore be supposed that the negative effect of hCG is only found in Meishan pigs, and fewer TB and BA of induced ovulating sows obtained in this study resulted from the inappropriate insemination protocol.

In experiment 7, where the number of FT spermatozoa as well as the number of inseminations during standing estrus was increased to $1x10^9$ motile spermatozoa per dose (~$2.8x10^9$ total spermatozoa) and 2 to 3 times per sow in the induced ovulation group, respectively, the fairly good NR ($\geq$ 80%) were revealed. Nonetheless, 60 to 65% FR became observed. The possible reason behind this finding included early embryonic/fetal loss. In the present experiment, the incidence of irregular return to estrus, at 25 to 30 days after IUI, and abortion found in the spontaneous and induced ovulation groups was as high as 12.5% (2/16) and 23.5% (4/17), respectively. This problem is frequently observed after insemination with FT semen (Lwoff et al., 1987; Sailer et al., 1996; Hammadeh et al., 1999). During freezing and thawing of spermatozoa, reactive oxygen species (ROS) arising induce the presence of DNA strand breaks and increase the compactness of spermatozoa nuclei, which both are related to higher instability of sperm DNA (Twigg et al., 1998; Hamamah et al., 1990). These alterations in the chromatin structure could delay paternal nuclear formation during fertilization and induce early embryonic death or lower embryonic development, resulting in a low *in vivo* fertility (Evenson et al., 1980). Owing to the impact of the sperm chromatin state on fertility rates, this sperm parameter should be evaluated in conjunction with conventional parameters, particularly when using FT spermatozoa to insemination.

To our knowledge, the current study generated the first offspring produced in pigs housed in commercial herds by transcervical IUI with FT spermatozoa, albeit average numbers of piglets born per litter were not high. The possible reason for explaining the

the results included insufficient numbers of spermatozoa for each insemination dose ($1x10^9$ motile spermatozoa), rather than the inadequacy of the number of insemination per sow or sub-optimal time of insemination with respect to ovulation. In the present study, IOI of all farrowing sows were ≤ 6 h (data not shown). Therefore, a higher number of spermatozoa should be considered in order to obtain consistently high fertility results. Soede et al. (1992) found that hCG used to induce ovulation had no influence on the number of follicles on the ovaries but instead it could extend the duration of ovulation, leading to more success in fertilization following insemination. This might partly explain why both TB and BA in the hCG induced ovulating sows in experiment 7 were larger than those of spontaneously ovulating sows.

In conclusion, IUI using $800x10^6$ motile FT spermatozoa was not sufficient to provide acceptable fertility results in weaned sows, and a single fixed-time insemination with low numbers of FT spermatozoa seemed not to be an appropriate protocol for induced ovulating sows to achieve high fertility rates. Using multiple IUI with $1x10^9$ motile spermatozoa, the number of inseminations required for attaining reasonable fertility tended to be lower in the weaned sows with induced ovulation. However, further research is still needed to determine the minimal FT sperm number necessary to maximize *in vivo* fertility.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

Although the production of FT semen in pigs has been reported many years ago, the utilization of FT semen for insemination in the commercial swine herds at present is not prevailing yet (Polge, 1956; Wagner and Thibier, 2000). This is owing to the suboptimal fertility of FT spermatozoa when used for conventional AI (Johnson et al., 2000). However, the study on cryopreservation of boar semen is still likely to be important particularly for superior genetic animals. Consequently, the production of FT boar semen, the studies on factors affecting FT sperm quality, and *in vivo* fertility of FT boar semen were focused in this thesis.

1. Factors influencing FT boar sperm quality

The quality of the sperm after cryopreservation is influenced by a number of factors. A couple of external factors include the type of freezing package and the addition of a detergent Equex STM Paste which both were examined in this study. After thawing, the motility, viability and NAR acrosomes of spermatozoa evaluated with conventional methods were improved when Equex STM Paste was added into the freezing media. This finding confirms beneficial effects of the detergent on preventing/diminishing cell damage during the freeze-thaw process (Fraser and Strzezek, 2007; Ponglowhapan and Chatdarong, 2008). Equex STM Paste improves post-thaw survival of spermatozoa by acting as a surfactant to stabilize cell membranes, particularly acrosomal membranes, and to protect spermatozoa against the toxic effects of glycerol during cryopreservation (Arriola and Foote, 1987). However, since the positive effects of this substance are only observed in the present of egg yolk in the semen extender, it is suggested that Equex STM Paste exerts its beneficial action through the alteration of low-density lipoproteins in egg yolk rather than directly affects sperm membranes (Pursel et al., 1978a).

In theory, post-thaw spermatozoa loaded in 0.5-ml straws which have smaller surface-to-volume ratio should not have a better quality than those in 0.25-ml straws.

Nevertheless, based on the results of 12 ejaculates from 4 boars evaluated in this study, the viability and NAR acrosomes of FT spermatozoa packaged in 0.5-ml straws were superior to those in 0.25-ml straws despite being frozen and thawed with their own optimal protocols. The reason behind this is unknown, but it is interesting that similar results have also been observed in dog semen (Nizanski and Dubiel, 2003; Nothling and shuttleworth, 2005). Therefore, in order to find the reason and draw conclusions with boar spermatozoa, more investigations in this aspect might have to be performed.

The present study found the difference of FT sperm quality between individual boars and also discovered that cryosensitivity of their spermatozoa seemed to be consistent, indicated through no significant difference in post-thaw sperm quality between different ejaculates (1^{st} to 3^{rd}) of each boar, and could not be predicted by evaluation of fresh sperm quality using conventional methods. These results are in agreement with what have been reported elsewhere that freezability of boar spermatozoa is a consistent feature of individuals and those individuals differ in susceptibility (Larsson and Einarsson, 1976; Holt et al., 2005). There has been evidence that freezability and post-thaw quality of boar spermatozoa correlate with the genomic differences between individual boars which may lead to an improvement in the predictability of both post-thaw semen quality and fertility of individual males (Thurston et al., 2002). Furthermore, when considering the post-thaw sperm quality in concomitant with the breed of boar (L, Y and D), we found that FT sperm quality (i.e., motility, viability, NAR acrosomes, plasma membrane integrity assessed with sHOST and SYBR) in the Y boars was always lowest. This finding is different from the results of Johnson et al. (1981) who stated that cryotolerance of spermatozoa of Y boars were superior to L boars, resulting in higher FT sperm quality of Y boars.

2. Insemination techniques and *in vivo* fertility of FT boar semen

Cervical AI with FT semen usually results in suboptimal fertility; thereby, deep AI using IUI and DIUI procedures was developed. The present study evaluated fertility (fertilization rate, FR and LS) of FT boar semen after IUI, with $2x10^9$ total spermatozoa per dose, and DIUI, with $1x10^9$ per dose, in spontaneously ovulating weaned sows. The methods yielding better results would be used further. The results revealed that at approximately 2 days following inseminations either with IUI or DIUI, embryo(s) could be

recovered from both sides of the oviducts. This observation, the first report in FT semen, was consistent with previous studies where the extended fresh semen was used (Martinez et al., 2002; Sumransap et al., 2007; Tummaruk et al., 2007). It was demonstrated that both transuterine and transperitoneal migrations were involved in transport of spermatozoa inseminated using DIUI to reach the other side of the oviduct (Martinez et al., 2002). Nonetheless, comparing between techniques, fertilization rate in the IUI group was significantly higher than the DIUI group. The reason for this finding might not associate with the insemination techniques, but rather it was a result of insemination time relative to the moment of ovulation which was not appropriate in the DIUI group (≥ 8 h before ovulation).

After AI using the same procedures (IUI and DIUI) and same numbers of FT spermatozoa (1 to 2 $x10^9$ per dose), acceptable fertility (67% FR and 7.7 to 10.5 LS) were obtained in both groups (P>0.05); however, TB in the DIUI group was about 3 piglets fewer than the IUI group. This was probably the consequence of inadequate numbers of functional spermatozoa used for DIUI ($400x10^6$ motile spermatozoa) which leaded to the unilateral and/or incomplete bilateral fertilization and resulted in the low LS.

Our results indicated that timing of insemination in relation to ovulation and sperm numbers per insemination dose are important factors for successful insemination regardless of insemination procedures and types of semen used. The time of insemination factor becomes more essential when using FT semen because the life span of FT spermatozoa in the female reproductive tract is relatively short compared with the fresh cells, i.e. 4 to 8 h vs about 24 h after insemination, respectively (Waberski et al., 1994; Bertani et al., 1997). It has been demonstrated that the number of spermatozoa per insemination dose relates to both the number of functional spermatozoa colonized in the oviductal sperm reservoir and fertilization rate (Watson, 2000; Flowers, 2001; Tummaruk et al., 2007). Insufficient sperm numbers in the DIUI group might account for the lower fertilization rate and thus smaller LS encountered in the present study.

3. Fertility and fecundity after AI with FT boar semen under field conditions

As insemination at/close to the time of ovulation is one of the key roles for achieving high fertility and ovulation time among individuals is variable, control of

ovulation in female pigs by hormonal treatments prior to insemination might be necessary. The present study assessed the effectiveness of hormones (hCG and GnRH) in controlling ovulation in naturally estrous weaned sows. It was found that EOI of treated sows was 43.2±5.5 h and 40.5±10.3 h in the hCG and GnRH groups, respectively. The results were similar to those of previous researches which studied in weaned sows with induced estrus and ovulation (Brussows et al., 1996; Roca et al., 2003). Moreover, variations in the EOI among treated sows were less than that in spontaneously ovulating sows (5.5 to 10.3 h SD vs 19.2 h SD, respectively) indicating that both hCG and GnRH had the efficacy to control ovulation in sows. However, when comparing between the hormones, hCG seemed to be more effective than GnRH since no incidence of ovarian cysts and less variation in EOI among sows could be observed in the hCG group. The reasons for the development of ovarian cysts in 23% (3/13) of the sows treated with GnRH were not known but, in our view, it could occur accidentally or it could be an effect of the hormone. In present study, GnRH-agonist (buserelin) at a dose of 10 μg were probably able to rapidly increase plasma LH but maximal concentration of released LH might not be high enough to induce ovulation in some treated sows. On the other hand, 9.1% (3/33 sows) of cyst incidence investigated seem to be common and were not a consequence of the given hormone because up to 40% of female pigs in any herd could be affected by ovarian cysts (Schmidt et al., 1995; Castagna et al., 2004). Depended on better results in the present study, the hCG hormone was therefore used to induce ovulation in weaned sows, for insemination with FT semen.

To study feasibility of the use of FT boar semen for insemination in sows under field conditions, the fertility (FR ans LS) of spontaneous and induced ovulating weaned sows after IUI with FT boar semen were evaluated. The results revealed that fertility of induced ovulating sows after a single IUI at the expected ovulation time with low numbers of FT spermatozoa (~$800x10^6$ motile sperm per dose) tended to be lower, despite no significant difference, than that of spontaneously ovulating sows following multiple IUI. Nevertheless, suboptimal fertility results (20 to 40% FR and 7.5 to 9.3 TB) were still demonstrated in both groups. It was thereby suggested that the number of FT spermatozoa per dose and the number of inseminations performed in the induced ovulating sows should be increased for achieving acceptable fertility results. Following increasing FT sperm

numbers per dose to approximately $1x10^9$ motile spermatozoa and increasing insemination times during estrus in the induced ovulating sows, we found that reasonable fertility results (60 to 65% FR and 8.0 to 9.4 TB) were obtained in both spontaneous and induced ovulation groups ($P>0.05$). Interestingly, owing to a less variation in EOI among hormonally treated sows, the number of insemination per estrus conducted in this group was fewer in comparison to the naturally ovulating group. This means more sows are able to be inseminated with one ejaculate of any boar. This aspect should be more considered when using semen prepared from valuable animals. Although fertility results were somewhat low, the current study generated the first offspring produced in pigs using transcervical IUI with FT spermatozoa. However, in order to apply this technology to field conditions especially commercial pig farms where production levels are highly concerned, sperm numbers per insemination dose may have to be increased for better fertility rates.

The results of all trials indicated that the production of cryopreserved boar semen and artificial insemination with FT boar semen could be successfully performed in Thailand. An IUI procedure was considered to be suitable for FT boar semen to produce acceptable fertility rates and when conducting in sows with induced ovulation, the number of insemination during estrus could be decreased without lowering the results. Taking all of them together, it is very useful for the conservation and/or production of animal with high genetic merits.

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APPENDIX

List of publications and conferences

Local:

1. **Buranaamnuay, K.**, Singlor, J., Sirivaidyapong, S., Tummaruk, P. and Techakumphu, M. 2005. The effect of collection frequency on semen quality in Holstein Friesian crossbred-bulls. In: Proc 43rd Kasetsart University Annual Conference. Kasetsart University, Thailand: 119-127.
2. **Buranaamnuay, K.**, Singlor, J., Wongtawan, T., Tummaruk, P. and Techakumphu, M. Intra-uterine artificial insemination with frozen-thawed boar semen in sows in Thailand. 2006. The 2nd Symposium on Animal Reproduction; Trend of Biotechnology for Animal Reproduction: From basic to advance, 27-28 March 2006. Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand: 4.
3. **Buranaamnuay, K.**, Singlor, J., Tummaruk, P. and Techakumphu, M. 2006. The success of boar semen cryopreservation in Thailand: The impact of straw volume and Equex-STM. Thai J Vet Med 36: 64 (Abstract).
4. **Buranaamnuay, K.**, Wongtawan, T., Kaeoket, K., Tummaruk, P. and Techakumphu, M. 2006. Piglets born after intra-uterine artificial insemination with frozen-thawed boar semen in Thailand. Thai J Vet Med 36: 65 (Abstract).
5. **Buranaamnuay, K.**, Singlor, J., Tummaruk, P. and Techakumphu, M. 2006. Effects of the breed and individual differences on boar sperm quality after cryopreservation. RGJ Seminar Series XLVII; Reproductive Biotechnology for Improving Animal Breeding Strategies, 20 October 2006. Chulalongkorn University, Nan Province, Thailand.
6. **Buranaamnuay, K.**, Panyaboriban, Y., Satrakulwong, W., Pholnuengma, H., Tummaruk, P. and Techakumphu, M. 2008. Fertilization rate after intra-uterine insemination and deep intra-uterine insemination with frozen-thawed boar semen. RGJ-Ph.D. Congress IX, 4-6 April 2008. Chonburi, Thailand.

7. **Buranaamnuay, K.**, Panyaboriban, Y., Satrakulwong, W., Pholnuengma, H., Wongtawan, T., Tummaruk, P. and Techakumphu, M. 2008. *In vivo* fertility of frozen-thawed boar semen after transcervical artificial insemination in pig. 2008 CU VET Graduate Seminar: Innovations for Animal Health and Production, 8-9 April 2008. Chulalongkorn University, Bangkok, Thailand.
8. Tretipskul, C., **Buranaamnuay, K.**, Koonjaenak, S., Tummaruk, P. and Techakumphu, M. 2009. The use of computer-assisted sperm analysis (CASA) for discriminating a series of motility pattern of frozen-thawed boar semen. (in preparation)

International:

1. **Buranaamnuay, K.**, Wongtawan, T., Kaeoket, K., Tummaruk, P. and Techakumphu, M. 2006. Intra-uterine artificial insemination with frozen-thawed boar semen in sows in Thailand. In: Proc 19th Inter Pig Vet Soc Congr 2. Copenhagen, Denmark: 526.
2. **Buranaamnuay, K.**, Singlor, J., Tummaruk, P. and Techakumphu, M. 2006. Effects of straw volume and Equex-STM® on boar semen quality after cryopreservation. In: Proc 19th Inter Pig Vet Soc Congr 2. Copenhagen, Denmark: 548.
3. Tejerina, F., **Buranaamnuay, K.**, Saravia, F., Wallgren, M. and Rodriguez-Martinez, H. 2008. Assessment of motility of ejaculated, liquid-stored boar spermatozoa using computerized instruments. Theriogenology 69: 1129-1138.
4. **Buranaamnuay, K.**, Tummaruk, P. and Techakumphu, M. 2008. Conception rate, farrowing rate and litter size after intrauterine insemination with cryopreserved boar semen in spontaneous and induced ovulating sows. In: Proc 15th Congr Fed Asian Vet Assoc& OIE Symposium. Bangkok, Thailand: 163-164.
5. Panyaboriban, Y., Satrakulwong, W., Pholnuengma, H.**, Buranaamnuay, K.**, Tummaruk, P. and Techakumphu, M. 2008. Number of embryos and unfertilized ova after intra-uterine and deep intra-uterine insemination with cryopreserved boar semen in sows. In: Proc 15th Congr Fed Asian Vet Assoc& OIE Symposium. Bangkok, Thailand: 195-196.
6. **Buranaamnuay, K.**, Singlor, J., Tummaruk, P. and Techakumphu, M. 2008. The establishment of boar semen cryopreservation in Thailand: post-thaw semen quality, sperm concentration and variation among ejaculates. Thai J Agri Sci 41(3-4): 135-141.

7. **Buranaamnuay, K.**, Tummaruk, P., Singlor, J., Rodriguez-Martinez, H. and Techakumphu, M. 2009. Effects of straw volume and Equex-STM® on boar semen quality after cryopreservation. Reprod Dom Anim 44: 69-73.
8. **Buranaamnuay, K.**, Großfeld, R., Struckmann, C. and Rath, D. Use of glycerol or amides, along with antioxidants fails to improve quality of frozen-thawed boar sperm. 2009. The 13th Conf European Soc Dom Anim Reprod. Ghent, Belgium.
9. **Buranaamnuay, K.**, Wongkaweewit, K., Raksasub, R, Prommachart, P., Tummaruk, P. and Techakumphu, M. Control of ovulation in sows in the tropical environment using human chorionic gonadotrophin in comparison to gonadotrophin releasing hormone analog. 2010. The 36th Annual Conference of the IETS/the 23rd Annual Meeting SBTE. Cordoba, Argentina.
10. **Buranaamnuay, K.**, Tummaruk, P. and Techakumphu, M. Intra-uterine insemination using frozen-thawed boar semen in spontaneous and induced ovulating sows under field conditions. 2010. The 36th Annual Conference of the IETS/the 23rd Annual Meeting SBTE. Cordoba, Argentina.

BIOGRAPHY

Miss Kakanang Buranaamnuay was born on September 4th, 1981 in Bangkok province, Thailand. She graduated with Degree of Doctor of Veterinary Medicine (DVM) (1st Class Honour) from the Faculty of Veterinary Science, Chulalongkorn University, in 2003. In 2004, she received a scholarship from the Thailand Research Fund through the Royal Golden Jubilee Ph.D Program (Grant No. 5V.CU/46/A.1) to perform a Ph.D program at the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University.