ความแตกต่างของโปรตีนบางชนิดในเซลล์อสุจิและเซมินอลพลาสมา ในน้ำเชื้อพ่อสุกรที่มีความสามารถในการถูกแช่แข็งสูงและต่ำ

นางจรรยาพร รุ่งเรืองศักดิ์

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Difference of some spermatozoa and seminal plasma proteins
in good and poor freezability boar ejaculates

Mrs. Janyaporn Rungruangsak

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
Faculty of Veterinary Science
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จรรยาพร รุ่งเรืองศักดิ์ : ความแตกต่างของโปรตีนบางชนิดในเซลล์อสุจิและเซมินอลพลาสมาในน้ำเชื้อพ่อสุกรที่มีความสามารถในการถูกแช่แข็งสูงและต่ำ (Difference of some spermatozoa and seminal plasma proteins in good and poor freezability boar ejaculates) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. น.สพ. ดร. เผด็จ ธรรมรักษ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: สพ.ญ. ดร. คคนางค์ บูรณะอำนวย, 59 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาความแตกต่างของโปรตีนบางชนิดในเซมินอลพลาสมา ได้แก่ GPX5 และ FN1 และ โปรตีนบางชนิดในเซลล์อสุจิ ได้แก่ TPI และ ACRBP ในน้ำเชื้อพ่อสุกรที่มีความทนต่อการถูกแช่แข็งสูงและต่ำ ทำการศึกษาโดยใช้น้ำเชื้อที่รีดเก็บจากพ่อสุกรจำนวน 38 ครั้งในการศึกษาโปรตีนในเซมินอลพลาสมา และ จำนวน 32 ครั้งในการศึกษาโปรตีนในเซลล์อสุจิ โดยตัวอย่างทั้งหมดเก็บจากพ่อสุกร 27 ตัว (ดูร็อค= 13 ตัว แลนด์เรซ = 8 ตัว ยอร์คชาย= 6 ตัว) ทำการแบ่งน้ำเชื้อเป็น 2 ส่วน โดยส่วนแรกทำการปั่นแยกเซมินอลพลาสมา และ เซลล์อสุจิ หลังจากนั้นจะทำการเก็บตัวอย่างเซลล์อสุจิ และ เซมินอลพลาสมาที่อุณหภูมิ -80 องศาเซลเซียส เพื่อนำไปสกัดและตรวจหาโปรตีน น้ำเชื้อพ่อสุกรส่วนที่สองนำไปแช่แข็ง ภายหลังการแช่แข็งและทำละลายแบ่งน้ำเชื้อพ่อสุกรตามอัตราการเคลื่อนที่ของเซลล์อสุจิออกเป็น 3 กลุ่ม ได้แก่ น้ำเชื้อที่มีความสามารถในการแช่แข็งสูง ปานกลาง และ ต่ำ โดยแต่ละกลุ่มมีอัตราการเคลื่อนที่ของอสุจิภายหลังการทำละลาย เฉลี่ย 60.2 ± 1.67% 29.3 ± 2.02% และ 16.6 ± 2.17% ตามลำดับ ปริมาณโปรตีนในตัวอย่างน้ำเชื้อพ่อสุกรถูกวิเคราะห์ด้วยวิธีวิเคราะห์แบบเวสเทิร์นบลอท ผลการทดลองพบว่า ในเซมินอลพลาสมา FN1 มีปริมาณสูงในน้ำเชื้อพ่อสุกรที่มีความทนต่อการถูกแช่แข็งสูงกว่ากลุ่มที่มีความทนต่อการถูกแช่แข็งต่ำ (0.08±0.01 กับ 0.05±0.01, $P < 0.05$) แต่ไม่แตกต่างกับกลุ่มที่มีความทนต่อการถูกแช่แข็งปานกลาง (0.07±0.01, $P>0.05$). นอกจากนี้ยังพบว่า FN1 มีความสัมพันธ์ในเชิงบวกกับอัตราการเคลื่อนที่ของอสุจิ (r =0.43, P=0.007) อัตราการเคลื่อนที่ไปข้างหน้าของเซลล์อสุจิ (r=0.42, P=0.009) ลักษณะการเคลื่อนที่ของอสุจิแบบวีเอพี (r= 0.34, P=0.04) สัดส่วนของเซลล์อสุจิที่มีความผิดปกติแบบหางงอ (r =0.36, P = 0.03) สัดส่วนของเซลล์อสุจิที่มีความผิดปกติแบบหางม้วน (r= 0.31, P=0.05) ไม่พบความแตกต่างของปริมาณโปรตีนชนิด GPX5 ในแต่ละกลุ่ม แต่พบว่าปริมาณโปรตีนชนิด GPX5 มีความสัมพันธ์ในเชิงบวกกับปริมาณของ FN1 (r = 0.35, P = 0.03) ในเซลล์อสุจิพบว่าปริมาณโปรตีนชนิด TPI มีความสัมพันธ์ในเชิงลบกับทั้งอัตราการเคลื่อนที่ของเซลล์อสุจิ (r = -0.43, P = 0.01) และ อัตราการเคลื่อนที่ไปข้างหน้าของเซลล์อสุจิ (r = -0.35, P = 0.05) นอกจากนี้ยังพบว่าปริมาณโปรตีนชนิด TPI ในน้ำเชื้อที่มีความทนต่อการถูกแช่แข็งปานกลาง (4.4 ± 0.46, $P<0.05$) สูงกว่ากลุ่มที่มีความทนต่อการถูกแช่แข็งสูง (3.2 ± 0.36) และต่ำ (4.1 ± 0.39) ปริมาณของ ACRBP พบว่าไม่มีความแตกต่างในแต่ละกลุ่มของน้ำเชื้อ อย่างไรก็ดีปริมาณของโปรตีนชนิด ACRBP มีความสัมพันธ์ในเชิงบวกกับอัตราการเคลื่อนที่ของอสุจิ (r =0.38, P = 0.03) การศึกษานี้สรุปได้ว่า น้ำเชื้อของพ่อสุกรที่มีปริมาณโปรตีนชนิด FN1 สูงมีความทนต่อการถูกแช่แข็งสูงกว่าน้ำเชื้อของพ่อสุกรที่มีปริมาณโปรตีน FN1 ในน้ำเชื้อต่ำ อัตราการเคลื่อนที่ของเซลล์อสุจิหลังการแช่แข็งและทำละลายมีความสัมพันธ์ในเชิงบวกกับปริมาณโปรตีนชนิด FN1 ในเซมินอลพลาสมา และโปรตีนชนิด ACRBP ในเซลล์อสุจิ และมีความสัมพันธ์ในเชิงลบกับโปรตีนชนิด TPI ในเซลล์อสุจิ

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5875328431 : MAJOR THERIOGENOLOGY
KEYWORDS: BOAR,FROZEN SEMEN, SEMINAL PLASMA, SPERM, PROTEIN

JANYAPORN RUNGRUANGSAK: Difference of some spermatozoa and seminal plasma proteins in good and poor freezability boar ejaculates. ADVISOR: PROF. PADET TUMMARUK, Ph.D., CO-ADVISOR: KAKANANG BURANAAMNUAY, Ph.D., 59 pp.

The present study was performed to determine the differences of some seminal plasma proteins, i.e., glutathione peroxidase 5 (GPX5) and fibronectin 1 (FN1), and some spermatozoa proteins, i.e., triosephosphate isomerase (TPI) and ACRBP (acrosin binding protein), in the boar semen having good and poor freezability. The study was conducted by including 38 ejaculates in seminal plasma study and 32 ejaculates from spermatozoa study from 27 boars (Duroc=13, Landrace= 8, Yorkshire= 6). The semen was split into two portions. The first portion was centrifuged to separate the pellet of sperm from the seminal plasma. The samples were kept at −80 °C until protein extraction. The second portion was cryopreserved. After thawing, the ejaculates were classified into 3 groups according to their post-thawed sperm motility, i.e., good, moderate and poor. Post-thawed sperm motility in each group were 60.2 ± 1.67%, 29.3 ± 2.02% and 16.6 ± 2.17%, respectively. The samples were determined by using Western blot analysis. It was found that the level of FN1 in seminal plasma was higher in the semen with good than poor freezability (0.08±0.01 *vs* 0.05±0.01, respectively, $P < 0.05$), but did not differ significantly compared to semen with moderate freezability (0.07±0.01, $P > 0.05$). Additionally, the level of FN1 in the seminal plasma was positively correlated with the post-thawed sperm progressive motility (r = 0.42, P = 0.009), total motility of the boar spermatozoa (r = 0.43, P = 0.007), VAP (r= 0.34, P=0.04), the proportion of bent tail sperm (r = 0.36, P = 0.03) and the proportion of coil tail sperm (r = 0.31, P = 0.05). GPX5 was not correlated with the post-thawed sperm qualities ($P > 0.05$). However, the levels of GPX5 was positively correlated with FN1 (r = 0.35, P = 0.03). For spermatozoa proteins, the level of TPI was negatively correlated with the post-thawed sperm progressive motility (r = -0.35, P = 0.05) and total motility (r = -0.43, P = 0.01). Additionally, the level of TPI was higher in moderate (4.4 ± 0.46, $P < 0.05$) than good (3.2 ± 0.36) and poor (4.1 ± 0.39) freezability ejaculates. The level of ACRBP did not differ significantly among semen freezability groups. However, the level of ACRBP was positively correlated with post-thawed total sperm motility (r = 0.38, P = 0.03). It could be concluded that boar semen containing a high level of FN1 in seminal plasma had a higher freezability than those containing low level of FN1. The post-thawed sperm motility were positively correlated with the level of FN1 in boar seminal plasma and the level of ACRBP in spermatozoa and negatively correlated with TPI in boar spermatozoa.

Department:	Obstetrics Gynaecology and Reproduction	Student's Signature ....
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ABBREVIATIONS

AI	artificial insemination
ACRBP	acrosin binding protein
ALH	amplitude of lateral head displacement
BHT	butylated hydroxyltoluene
BCF	beat cross frequency
CASA	computer-Assisted Sperm Analysis
CP	cryoprotectant
CLC	Cholesterol loaded cyclodextrin
COD	catalase
DHA	docosahexaenoic acid
EthD-1	ethidium homodimer-1
FITC-PNA	fluorescein isothiocyanate-labeled peanut agglutinin
FT	frozen-thawed
FN1	fibronectin1
GSH	glutathione
GPX5	glutathione peroxidase5
GFE	good freezability
HSP90AA1	heat shocked protein 90AA1
HSP70	heat shocked protein 70
H_2O_2	Hydrogen peroxide
IVF	In-vitro fertilization
LEY	lactose egg yolk
LIN	linearity movement
LN_2	liquid nitrogen
MFE	moderate freezability
ODF	outer dense fiber
PMOT	progressive motility
PCV2	porcine circovirus
PFE	poor freezability

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PRRS	porcine reproductive and respiratory syndrome
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis
sHOST	short hypo osmotic swelling test
SOD	superoxide dismutase
STR	straightness; ratio of VSL/VCL
TBS	Tris-buffered saline
TPI	Triosephosphate isomerase
TMOT	total motility, percentage of sperm moving with straightness
VAP	average velocity path
VCL	curvilinear velocity
VDAC2	voltage-dependent anion channel 2
VSL	straight-line velocity
WOB	wobble of the curvilinear trajectory; ratio of VAP/VCL

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CHAPTER I
INTRODUCTION

Importance and Rationale

In current swine industry worldwide, more than 90% of mating is performed by artificial insemination (AI) and over 99% of the semen used for AI in practice is extended fresh semen. In general, the extended fresh semen is stored at 15–20°C for 0 to 3 days before use. In practice, 85% of insemination is conducted on the day of semen collection and the following day (Johnson et al., 2000). Up to date, less than 1.0% of insemination in pig was carried out by using frozen-thawed semen (Yeste, 2015). The reason is due to the fact that AI using extended fresh semen results in a higher conception rate and litter size compared to frozen-thawed semen (Chanapiwat et al., 2014). The extended fresh semen cannot maintain their qualities for a long period of time. Although many extenders have been developed to increase lifespan of the extended fresh semen, boar semen quality can be maintained for only few days. Thus, superior genetic boars cannot be preserved for a long period of time by using conventional extended fresh semen. Therefore, cryopreservation of boar semen is the only one method to preserve superior genetic boar semen for a long period of time.

The cryopreservation of boar semen has been developed for commercial swine herds both in pellet form and in straws (Eriksson et al., 2002; Men et al., 2012). The cryopreserved boar semen can be exported across countries for improving swine genetics. However, low fertility rate of sows following AI using frozen-thawed boar semen is still commonly observed (Buranaamnuay et al., 2009; Chanapiwat et al., 2014). Another advantage of using frozen-thawed boar semen is to control the transmission of diseases among herds. The study of boar semen extender, antioxidants and cryoprotective agent for frozen boar semen revealed that 2.0 - 3.0% glycerol gave the best results in the production of frozen boar semen (Kim et al., 2011; Zeng et al.,

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2014). In addition, an addition of some antioxidants, e.g., vitamin E, alpha-tocopherol, glutathione (GSH), taurine, cysteine, butylated hydroxyltoluene (BHT), superoxide dismutase (SOD) and catalase, in the semen extender improved the quality of frozen-thawed boar semen (Pena et al., 2003; Roca et al., 2005). Moreover, a supplement of docosahexaenoic acid (DHA), from fish oil, in the semen extender improved the sperm cell membrane integrity and increased progressive motility of the boar spermatozoa post-thawing (Kaeoket et al., 2010b). Nevertheless, the supplement of various substances can improve the survival rate of frozen-thawed boar spermatozoa for only 5-10% compared to the control.

In fact, factors affecting frozen-thawed boar semen are composed of both internal and external factors. The control of some external factors can only slightly improve the frozen-thawed boar semen. Thus, some internal factors, e.g., protein contents in the spermatozoa and/or seminal plasma, are needed to be further investigated. During recent years, studies have been focused on some biological markers including the protein contents in the boar seminal plasma and spermatozoa as indicators of freezability, fertility and fecundity markers of the boar semen. It has been demonstrated that some proteins had a positive effect on fertility and freezability, while some of them have a negative impact. The purpose of this study is to investigate the differences of some protein contents in the boar spermatozoa and seminal plasma in good and poor freezability boar semen. The research question is whether or not the spermatozoa and/or seminal plasma proteins have a significant impact on freezability of boar semen.

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Objectives

To investigate the differences of some protein contents in the spermatozoa (i.e., ACRBP, TPI) and seminal plasma (i.e., GPX5, FN1) in good and poor freezability boar semen.

Expected output

1. To obtain a novel knowledge on the effect of seminal plasma and sperm proteins on freezability of boar semen.
2. To be able to predict freezability of boar semen.

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CHAPTER II
LITERATURE REVIEW

Cryopreservation of boar semen

The use of fresh extended semen in swine industry includes a number of advantages such as genetic improvement and reduce costs. However, fresh semen cannot be stored for a long period of time. Thus, the frozen boar semen is an option to keep the genetic materials for a long period of times. Cryopreservation has numerous potentials, such as the control of transmitted diseases i.e. PCV2, PRRS compared with extended semen, the reduction of the sudden disease outbreak or natural disaster, and the worldwide distribution of desirable genetics (Bailey et al., 2008). Boar semen cryopreservation is important for global markets. However, the limitation of boar frozen semen is the reduction of the frozen-thawed sperm motility and all sperm quality parameters. It is because of the sensitive to cold shock of boar spermatozoa (Casas and Althouse, 2013).

The first protocol of frozen-thawed boar semen has been established since 1975 (Pursel and Johnson, 1975). Thereafter, studies have been intensively investigated various parameters to improve the cryopreservation process (Johnson et al., 2000). Nonetheless, a protocol of frozen-thawed boar semen developed by Westendorf et al. (1975) is still being used (Carvajal et al., 2004). Nowadays, the use of frozen-thawed boar semen is limited due to the reduction of fertility rate compared to the extended fresh semen (Knox, 2015). The cryopreservation process is a cause of decrease in the motility, viability, acrosome integrity, DNA integrity and fertilizing ability of FT sperm (Yeste, 2016). Studies have been done to improve the boar cryopreservation process. The main problem of frozen-thawed boar semen is cryoinjury. The viability of sperm cell after cryopreservation process is associated with both freezing and thawing process. The plasma membrane of boar sperm includes high proportion of polyunsaturated fatty acids (PUFA). Then, restriction movements of phospholipids membrane occur when temperatures are below 5 °C (Cerolini et al., 2001). As a result,

the boar spermatozoa are susceptible to cold shock and the lipid peroxidation that correlate with reactive oxygen species (ROS) attacked from cryopreservation process (Alvarez and Storey, 1992; White, 1993), Therefore, the motility and capacity for fertilization of the boar spermatozoa are decreased (Aitken, 1989).

To minimize the level of ROS from cryodamage, many antioxidants in boar semen extender have been studied such as glutathione (GSH) (Yeste et al., 2013b). Yeste et al. (2013b) revealed that both addition of GSH and procaine hydrochloride had a stabilizing effect on the nucleoprotein structure of FT-sperm but only GSH affected sperm viability. Moreover, the supplementation of antioxidants e g., GSH, vitamin E, L-cysteine and DHA from fish oil into freezing extender improved FT-sperm quality (Kaeoket et al., 2008; Kaeoket et al., 2010a). In case of GSH supplementation, a combination of GSH and Ascorbic acid was beneficial for FT- boar sperm (Giaretta et al., 2015). Furthermore, previous studies found that supplementation of superoxide dismutase (SOD) and catalase (COD) or only COD could improve the FT-sperm quality (Roca et al., 2005). The addition substance such as cholesterol-loaded cyclodextrins (CLC) which can stabilize plasma lemma may improve FT sperm quality in mammal (Purdy and Graham, 2004). In the previous studies, the addition of CLC to semen extender improves FT sperm quality and in vitro fertilizing ability (Tomas et al., 2011). Moreover, Hu et al. (2014) found that the alginate maintained the acrosome integrity, increased the activities of SOD and GPX and decreased malondialdehyde levels.

The most common sugar used for boar semen cryopreservation is lactose (Roca et al., 2004; Buranaamnuay et al., 2011). Studies demonstrated that the cryoprotective effect of sugars is different among species (Gomez-Fernandez et al., 2012). Nonetheless, results in boar FT-semen suggest that disaccharides have higher cryoprotective effect than monosaccharide. In common, the main permeable CPA is glycerol with an optimal concentration of 2-3%. Okazaki et al. (2009) found that the hyperosmotic (400 mOsm/kg) and 2% glycerol freezing extender are beneficial for the FT boar semen.

For thawing process, the most widely used thawing rate in boar sperm is 37°C for 20–30 second (Gomez-Fernandez et al., 2012). However, the different protocol (50°C for 12 second) is also available (Buranaamnuay et al., 2009). Some studies found the FT-motility in rapid thawing rate (70 °C for 8 second) is greater than in slow thawing rate (37 °C for 20–30 second) (Tomas et al., 2014).

Moreover, breed of boars is another factor affecting FT sperm quality (Waterhouse et al., 2006a; Chanapiwat and Kaeoket, 2015). In addition, the external factors that affect FT sperm quality also includes season. Barranco et al. (2013) found the FT sperm quality was lower in summer and autumn, than in winter and spring. This indicates that the season of ejaculate collection influences sperm freezability.

Previous studies demonstrated that even though there are many optimization of cryopreservation protocols and modification of extenders, FT sperm quality is still limited. Nowadays, there are a major attention to identify freezability markers in boars (Yeste, 2016).

Associations between semen proteins and freezability

Freezability is defined as "the ability of the sperm to withstand cryopreservation procedures" (Mazur et al., 2008; Yeste, 2016). Boar ejaculates are classified into good and poor freezability (Casas et al., 2009). Not all ejaculates have the same freezability (Dyck et al., 2011; Yeste, 2016). In the previous studies, Pena et al. (2006) found that the FT sperm motility and sperm membrane integrity from sperm rich fraction are better than other fractions. However, the individual boar response is not only one factor related to sperm freezability, the difference of semen-processing techniques has also been reported to be greatly influenced (Parrilla et al. (2012). Although, the differences between good freezability ejaculate (GFE) and poor freezability ejaculate (PFE) remain unknown, some studies have been classified the variation between boar ejaculates into GFE and PFE by using amplified fragment length polymorphism markers. The differences of genetic could be responsible for these freezability differences (Thurston et al., 2002). Moreover, Yeste et al. (2013a) found that PFE are less resistant than GFE to cryopreservation in terms of the integrity of

nucleoprotein structure. Although, the mechanisms of different ejaculate freezability remain unknown, several studies report the correlation between sperm, seminal plasma proteins and boar freezability (Casas et al., 2010; Vilagran et al., 2013; Vilagran et al., 2014; Vilagran et al., 2015).

For boar semen cryopreservation, the main problem is to classify of GFE and PFE before starting cryopreservation procedures. Because the conventional sperm parameters in fresh or extended semen cannot predict the ejaculate freezability or fertility in boars, identification of genetic and protein markers are still needed (Hu et al., 2008; Dyck et al., 2011; Yeste et al., 2013a).

During recent years, the study of potential biomarkers is seminal plasma, because it is a complex secretion from testis, epididymis, and accessory sex glands. In the previous studies, the addition of 50% of boar seminal plasma to frozen-thawed sperm has been found to have beneficial effects on frozen-thawed sperm (Kaeoket et al., 2011; Fernandez-Gago et al., 2013). Seminal fluid is composed of several seminal plasma proteins that influence on sperm parameters i.e., motility, capacitation, sperm transport, survival and longevity, protection against damages, and the formation of the sperm reservoir inside the female reproductive tract (Novak et al., 2010).

In human, the potential markers used to classify male fertility consist of a number of seminal plasma proteins (Yamakawa et al., 2007; Martinez-Heredia et al., 2008). In animals, proteomic analyses in seminal plasma proteins have been initiated in the bull (Moura et al., 2007) and stallion semen (Fouchécourt et al., 2000). In boar semen, Vilagran et al. (2015) found that fibronectin 1 (FN1) is a positive freezability marker of the boar semen. Another seminal plasma, i.e., GPX5 is also related to protection of the sperm and plasma membrane against peroxides (Chabory et al., 2009; Noblanc et al., 2011) and is positively correlated with farrowing rates (Novak et al., 2010). However, GPX5 cannot predict boar freezability (Vilagran et al., 2016). Some studies found that N-acetyl-ß-hexosaminidase protein in boar seminal plasma is negatively correlated to sperm viability, motility, and lipid peroxidation (Wysocki et al., 2015).

With regard to the sperm freezability, Vilagran at al. (2013) found positive relationship between the amount of Acrosin binding protein (ACRBP) and post-thawed

motility in boar sperm. The mechanism of ACRBP controlling of acrosin was released from the matrix during the acrosome reaction (Baba et al., 1994). On the other hand, Triosephosphateisomerase (TPI) has been described as a negative freezability marker (Vilagran et al., 2013). Furthermore, voltage-dependent anion channel 2 (VDAC2) sperm proteins that classify as positive boar freezability marker, play an important role in osmotic and thermal stress include fluidity of sperm membrane (Vilagran et al., 2014). Chen et al. (2014) found the levels of A kinase anchoring protein 3 (AKAP3), superoxide dismutase (SOD1), triosephosphate isomerase 1 (TPI1), and outer dense fiber 2 (ODF2) proteins were increased in FT boar spermatozoa when compared with those in the fresh sample. These may be related to alteration of proteins which is a cause of premature capacitation in FT sperm.

Casas et al. (2009) had studied in 10 boar ejaculates, found that the protein levels of Cu/ZnSOD at 240 min post-thaw and of HSP90AA1 at 17 °C and 5 °C were significantly different between groups. The lower expression of HSP90AA1 and the higher expression of Cu/ZnSOD were found in PFE compared to GFE. This seemed to be related to the higher sensitivity to cold-shock in PFEs. A study of Yeste et al. (2014) determined the effect of different holding times (3 and 24 hours) on FT-sperm quality parameters. They found that a holding time of 24 h showed a higher sperm resistance to freeze-thawing procedures. It was correlated with changes in the phosphorylation levels of serine residues in GFE groups.

In addition, proteomic studies have found that several proteins such as cytochrome b-c1 complex subunit 2 was highly expressed in high-litter size whereas cytochrome b-c1 complex subunit 1 , beta-tubulin, Ras-related protein Rab-2A, spermadhesin AQN-3, and speriolin were highly expressed in low-litter size spermatozoa (Kwon et al., 2015). GPX5 and TPI affected boar fertility and GPX5 seemed to be a negative marker of boar semen quality. (Vilagran et al., 2016). Nonetheless, Novak et al. (2010) found positive correlation between GPX5 and litter size index. Some sperm proteins, such as TPI involved in zona pellucida binding (Auer et al., 2004; Petit et al., 2013), correlated to low litter sizes (Kwon et al., 2015b; Rahman et al., 2017) and found in asthenozoospermic human (Siva et al., 2010). In addition, Vilagran et al.

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(2016) found that TPI was significantly higher in the low than in the high sperm quality group and was found negatively correlated with fresh semen quality.

Results from previous studies showed a relationship both positive and negative on freezability in boars as shown in Table 1. Thus, it is uncertain how to predict the freezability of boars. The purpose of the present study was to investigate the difference of some seminal plasma and sperm proteins on boar sperm freezability. The relationship between amount of protein in seminal plasma (FN1, GPX5) and sperm proteins (ACRBP, TPI) and frozen-thawed sperm motility patterns was determined.

Table 1 Sperm and seminal plasma proteins associated with freezability of the boar semen

Type of protein	Direction	Reference
Sperm protein		
HSP90AA1	positive	Casas et al. (2010)
HSP70	positive	Yeste et al. (2014)
ACRBP	positive	Vilagran et al. (2016)
VDAC2	positive	Vilagran et al. (2013)
TPI	negative	Vilagran et al. (2014)
Seminal plasma protein		
Fibronectin 1	positive	Vilagran et al. (2015)
N-acetyl-β-hexosaminidase	negative	Wysocki et al. (2015)

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CHAPTER III
MATERIALS AND METHODS

This project proposal was approved by the Institution of Animal Care and Use Committee (IACUC)-protocol no. 1631032.

Animals and semen collection

The present study was performed at Swine Research and Development center, Nakhon Ratchasima, Thailand. A total of 30 boars (D=35, L=34, Y=25) with proven fertility routinely used in the farm were included. A total of 38 ejaculates were used in seminal plasma study and 32 ejaculates were used in spermatozoa study. All samples used for protein analysis are from 27 boar ejaculates (D=13, L=8, Y=6). All boars were selected from their semen quality. The ejaculates included in the study contained more than 70% subjective motility and more than 75% normal morphology (Chanapiwat et al., 2009). The boars were housed in conventional system and allocated in individual pens. The boars were collected semen with the gloved-hand technique and interval of collection was twice a week.

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Preparation of seminal plasma and sperm samples

The semen was filtered through semen filter bag and split equally into two portions. The first portion was prepared for the evaluation of seminal plasma proteins (GPX5 and FN1) and sperm proteins (TPI and ACRBP). The second portion was used to analyze the quality of semen (subjective motility) and for cryopreservation. The first portion was centrifuged at 800× g and 15 °C for 10 min, the pellet of sperm was separated from the seminal plasma. Following this step, the supernatant was centrifuged for 15 min at 10,000× g and 4 °C and the supernatant was investigated under a phase contrast microscope (Olympus, Ham-burg, Germany) at 200x magnification to verify that they contain no sperm. Then, seminal plasma sample was

collected in cryotube and carried in liquid nitrogen. After that, both samples were stored at −80°C in a freezer before protein extraction and Western blot analysis. The second portion was continued to cryopreservation.

Semen cryopreservation

The second portion of semen was cryopreserved as suggested by Buranaamnuay et al. (2009) with some modifications. Briefly, the semen was diluted (1:1, v/v) in extender. The diluted semen was stored at 15 °C for 2 hours. After that, the semen was centrifuged at 15 °C, 800 xg for 10 min. Then, the sperm pellet was resuspended in lactose-egg yolk (LEY) extender (80 ml of 11% lactose solution and 20 ml egg yolk) (extender II) to achieve 1.5 x 10^9 sperm per ml in concentration and cooled to 4°C for 2 hours period. After that, the resuspended sperm were added with extender III (LEY extender plus 10% glycerol and 1.5% Equex STM Paste; Nova Chemical Sales Inc., MA, USA) to a final sperm concentration of 1x10^9 per ml and loaded into 0.5 PVC-French straws (IMV, IMV Technologies, France). In freezing process, the straws were placed at approximately 4 cm over the liquid nitrogen level for 15 min (-120°C) and dropped into liquid nitrogen (-196°C).

Thawing protocol

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The straws were plunged in 50°C water for 12 second. After thawing, the semen was diluted in Duragen® extender (Magapor, Zalagoza, Spain) 1:10 (v:v) and evaluated post-thawed sperm qualities. The post-thawed semen was classified into 3 classes according to the post-thawed total motility, i.e., poor (≤20%), moderate (21-40%) and good (>40%).

Semen evaluation

The sperm concentration of the frozen semen was evaluated by using a Bürker hemacytometer (Boeco, Germany). The total motility and sperm kinematic parameters were evaluated at 37°C by using a computer assisted sperm analysis system (CASA) (IVOSII, Hamilton-Thorne®, Beverly, USA). The semen parameters

evaluated included total sperm motility, progressive motility and kinematic characteristic parameters. The sperm kinematic characteristics included VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF.

Sperm viability

The viability of sperm was determined by using SYBR-14/ Ethidiumhomodimer-1 (EthD-1) (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, Netherland) (Axnér et al., 2004). Briefly, 10 µl of semen sample was diluted using 140 µl of thawing medium. Thereafter, 50 µl of diluted semen was mixed with 2.7 µl of SYBR-14 and 10 µl of EthD-1. After incubation at 37 °C for 20 min, 200 sperm were assessed (1000x) under fluorescent microscope. The nuclei of sperm with intact plasma membrane are stained green with SYBR-14, while those with damaged membranes are stained red with EthD-1. Sperm were classified into two types; live sperm stained green and dead sperm stained red.

Acrosome integrity

An aliquot (10 µl) of diluted semen was mixed with an equal volume of EthD-1 and incubated at 37°C for 15 min. Next, a 5 µl of mixture was spread on a slide and fixed with 95% ethyl alcohol for 30 second. Next, each sample was smeared with 15 µl of FITC-PNA solution [FITC-PNA in PBS (1:10, v/v)]. The slide was placed in a moist chamber at 4 °C for 30 min. After that, the sample was rinsed gently with PBS and dried. For sperm assessment, 200 sperm were observed under a fluorescent microscope at 1000x magnification. The acrosome of sperm was categorized as intact acrosome, reacted acrosome and loose acrosome (Chanapiwat et al., 2009).

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Plasma membrane integrity

The integrity of plasma membrane was assessed using short hypo-osmotic swelling test (sHOST). Briefly, a 100 µl semen sample was incubated with 1,000 µl of hypo-osmotic solution at 38°C for 30 min. After incubation, a 200 µl of semen and hypo-osmotic solution (composition and how to prepare) was loaded on 1,000 µl of hypo-osmotic solution plus 5% formaldehyde (Merck, Germany). A 10 µl of sample was placed on a warm slide with cover slip. After that, 200 sperm were evaluated using bright-field microscopy (x400). The positive result (coiled tail) was classified as sperm having active plasma membrane function (Buranaamnuay et al., 2008).

Mitochondrial activity

The mitochondrial activity was assessed by JC-1 (Molecular Probes, Molecular Probes Inc., Eugene, OR). The stock solutions were prepared 0.153 mM JC-1 in DMSO, 0.02mM SYBR-14 and 2.4 mM PI. The staining solution contained 1.6 µl of 0.153 mM JC-1, 1 µl of 0.02 mM SYBR-14, 1.6 µl of 2.4mM PI in 100 µl Hepes-buffered medium. A 12.5 µl of FT boar semen incubated with the staining solution (25 µl) at 37°C for 30 min. After that, the stained spermatozoa were examined under a fluorescent microscope at 1000x magnification. Spermatozoa stained with JC-1 display either green fluorescence for mitochondria with low to medium membrane potential, or red-orange fluorescence for mitochondria with high membrane potential in tail (Cossarizza et al., 1996; Garner et al., 1997).

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Quantification of total protein

Each seminal plasma portion sample was separated from sperm by centrifugation at 800 ×g and 15°C for 10 min. After that, the supernatant was centrifuged at 10,000 × g and 4°C for 15 min for eliminating pellet of sperm, and the supernatant was investigated under a phase contrast microscope to verify there were no sperm. Then, total protein content was quantified by using the QuickStart^{TM} Bradford Protein Assay (BioRad) as suggested by (Vilagran et al., 2016) with some modifications. Next, the measurement was repeated three times.

For sperm proteins, boar semen was centrifuged 800 ×g to separate the seminal plasma from sperm at 15 $^{\circ}$C for 10 min. The sperm pellet was washed with PBS to eliminate remaining traces of seminal plasma proteins and centrifuged at 800 ×g at 15°C for 10 min. Then, the sperm pellet was resuspended with 1XPBS with protease inhibitor (Gibco BRL, Life Technologies Ltd.; Paisley, UK). After that, protein was extracted according to the protocol described by Vilagran et al. (2013) with some modifications. Briefly, after washing three times with 1XPBS at 400xg, the sperm pellets were resuspended in PBS at the same sperm concentration as before protein extraction (50 × 10^{6}sperm per sample) (adjusted through the Makler counting chamber).The pellet of sperm were mixed with 400 µL of lysis buffer, made up of 2%Sodium Dodecyl Sulfate (Affymetrix/USB; Thermoscientific; USA), 1M Triethylammonium bicarbonate buffer (Sigma-Aldrich; USA) and protease inhibitor. Samples were lysed by Homogenizers (VCX750,Vibra-Cell™,USA) on ice condition :on 10 s off 5 s amp 30% for 5 min and subsequently centrifuged for 10 min at 10000 × g and 4°C. Supernatants were stored at−80°C for subsequent analysis. The total proteins were quantified by using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). The measurement was repeated three times.

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Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (SDS-PAGE)

Seminal plasma protein (20 µg) was solubilized by 5X Laemmli reducing buffer (0.5 M Tris-Hcl pH6.8, glycerol, SDS, Bromophenol blue, ß-mercaptoethanol) and denatured at 95°C for 10 min. Protein samples were loaded in polyacrylamide gel electrophoresis (1-mm, SDS-PAGE). Proteins FN1 and GPX5 were loaded onto the separating and stacking gels containing 12.5% (w/v) and 4% (w/v) acrylamide, under the running condition: 130 Volts for 80 min. For GPX5 protein, protein was transferred from gel to nitrocellulose membranes (Biorad) under the running condition: 25 Volts for 15 min. (Trans-Blot® Turbo™ Blotting System - Bio-Rad). For FN1 proteins, protein was transferred from gel to nitrocellulose membranes (Biorad) under wet condition: 25 Volts for overnight. Next, the nitrocellulose membrane was washed by 1×Towbin's buffer [Glycine (Omnia, USA), Tris (Affymatrix/USB, Thermofisher scientific, USA) and 0.01% Tween-20 (Omnia, USA)] at room temperature for 5 min.

For sperm protein, TPI protein analysis, 20 µg of protein samples was loaded in polyacrylamide gel electrophoresis (1-mm, SDS-PAGE) which had the separating and stacking gels of 12.5% (w/v) and 4% (w/v) acrylamide, respectively. Each sample was solubilized by 5X Laemmli reducing buffer and denatured protein at 95°C for 10 min. Then, the samples were loaded and separated under running condition: 130 volts for 60 min. After gel electrophoresis, the gel containing TPI protein was transferred to nitrocellulose membranes (biorad) under the running condition: 25 Volts for 30 min (Trans-Blot® Turbo™ Blotting System - Bio-Rad). After that, the membrane was washed by 1×Towbin's buffer solution for five times (5 min per wash). For ACRBP protein analysis, 40 µg of protein samples were loaded in polyacrylamide gel electrophoresis (1-mm, SDS-PAGE) which had separating and stacking gels of 10% (w/v) and 4% (w/v) acrylamide, respectively. The samples were solubilized by 5X Laemmli reducing buffer and denatured protein at 95°C for 10 min. Then, samples were loaded and separated under running condition: 130 volts for 80 min. After gel electrophoresis, the gel containing ACRBP protein was transferred into nitrocellulose membranes (biorad) under the running condition: 25 volts for 30 min (Trans-Blot® Turbo™ Blotting System

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- Bio-Rad). After that, the membrane was washed by 1XTowbin's buffer solution for five times (5 min per wash).

Western blot analysis

Each membrane was subsequently incubated with Odyssey Blocking Buffer (1xTBS) solutions (Odyssey®, Licor, United States) at room temperature for 1 h. Then, the membrane was washed by 1×Towbin's buffer and incubated with the primary antibodies under agitation for overnight at 4°C. Anti-FN1 rabbit (ref.Fab2413, Abcam; dilution: 1:1,000) and anti-GPX5 rabbit (ref. SC50498, Santacruz Biotechnology, Chicago, IL, USA; dilution 1:200) were primary antibodies. Next, membrane was washed three times (15 min each) by 1×Towbin buffer, and subsequently incubated with the secondary antibody (Ref. IRDye® 680RD Donkey anti-Rabbit IgG, United States; final dilution 1:10,000) in the dark at room temperature for 60 min. These antibodies are universal 680 nm channel antibodies. Membranes were washed five times (5 min per wash) by 1×Towbin's buffer.

For sperm proteins, the membrane was blocked, nonspecific proteins by blocking solution and washed by 1×Towbin's buffer. Then, the membrane was incubated with the primary antibodies under agitation for overnight at 4°C. These primary antibodies were anti-ACRBP rabbit (ref. ab64809, Abcam) and anti-TPI rabbit (ref. ab96696, Abcam). Next, the membrane was washed by 1×TBS-Tween20 solution for five times (5 min per wash), and subsequently incubated in the dark at room temperature for 60 min under agitation. Then, the membrane was incubated with the fluorescent tagged secondary antibody, IRDye® 680RD Donkey-anti-Rabbit Antibody (926-68073, 925-68073, Licor,USA) final dilution 1:10,000 in blocking buffer with 50% 1XTBS. After that, the membrane was washed for five times (5 min per wash) by 1xTowbin'S buffer. After that, the membranes were washed with washing buffer, then stripping-probing membranes as suggested by Prieto-Martínez et al. (2014) with some modifications. Briefly, the membranes were stripped under agitation at 38° C for 40 minutes by buffer containing 0.2 M glycine and 0.05% (v:v) Tween 20 (pH 2.2). Anti α-tubulin antibody 11H10, Rabbit monoclonal antibody (cell signaling technology, USA)

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was diluted 1:1000 in blocking solution and 1XTBS. The membrane was incubated with Anti α-tubulin antibody for 1 hour at room temperature and under agitation. Then, the membrane was incubated with the fluorescent tagged secondary antibody, IRDye® 680RD Donkey-anti-Rabbit Antibody (925-68073, Licor, USA) final dilution 1:10,000 in blocking buffer and 1XTBS. After that, the membrane was washed for five times (5 min per wash) by 1xTowbin'S buffer.

Quantification the density of protein

The reactive protein band was developed by fluorescent assay (Samantha et al. 2014). Briefly, the reactive protein band was visualized by Odyssey® (Odyssey Fc infrared imaging system, LI-COR Biosciences). Then, the membrane was scanned by Odyssey®. Next, the band density was normalized by Coomassie staining (Welinder and Ekblad, 2011) with some modifications. Briefly, membranes were subsequently stained for 1 min at room temperature with coomassie brilliant blue R-250 (Imperial protein stain, Thermo fisher Scientific, USA) and then the membranes were destainned with methanol-water (1:1, v:v). After destaining for 20 min with 1:5:4 (v:v:v) acetic acid-ethanol-water solution, membranes were washed twice with double-distilled water. Membranes were air dried and then take pictured with ChemiDoc Imaging System (Biorad) and analyzed by Image J Software (Version 1.6; NIH).

The density of each band was calculated as suggested by Vilagran et al. (2015) with some modifications.

$$\text{Seminal plasma protein content} = \frac{band\ of\ interest(intensity)}{total\ lane\ intensity\ (Coomassie's\ blue)}$$

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For sperm proteins, the band densities of interested protein (ACRBP and TPI) were normalized by using α-tubulin as an internal standard.

$$\text{Sperm protein content} = \frac{band\ of\ interest(intensity)}{\alpha{-}tubulin\ (intensity)}$$

Each sample was normalized by divided the density of the band corresponding to α-tubulin by Image J Software (Version 1.6; NIH).

Statistical analyses

SAS version 9.0 (SAS Inst. Inc., Cary, NC. USA.) was used for the statistical analyses. Descriptive statistics on the boar semen qualities and the amount of proteins were calculated. Continuous data was presented as means ± SEM. Discrete data was presented as percentage. The continuous data included total motility, progressive motility, subjective motility, sperm kinematic characteristic parameters (i.e., VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF), sperm viability, acrosome integrity and functional integrity of the sperm plasma membrane. The post-thawed semen was classified into 3 classes according to the post-thawed total motility of the spermatozoa, i.e., poor (≤20%), moderate (21-40%) and good (>40%). The amount of protein contents in the seminal plasma (i.e., FN1 and GPX5) and spermatozoa (i.e., ACRBP and TPI) was compared between groups by using ANOVA. Least square means was calculated and compared among groups by using Tukey-Kramer adjustment for multiple comparisons. Pearson's correlation coefficients were calculated between sperm quality parameters and levels of protein contents in the boar sperm (ACRBP and TPI) and seminal plasma (FN1 and GPX5). $P<0.05$ was regarded as statistically significant.

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CHAPTER IV
RESULTS

Descriptive statistics

Descriptive statistics on sperm parameters measurements of fresh and FT boar semen are demonstrated in Table 2.

Table 2 Descriptive statistics (mean ± SD) on sperm parameters measurements of fresh and FT boar semen from 94 ejaculates

Sperm quality parameters	Fresh semen	FT-semen
Sperm concentration($x10^6$ cell/ml)	314.2 ± 162.67	757.7 ± 243.35
Total motility (%)	82.2 ± 6.59	40.6 ± 16.13
Progressive motility (%)	37.1 ± 13.31	19.3 ± 10.51
Static cell	17.8 ± 6.59	59.4 ±16.13
Sperm kinematic characteristics		
VSL (µm/s)	68.1 ± 13.70	56.0 ± 8.50
VCL (µm/s)	168.5 ± 27.84	117.5 ± 27.7
VAP (µm/s)	91.1 ± 15.38	69.6 ± 11.83
% LIN	42.5 ± 7.58	51.7±9.39
% STR	74.4 ± 6.17	80.7±7.08
WOB	55.4±5.85	62.1±7.11
ALH (µm)	6.60±1.13	4.81±1.11
BCF (Hz)	37.3±3.29	34.4±3.16
Sperm morphology		
Normal sperm	82.7±7.62	74.46±11.10
Coil tail	1.1±1.00	1.86±1.41
Bent tail	12.6±5.54	21.0±7.31

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VSL (μm/s): velocity straight line, VCL (μm/s): velocity curved line, VAP (μm/s): velocity average path, LIN (%): linearity (VSL divided by VCL), STR (%): straightness (VSL divided by VAP), WOB (μm/s): wobble ALH (μm): Amplitude of lateral head displacement BCF (Hz): beat-cross frequency

The FT boar semen parameters, i.e., sperm viability, acrosome integrity, plasma membrane integrity and mitochondrial activity are presented in Table 3.

Table 3 Descriptive statistics (number of observation, mean, SD, median and range) on sperm parameter measurements of FT boar semen

Sperm quality parameters	N	Mean ± SD	Median	Range
Viability	78	59.0 ± 8.92	60.0	27.5 - 76.0
Acrosome integrity	78	60.9 ± 12.73	60.7	31.0 - 94.0
Plasma membrane integrity	77	55.9 ± 11.00	53.0	16.0 - 71.0
Mitochondrial activity	38	63.5 ± 10.66	65.0	42.0 - 87.0

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Table 4 Pearson's correlation among frozen-thawed sperm parameters measured by computer assisted sperm analysis system (CASA), i.e., total sperm motility, progressive motility of the sperm, kinematic characteristics of the sperm morphology (n = 94)

Sperm quality parameter	Total sperm motility	Progressive motility
Sperm viability	0.35**	0.22*
Acrosome integrity	0.40***	0.34*
Plasma membrane integrity	0.37**	0.25*
Mitochondrial activity	NS	NS
Sperm kinematic characteristics		
VSL (μm/s)	0.53***	0.69***
VCL (μm/s)	0.27**	NS
VAP (μm/s)	0.50***	0.41***
% LIN	NS	0.37***
% STR	NS	0.24**
WOB	NS	0.45
ALH (μm)	NS	NS
BCF (Hz)	NS	NS
Sperm morphology (%)		
Normal sperm	0.34***	0.39***
Proximal cytoplasmic droplets	-0.64***	-0.61***
Distal cytoplasmic droplets	-0.58***	-0.52***
Coil tail	-0.64***	-0.52***
Bent tail	-0.57***	-0.45***

* $P < 0.05$, ** $0.05 < P < 0.01$, *** $P < 0.001$, NS $P > 0.05$

VSL (μm/s): velocity straight line, VCL (μm/s): velocity curved line, VAP (μm/s): velocity average path, LIN (%): linearity (VSL divided by VCL), STR (%): straightness (VSL divided by VAP), WOB (μm/s): wobble ALH (μm): Amplitude of lateral head displacement BCF (Hz): beat-cross frequency

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Table 5 Pearson's correlation among frozen-thawed sperm parameters measured by computer assisted sperm analysis system (CASA), i.e., post-thawed total sperm motility, progressive motility and post-thawed sperm quality parameters (n = 94)

Sperm quality parameter	Viability	Mitochondrial activity
Sperm viability (%)	-	0.74***
Acrosome integrity (%)	0.54***	0.46***
Plasma membrane integrity (%)	0.64***	0.51***
Mitochondrial activity (%)	0.74***	-
Total motility (%)	0.35**	NS
Progressive motility (%)	0.22*	NS

* $P < 0.05$, ** $0.05 < P < 0.01$, *** $P < 0.001$, NS = $P > 0.05$

Sperm kinematic characteristics

The association among sperm parameters i.e., total sperm motility, progressive motility of the sperm, kinematic characteristics of the sperm and post-thawed sperm quality parameters are presented in Table 4. The total sperm motility was correlated with sperm viability (r = 0.35, $P < 0.01$), acrosome integrity (r = 0.40, $P < 0.001$), plasma membrane integrity (r = 0.37, $P < 0.01$) and sperm kinematic characteristics i.e., VSL (r = 0.53, $P < 0.001$), VCL (r = 0.27, $P < 0.01$). Furthermore, the percentages of sperm with bent tail (r= -0.57, $P < 0.001$), coiled tail (r=-0.63, $P < 0.001$), proximal cytoplasmic droplets (r= -0.65, $P < 0.001$) and distal cytoplasmic droplets (r=-0.58, $P < 0.001$) were also significantly associated with the total sperm motility (Table 4). Moreover, the post-thawed sperm viability was correlated with acrosome integrity (r = 0.54, $P < 0.001$), plasma membrane integrity (r = 0.64, $P < 0.001$) and mitochondrial activity (r = 0.74, $P < 0.001$). In addition, the mitochondrial activity of the FT sperm was correlated with acrosome integrity (r = 0.46, $P < 0.001$) and plasma membrane integrity (r = 0.51, $P < 0.001$) (Table 5).

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Table 6 Sperm quality parameters (least squares means ± SEM) of FT semen classified by freezability i.e., good (>40% total motility), moderate (20 - 40% total motility), poor (≤20% total sperm motility) (A total of 94 samples was used in sperm analysis)

Sperm quality parameter (total ejaculates)	Sperm freezability		
	Good (n = 44)	Moderate (n = 37)	Poor (n = 13)
Fresh semen			
Total motility (%)	84.5±0.95[a]	80.6±1.04[b]	79.2±1.75[b]
Normal morphology (%)	85.6±1.08[a]	79.8±1.18[b]	81.1±1.99[ab]
Frozen semen			
Total motility (%)	54.3 ± 1.21[a]	32.9 ± 1.32[b]	16.07 ± 2.23[c]
Progressive motility (%)	27.2 ± 1.06[a]	14.7 ± 1.16[b]	6.2 ± 1.95[c]
Static cell (%)	45.7±1.21[a]	67.1±1.32[b]	83.9±2.32[c]
Sperm viability (%)	60.4± 1.34[a]	59.8 ± 1.44[a]	47.8 ± 3.13[b]
Acrosome integrity (%)	63.9 ± 1.98[a]	60.0 ± 2.12[a]	48.9 ± 4.60[b]
Plasma membrane integrity (%)	60.0 ± 1.71[a]	52.8 ± 1.81[a]	43.2 ± 3.93[b]
Mitochondrial activity (%)	65.3 ± 2.28	64.1 ± 3.32	57.1 ± 3.97
Sperm concentration ($x10^6$spz/ml)	789.2 ± 36.8	720.1 ± 40.10	757.8 ± 67.6
Sperm kinematic characteristics			
VSL (μm/s)	60.1 ± 1.09[a]	54.1 ± 1.19[b]	47.0 ± 2.01[c]
VCL (μm/s)	124.4 ± 4.00[a]	116.1 ± 4.36[a]	97.6 ± 7.35[b]
VAP (μm/s)	74.9 ± 1.55[a]	67.6 ± 1.69[b]	57.1 ± 2.85[b]
LIN (%)	51.5 ± 1.42	51.4 ± 1.55	53.4 ± 2.62
STR (%)	79.8 ± 1.07	80.9 ± 1.16	83.0 ± 1.96
WOB (μm/s)	62.6 ± 1.07	61.4 ± 1.18	62.04 ± 1.99
ALH (μm)	5.0 ± 0.16[a]	4.7 ± 0.18[ab]	4.3 ± 0.30[b]
BCF (Hz)	34.4 ± 0.48	34.8 ± 0.52	33.8 ± 0.88
Sperm morphology (%)			

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Normal sperm	78.5 ± 1.59^{a}	70.8 ± 1.73^{b}	71.0 ± 2.92^{b}
Proximal cytoplasmic droplet	10.2 ± 0.60^{a}	14.4 ± 0.64^{b}	19.7 ± 1.10^{c}
Distal cytoplasmic droplet	9.6 ± 0.42^{a}	11.3 ± 0.46^{b}	14.4 ± 0.77^{c}
Coil tail	1.1 ± 0.17^{a}	2.3 ± 0.19^{b}	3.3 ± 0.33^{c}
Bent tail	17.1 ± 0.92^{a}	23.2 ± 1.01^{b}	28.3 ± 1.71^{c}

Table 6.1 Sperm quality parameters (least squares means ± SEM) of FT semen classified by freezability i.e., good (>40% total motility), moderate (20 - 40% total motility), poor (≤20% total sperm motility) (A total of 38 seminal plasma samples was used in seminal plasma analysis)

Sperm quality parameter (seminal plasma)	Sperm freezability		
	Good (n = 15)	Moderate (n = 13)	Poor (n = 10)
Total motility (%)	60.8 ± 2.12[a]	29.4 ± 2.29[b]	16.4 ± 2.61[c]
Progressive motility (%)	31.0 ± 1.77[a]	12.8 ± 1.91[b]	6.0 ± 2.17[c]
Sperm viability (%)	61.8± 2.40[a]	58.4 ± 2.61[a]	45.1 ± 3.88[b]
Acrosome integrity (%)	66.9 ± 2.97[a]	60.6 ± 3.23[a]	44.7 ± 4.78[b]
Plasma membrane integrity (%)	57.7 ± 3.04[a]	52.1 ± 3.30[a]	38.9 ± 4.90[b]
Mitochondrial activity (%)	66.8 ± 4.01	55.8 ± 8.02	53.9 ± 5.07
Sperm concentration ($x10^6$spz/ml)	859.2 ± 65.0	694.4 ± 69.8	747.2 ± 79.6
Sperm kinematic characteristics			
VSL (μm/s)	61.7 ± 1.64[a]	53.5 ± 1.77[b]	45.3 ± 2.02[c]
VCL (μm/s)	127.7 ± 5.84[a]	111.5 ± 6.28[b]	95.1 ± 7.16[b]
VAP (μm/s)	77.3 ± 2.28[a]	65.0 ± 2.45[b]	55.0 ± 2.80[c]
LIN (%)	52.0 ± 2.40	51.8 ± 2.59	52.8 ± 2.94
STR (%)	79.7 ± 1.74	82.5 ± 1.87	83.3 ± 2.13
WOB (μm/s)	63.2 ± 1.82	61.0 ± 1.95	61.2 ± 2.23
ALH (μm)	5.1 ± 0.26[a]	4.6 ± 0.28[ab]	4.1 ± 0.32[b]
BCF (Hz)	34.5 ± 0.83	34.5 ± 0.89	34.7 ±1.02
Sperm morphology (%)			
Normal sperm	82.1 ± 2.51[a]	68.8 ± 2.70[b]	70.8 ± 3.07[b]
Coil tail	0.83 ± 0.32[a]	2.6 ± 0.34[b]	3.4 ± 0.39[b]
Bent tail	15.3 ± 1.74[a]	24.7 ± 1.87[b]	29.0 ± 2.13[b]

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Table 6.2 Sperm quality parameters (least squares means ± SEM) of FT semen classified by freezability i.e., good (>40% total motility), moderate (20 - 40% total motility), poor (≤20% total sperm motility) (A total of 32 sperm samples was used in sperm analysis)

Sperm quality parameter (sperm sample)	Sperm freezability		
	Good (n = 13)	Moderate (n = 8)	Poor (n = 11)
Total motility (%)	60.8 ± 1.70[a]	29.3 ± 2.16[b]	17.4 ± 1.85[c]
Progressive motility (%)	30.1 ± 1.40[a]	13.0 ± 1.78[b]	6.7 ± 1.52[c]
Sperm viability (%)	58.8± 2.95	59.1 ± 3.86	48.7 ± 4.17
Acrosome integrity (%)	65.2 ± 4.25[a]	63.5 ± 5.56[a]	50.5 ± 6.01[b]
Plasma membrane integrity (%)	53.4 ± 3.74	53.4 ± 4.90	42.9 ± 5.29
Mitochondrial activity (%)	56.9 ± 4.82	50.0 ± 11.82	59.3 ± 4.82
Sperm concentration ($x10^6$spz/ml)	792.7 ± 56.90	783.48 ± 72.53	788.35±61.85
Sperm kinematic characteristics			
VSL (μm/s)	60.9 ± 1.68[a]	54.4 ± 2.14[b]	47.8 ± 1.82[c]
VCL (μm/s)	124.0± 6.70	114.5 ± 8.55	100.9 ± 7.29
VAP (μm/s)	75.7 ± 2.46[a]	66.9 ± 3.14[b]	58.8 ± 2.68[c]
LIN (%)	52.2 ± 2.70	49.89 ± 3.44	53.0 ± 2.93
STR (%)	80.1 ± 2.06	80.5 ± 2.62	82.2 ± 2.24
WOB (μm/s)	63.2 ± 2.00	60.16 ± 2.55	62.08 ± 2.18
ALH (μm)	4.9 ± 0.32	4.8 ± 0.41	4.5 ± 0.35
BCF (Hz)	34.7 ± 0.91	34.8 ± 1.16	33.4 ±0.99
Sperm morphology (%)			
Normal sperm	79.8 ± 3.00[a]	63.1 ± 3.83[b]	70.1 ± 3.27[b]
Coil tail	0.7 ± 0.26[a]	2.7 ± 0.33[b]	2.89 ± 0.28[b]
Bent tail	14.5 ± 1.91[a]	26.11 ± 2.43[b]	27.7 ± 2.08[b]

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VSL (μm/s): velocity straight line, VCL(μm/s): velocity curved line, VAP (μm/s): velocity average path, LIN (%): linearity (VSL divided by VCL), STR(%): straightness (VSL divided by VAP), WOB (μm/s): wobble ALH (μm): Amplitude of lateral head displacement BCF (Hz): beat-cross frequency , a,b different superscript within row differ significantly ($P < 0.05$)

The post-thawed sperm total motility, progressive motility and VSL in good freezability ejaculate were higher than moderate and poor freezability ejaculates ($P < 0.05$). Moreover, sperm viability, acrosome integrity, plasma membrane integrity were different between good and poor freezability ejaculates (Table 6). Whereas mitochondrial activity and sperm kinematic characteristics i.e., VCL, LIN, STR, WOB, ALH, BCF were not different among groups ($P > 0.05$) (Table 6).

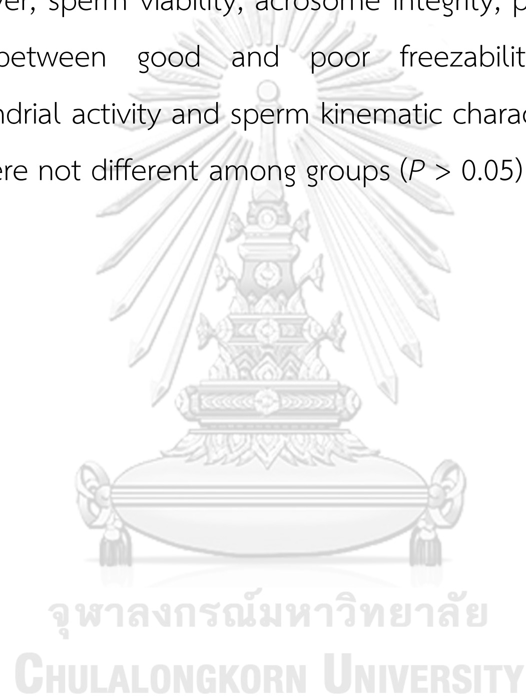

Table 7 Correlation coefficients between frozen-thawed sperm quality parameters and sperm proteins and seminal plasma protein.

Sperm quality parameter	Sperm protein		Seminal plasma protein	
	ACRBP	TPI	FN1	GPX5
Sperm viability (%)	NS	-NS	NS	NS
Total motility (%)	0.38**	-0.43**	0.43**	NS
Progressive motility (%)	NS	-0.35*	0.42**	NS
Sperm kinematic characteristics				
VSL (μm/s)	NS	NS	NS	NS
VCL (μm/s)	NS	NS	NS	NS
VAP (μm/s)	NS	NS	0.34*	NS
LIN (%)	NS	NS	NS	NS
STR (%)	NS	NS	NS	NS
WOB (μm/s)	NS	NS	NS	NS
ALH (μm/s)	NS	NS	NS	NS
BCF (Hz)	NS	NS	NS	NS
Sperm morphology (%)				
Normal sperm	NS	NS	NS	NS
Coil tail	NS	0.38*	-0.31*	NS
Bent tail	-0.42**	NS	-0.36*	NS

* $P < 0.05$, ** $0.05 < P < 0.01$, *** $P < 0.001$, NS $P > 0.05$

VSL (μm/s): velocity straight line, VCL (μm/s): velocity curved line, VAP (μm/s): velocity average path, LIN (%): linearity (VSL divided by VCL), STR (%): straightness (VSL divided by VAP), WOB (μm/s): wobble ALH (μm): Amplitude of lateral head displacement BCF (Hz): beat-cross frequency

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Table 7 shows the correlation coefficients between the sperm proteins (ACRBP, TPI) and seminal plasma proteins (FN1, GPX5) with all of FT sperm quality parameters. FN1 was positively correlated with total motility, progressive motility and VAP ($P < 0.05$). ACRBP was positively correlated with only total motility ($P < 0.05$). In contrast, TPI was negatively correlated with both total motility ($P < 0.05$) and progressive motility ($P < 0.05$). GPX5 was not found to be correlated with any of FT sperm quality parameters.

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Table 8 Amount of protein (least squares means ± SEM) in good, moderate and poor sperm freezability groups

Amount of protein	Good	Moderate	Poor
Seminal plasma protein			
GPX5	0.14±0.03	0.17±0.03	0.11±0.03
FN1	0.08±0.01[a]	0.07±0.01[a]	0.05±0.01[b]
Sperm protein			
TPI	3.17±0.36[a]	4.37±0.46[b]	4.07±0.39[ab]
ACRBP	0.43±0.13	0.28±0.16	0.16±0.14

[a,b] different superscripts within row differ significantly ($P < 0.05$), FN1: Fibronectin 1, GPX5: Glutathione peroxidase 5, ACRBP: Acrosin binding protein, TPI: Triosephosphate isomerase.

Table 8 shows the levels of protein in good, moderate and poor sperm freezability groups. The levels of GPX5 in the seminal plasma were not significant differences ($P > 0.05$) between groups (Figure 1A). FN1 in seminal plasma in good freezability group was ($P < 0.05$) higher than in the poor freezability group (Figure 1B). The level of TPI was higher in moderate ($P < 0.05$) than good freezability groups (Figure 1C). On the other hand, TPI level did not significantly differed ($P > 0.05$) between good and poor freezability groups (Figure 1C) while the ACRBP level did not significantly differed between groups ($P > 0.05$).

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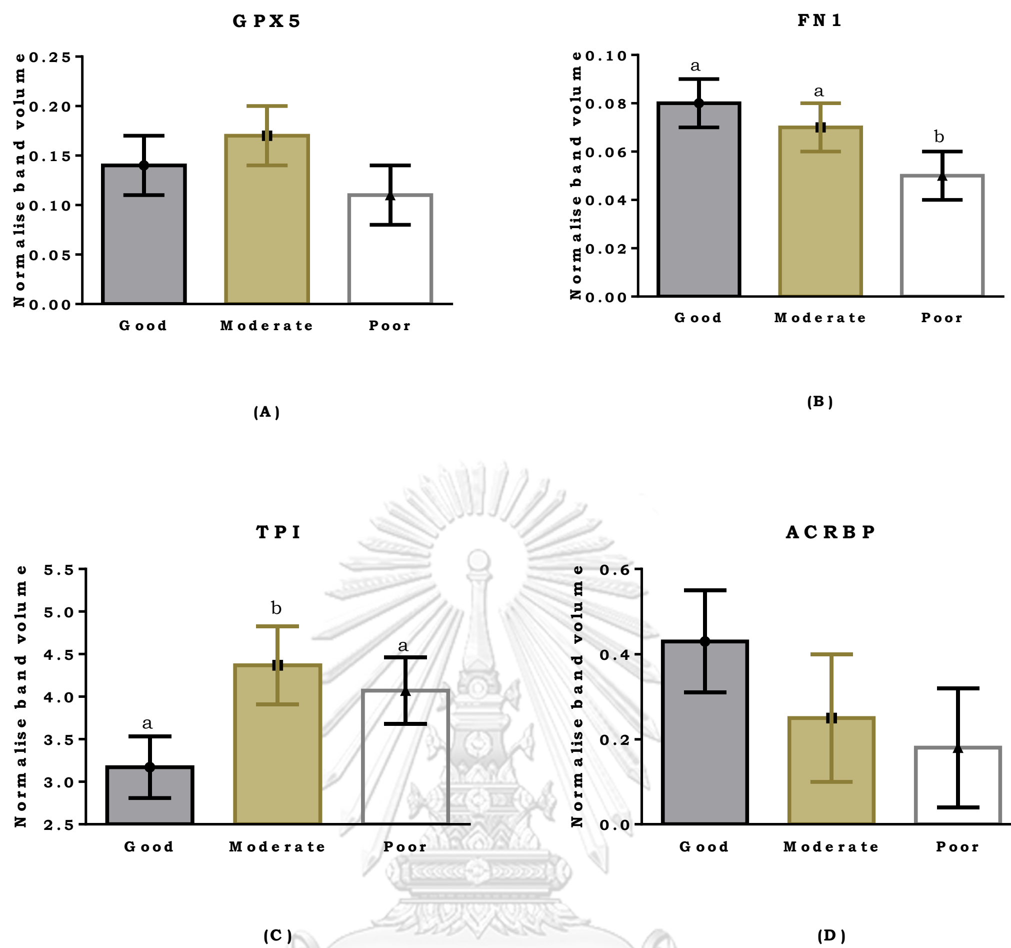

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Figure 1 Relative levels of seminal plasma proteins GPX5 (A), FN1 (B) Coommassie Blue staining was used as an internal standard, relative levels of sperm proteins TPI (C), ACRBP (D) in freezability groups, using α-tubulin as an internal standard, [a,b] different superscripts between groups differ significantly ($P < 0.05$).

Table 9 Sperm quality parameters (Least squares means ± SEM) of FT semen classified by breed, i.e., Duroc (n=35), Landrace (n=34), Yorkshire (n=25) (A total of 94 samples was used in sperm analysis)

Sperm quality parameter	Boar breed		
	Duroc	Landrace	Yorkshire
Total motility (%)	$33.0±2.48^{a}$	$49.3±2.51^{b}$	$39.6±2.93^{c}$
Progressive motility (%)	$14.0±1.58^{a}$	$25.5±1.60^{b}$	$18.4±1.87^{b}$
Sperm viability (%)	$55.6±1.53^{a}$	$60.0±1.58^{b}$	$63.2±2.00^{b}$
Acrosome integrity (%)	$59.0±2.24^{a}$	$59.4±2.32^{ab}$	$66.7±2.95^{b}$
Plasma membrane integrity (%)	$48.7±1.88^{a}$	$57.4±1.91^{b}$	$57.1±2.43^{b}$
Mitochondrial activity (%)	60.2±2.46	65.67±2.70	68.5±4.67
Kinematic characteristics			
VSL (μm/s)	$52.5±1.26^{a}$	$61.5±1.28^{b}$	$53.3±1.50^{a}$
VCL (μm/s)	$119.2±4.56^{a}$	$124.2±4.62^{ab}$	$105.7±5.40^{b}$
VAP (μm/s)	$67.0±1.82^{a}$	$76.1±1.85^{ab}$	$64.3±2.16^{b}$
LIN (%)	49.0±1.56	52.5±1.58	54.5±1.84
STR (%)	$79.3±1.18^{a}$	80.3±1.20	$83.2±1.40^{b}$
WOB (μm/s)	$59.5±1.16^{a}$	$63.5±1.18^{b}$	$63.8±1.38^{b}$
ALH (μm)	$5.0±0.18^{a}$	$5.0±0.19^{bc}$	$4.3±0.22^{c}$
BCF (Hz)	$35.2±0.53^{a}$	$34.1±0.54^{bc}$	$34.0±0.63^{c}$

[a,b,c] different superscripts within row differ significantly ($P < 0.05$)

n= ejaculates

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Table 9.1 Sperm quality parameters (Least squares means ± SEM) of FT semen classified by breed, i.e., Duroc (n=15), Landrace (n=13), Yorkshire (n=10) (A total of 32 sperm samples was used in sperm analysis)

Sperm quality parameter	Boar breed		
	Duroc	Landrace	Yorkshire
Total motility (%)	23.6±3.93[a]	55.0±4.36[b]	40.7±5.25[c]
Progressive motility (%)	9.1±2.27[a]	29.8±2.52[b]	17.5±3.03[c]
Sperm viability (%)	53.0±3.00	59.6±2.87	62.2±4.05
Acrosome integrity (%)	57.9±4.01	62.9±3.84	61.4±5.43
Plasma membrane integrity (%)	45.5±3.52	57.4±3.37	54.8±4.76
Mitochondrial activity (%)	52.0±5.00	65.1±3.95	67.5±7.90
Sperm concentration ($x10^6$spz/ml)	719.4±64.13	844.9±71.15	765.8±85.51
Kinematic characteristics			
VSL (μm/s)	48.9±1.78[a]	62.2±1.97[b]	53.7±2.37[a]
VCL (μm/s)	107.9±6.41	121.6±7.11	112.1±8.55
VAP (μm/s)	60.3±2.68[a]	76.1±2.98[b]	66.6±3.58[a]
LIN (%)	49.9±2.27	54.4±2.52	52.8±3.02
STR (%)	81.6±1.73	81.5±1.92	81.6±2.31
WOB (μm/s)	59.1±1.64[a]	65.0±1.83[b]	62.6±2.20[a]
ALH (μm)	4.7±0.27	4.83±0.30	4.5±0.36
BCF (Hz)	35.1±0.80	33.8±0.88	34.6±1.06

[a,b,c] different superscript within row differ significantly ($P < 0.05$)

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Table 9.2 Sperm quality parameters (Least square mean ± SEM) of FT semen classified by breed, i.e., Duroc (n=13), Landrace (n=8), Yorkshire (n=11) (A total of 38 seminal plasma samples was used in sperm analysis)

Sperm quality parameter	Boar breed		
	Duroc	Landrace	Yorkshire
Total motility (%)	27.5±4.65^{a}	53.0±6.00^{b}	41.0±6.36^{b}
Progressive motility (%)	12.6±2.62^{a}	27.9±3.38^{b}	16.5±3.60^{a}
Sperm viability (%)	53.9±2.90^{a}	53.6±3.80^{ab}	64.8±4.10^{b}
Acrosome integrity (%)	59.8±4.42	56.9±5.80	68.9±6.26
Plasma membrane integrity (%)	46.0±3.71	54.6±4.86	56.2±5.25
Mitochondrial activity (%)	57.3±4.12	52.9±5.45	67.5±7.71
Sperm concentration (x10^{6}spz/ml)	765.7±52.6	819.7±67.91	797.6±72.03
Kinematic characteristics			
VSL (μm/s)	51.1±1.83^{a}	61.5±2.36^{b}	54.0±2.50^{a}
VCL (μm/s)	108.5±6.65	120.9±8.60	115.2±9.11
VAP (μm/s)	62.8±2.65^{a}	75.6±3.42^{b}	67.8±3.63^{ab}
LIN (%)	51.6±2.53	52.9±3.26	51.4±3.46
STR (%)	81.5±1.93	80.2±2.49	80.6±2.64
WOB (μm/s)	61.1±1.86	64.0±2.40	61.7±2.55
ALH (μm)	4.8±0.30	4.8±0.40	4.6±0.41
BCF (Hz)	33.8±0.84	34.2±1.09	35.3±1.15

[a,b,c] different superscript within row differ significantly (*P* < 0.05)

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sperm kinematic characteristics were evaluated by a computer-assisted sperm analysis (CASA) system VSL (μm/s): velocity straight line, VCL(μm/s): velocity curved line, VAP (μm/s): velocity average path, LIN (%): linearity (VSL divided by VCL), STR(%): straightness (VSL divided by VAP), WOB (μm/s): wobble ALH (μm): Amplitude of lateral head displacement BCF (Hz): beat-cross frequency [a,b] different superscripts within row differ significantly ($P < 0.05$)

Breed effect on FT-sperm quality parameters

Sperm quality parameters were affected by breed (Table 9). The total motility was 33.0±2.48 in Duroc (n=35), 49.3±2.51 in Landrace (n=34) and 39.6±2.93 in Yorkshire (n=25) ($P<0.05$). Progressive motility was 14.0±1.58% in Duroc, 25.5±1.6 % in Landrace and 18.4±1.87 % in Yorkshire ($P < 0.05$). Sperm viability was 55.6±1.53% in Duroc, 60.0±1.58% in Landrace and 63.2±2.00 % in Yorkshire ($P < 0.05$). The post-thawed sperm parameters varied among individuals and among breeds. On average, the motility pattern and sperm kinematic characteristics including LIN, VSL, VAP, VCL and BCF after thawing are presented in Table 9. The FT-sperm parameters i.e., total motility, progressive motility, sperm viability, plasma membrane integrity and sperm kinematic parameter such as WOB in Landrace and Yorkshire were significantly higher than those in the Duroc.

Table 10 Amount of protein (least squares means ± SEM) in Duroc, Landrace and Yorkshire breeds

Amount of protein	Duroc	Landrace	Yorkshire
Seminal plasma protein			
GPX5	0.14±0.03	0.16±0.03	0.11±0.04
FN1	0.06±0.01	0.08±0.01	0.07±0.01
Sperm protein			
TPI	$4.54±0.31^{a}$	$3.05±0.40^{b}$	$3.18±0.42^{b}$
ACRBP	$0.06±0.11^{a}$	$0.54±0.14^{b}$	$0.50±0.14^{b}$

^{a,b} different superscripts within row differ significantly ($P < 0.05$), FN1: Fibronectin 1, GPX5: Glutathione peroxidase 5, ACRBP: Acrosin binding protein, TPI: Triosephosphate isomerase

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Table 10 shows the result as least squares means ± SEM in Duroc, Landrace and Yorkshire breeds. The amounts of FN1 and GPX5 in the seminal plasma were no significant differences (*P* > 0.05) between breeds. In contrast, the amounts of TPI in Duroc were significantly higher than Landrace and Yorkshire (*P* < 0.05) (Figure2). On the other hand, ACRBP was higher in Landrace and Yorkshire (*P* < 0.05) than Duroc breeds (Figure 2).

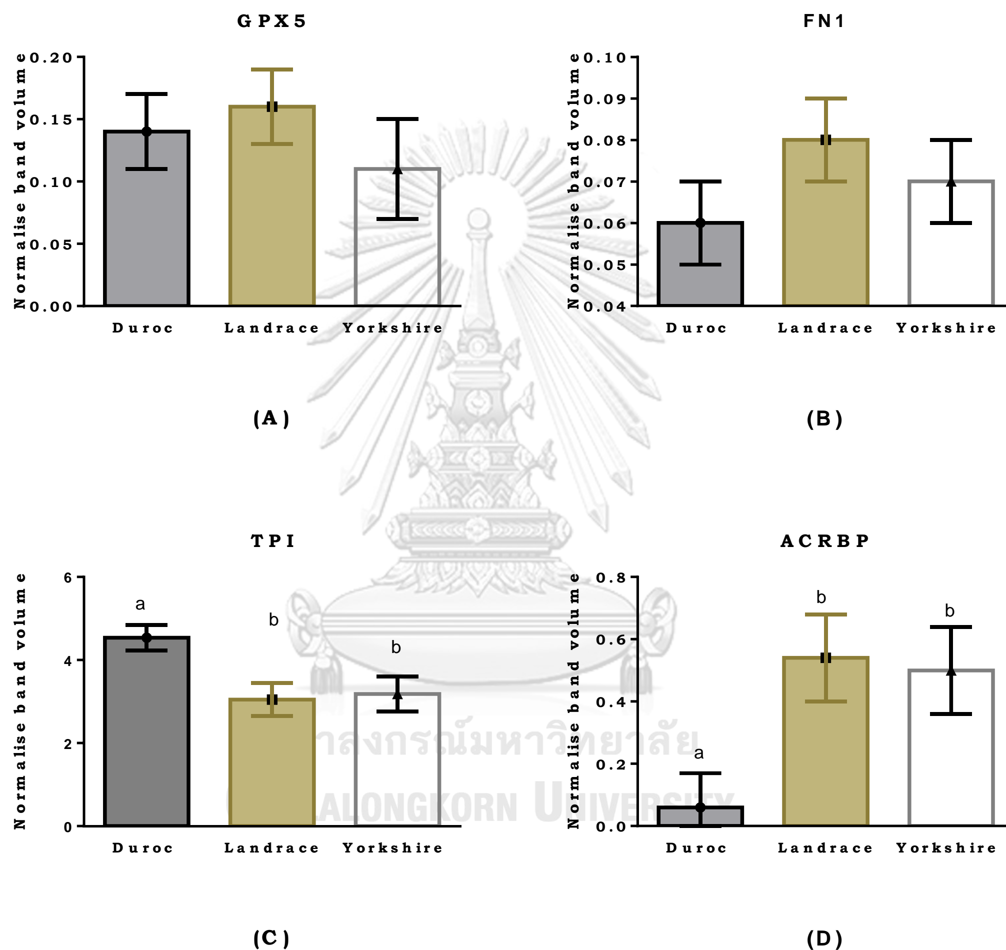

Figure 2 Relative levels of seminal plasma proteins GPX5 (A), FN1 (B) Coommassie Blue staining was used as an internal standard, relative levels of sperm proteins TPI (C), ACRBP (D) in three breeds, using α tubulin as an internal standard [a,b] different superscripts between groups differ significantly (P < 0.05).

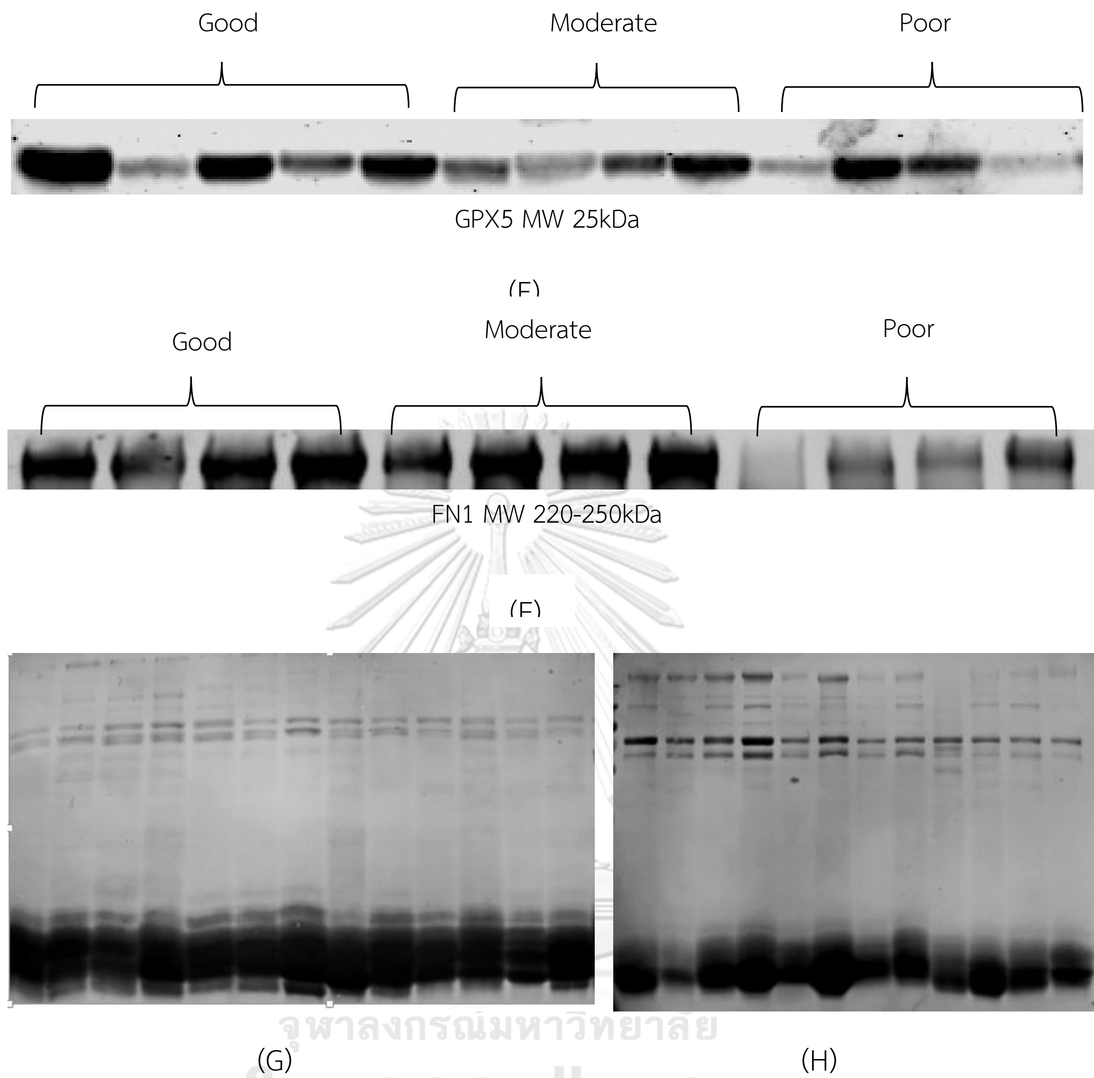

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Figure 3 Representative seminal plasma protein patterns of GPX5 (E), FN1 (F) in high sperm quality samples (good) , moderate sperm quality (moderate) and low sperm quality samples (poor),and Coomassie Brilliant Blue stained membranes were used to normalise the band intensity of seminal plasma proteins (G), (H)

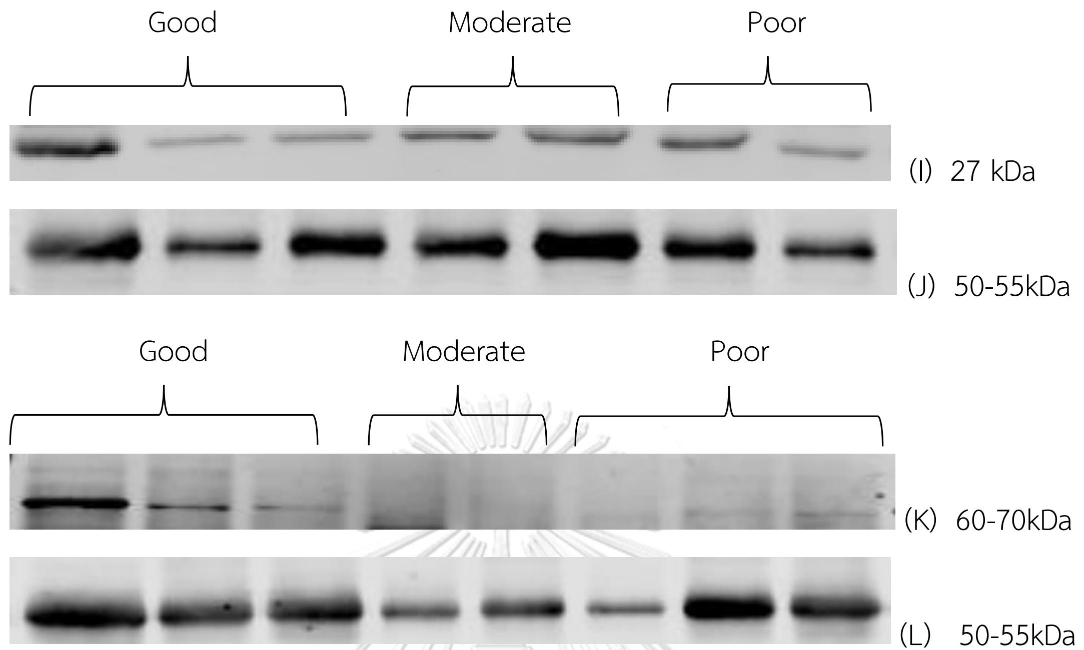

Figure 4 Representative sperm protein patterns of TPI (I), ACRBP (J) in high sperm quality samples (good), moderate sperm quality (moderate) and low sperm quality samples (poor) α–tubulin (K), (L) was used as an internal standard in order to normalise the intensity of sperm protein bands.

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CHAPTER V
DISCUSSION

The present study demonstrated differences of some seminal plasma (GPX5, FN1) and sperm protein (TPI, ACRBP) contents in good, moderate and poor freezability ejaculates, according to FT boar sperm total motility. FN1 content in boar seminal plasma was associated with sperm quality parameters. On the other hand, GPX5 was not correlated with any sperm quality parameters. In boar spermatozoa, TPI was negatively correlated with sperm freezability and ACRBP was positively correlated with the sperm quality parameters.

Fibronectin 1 is a high-molecular-weight (~250 kD) glycoprotein found in blood plasma, extracellular matrix, basal lamina, and on the cell surface (Kornblihtt and Gutman, 1988). This protein was also described as one of the most abundant proteins in boar seminal plasma (Druart et al., 2013). In addition, FN1 was negatively correlated with tail abnormalities in boar sperm, in agreement with Gonzalez-Cadavid et al. (2014). In human, FN was found high concentration in seminal plasma (Vuento et al., 1980) and involved in fertilization and that the capacity of sperm (Cooper, 1986). Studies found that FN binding domain act as a bridge between cell and surrounding media. Many binding molecules were located on fibronectin (Parrish et al., 1988). Miranda and Tezon (1992) found FN in soluble tissue extracts of epididymis, increasing from head of epididymis (18%) to distal of epididymis (64%) and suggested that FN in distal regions of the epididymis had a supportive role in sperm maturation.

Furthermore, the oocyte (cumulus, zona pellucida) also contained these compositions with potential FN affinity (Ball et al., 1982) and FN1 was presented in follicular and oviductal fluid (Makrigiannakis et al., 2009). Recent study suggested that fibronectin could stimulate human sperm capacitation in oviduct through the cyclic AMP/protein kinase pathway (Martinez-Leon et al., 2015). In addition, studies found that FN is involved in the process of compaction and blastocyst formation in bovine (Goossens et al., 2009).

In boar semen, FN1 was found as a freezability marker. In the present work, the normalized level of FN1 was positively correlated with their sperm quality parameters; this was in agreement with Vilagran et al. (2015). The mechanism of FN1 was still unknown. The important functions of boar seminal plasma proteins included the physiological modulations of sperm function during transport in the female reproductive tract, inhibiting and stabilizing activity against enzyme systems and sperm chromatin, immunosuppressive and immunomodulation properties, defense mechanism of spermatozoa and enzyme in sperm metabolism (Strzezek, 2002). The mechanism of FN1 on sperm therefore could stabilize and protect spermatozoa. GPX5 (approximately 25kDa) is one of GPX-family, which are composed of eight phylogenetically related enzymes; GPX1-8 (Toppo et al., 2008). Glutathione peroxidases (GPXs) and catalase (CAT) are neutralizing enzymes for converting H_2O_2 (one of ROS) to water (Noblanc et al., 2011). GPX5 is an epididymis-specific GPX in mice, rat, pig, monkey and human (Rejraji et al., 2002). It is controlled by androgenic hormone within adult epididymis (Vernet et al., 1997). The GPX5 is interesting for male reproduction because its amount is more than 95% of the total GPX presented in the epididymal fluid (Brigelius-Flohe and Maiorino, 2013). This protein is not only found in epithelial cells and in the lumen of the epididymis, but also associates to the head of spermatozoa when transiting from the epididymis to the vas deferens (Rejraji et al., 2002). Moreover, GPX5 could prevent premature acrosome reaction during the storage process in the cauda epididymis (Okamura et al., 1997). In previous studies, the GPX5 knock out male mice did not affect at young age. However, after mating with wild type female, higher incidence of miscarriages and development defects were observed. Therefore, they suggested that GPX5 was associated with maintenance integrity of sperm DNA (Chabory et al., 2009). In boar, Novak et al. (2010) found the positive correlation between GPX5 in boar seminal plasma and farrowing rate whereas Vilagran et al. (2016) found a negative correlation between GPX5 and semen quality. In our results, GPX5 was not significantly different between groups and the normalize level of GPX5 was not correlated with any of the post-thawed sperm qualities. However, the level of GPX5 was positively correlated with FN1, which had positively correlated with boar FT-sperm quality. Our result was correlated with the result of

Vilagran et al. (2015) which was described that GPX5 content was not significantly differ between good and poor freezability groups. On the other hand, Barranco et al. (2016) found that the GPX5 concentration was positively correlated with total sperm motility and farrowing rate and litter size. They demonstrated that the GPX5 expression in epididymis varies in epididymal and testicular factors, fibroblast growth factor and it also depends on androgen levels (Brigelius-Flohe and Maiorino, 2013).The differences in GPX5 concentration were also found among ejaculate portions, in a total of 15 ejaculates. This concentration differed among semen portion which was classified as sperm rich portion, the rest of the sperm rich portion and post sperm rich fraction. The result showed that first 10 ml of sperm rich fraction had a significantly lower GPX5 concentration than the rest and post sperm rich fractions. In previous studies, Rejraji et al. (2002) found GPX5 in epididymal cell lining, epididymal lumen. In addition, Barranco et al. (2016) used a specific polyclonal antibody and evaluated the genital tract of fertile boar by performing Western blot and Immunohistochemical examination (IHC). They demonstrated that GPX5 was presented in epithelial cells, vacuole membranes vascular endothelium of boar seminal vesicle, prostate and bulbourethral glands. This result could support that the concentration of GPX5 was higher in the rest of sperm rich fraction and post- sperm rich fraction. Nevertheless, the sperm rich fraction was only selected to use in this study. Then, the result might be related to the study of Vilagran et al. (2015). Also, the level of GPX5 had positively correlated with fibronectin1 which associated to the freezability of boar semen.

Two sperm proteins (TPI, ACRBP) were studied. TPI (Triosephosphate isomerase) is a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3 phosphate (Gracy, 1982). Moreover, TPI is an essential enzyme for sperm capacitation and the acrosome reaction (R Fraser and Quinn, 1981). In previous studies, the results showed that the inhibition of this enzyme using ornidazole in rat spermatozoa could block the capacitation process (Bone et al., 2001). Our result agreed with Kwon et al. (2015a) which revealed that the TPI level was higher in low sperm quality samples. In addition, a study of Vilagran et al. (2013) found that TPI level of poor freezability group was presented in higher amounts than good

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freezability group. It could be a cause of early capacitation in sperm. Chen et al. (2014) indicated the alteration of proteins, including TPI, was occurred after cryopreservation process. These might be mainly related to premature capacitation in FT-boar sperm. Moreover, in human, the asthenozoospermic samples had higher levels of TPI than normospermic samples (Siva et al., 2010). In Vilagran et al. (2016) study, they suggested that this defect, found in spermatozoa, was caused by the handling after ejaculation i.e., dilution techniques and cooling process. This could be explained that due to the lower of membrane integrity, the higher level of TPI protein level in head of spermatozoa was detected which correlated to the poor sperm freezability. In our study, the lower plasma membrane integrity after cryopreservation was shown in PFE group. It could be caused by an early capacitation of sperm. In the aspect of the normalized level of TPI in MFE which was higher than PFE, the result was quite unclear. This might be due to the variation in breed using in each sample.

Previous studies suggested that ACRBP (Acrosin binding protein) involved in the conversion of proacrosin to its mature form (Baba et al. 1989). This protein can bind to proacrosin and acrosin intermediates and plays an important role in the maturation of acrosin (Tardif et al., 2003; Tardif et al., 2010). Arcelay et al. (2008) reported that ACRBP can regulate sperm capacitation and fertilization. On the other hand, the mechanism of capacitation is not clear (Ficarro et al., 2003). In recent study, Dong et al. (2015) suggested that acrosomal membrane proteins might be modified and degraded during capacitation and the acrosome reaction. In addition, Sun et al. (2013) reported that the fresh semen and capacitation-like treated groups were in the proacrosin forms which failed to activate into active acrosin. Most proacrosin of the capacitation groups could be completely activated into acrosin. ACRBP began to express when sperm was capacitating. Moreover, capacitation and FT sperm groups had similar levels of protein phosphorylation. ACRBP phosphorylation levels could promote proacrosin to active acrosin. In our study, the level of ACRBP was not different among freezability groups. Nonetheless, ACRBP was significant positive correlated with the percentage of total motility. It was related to the acrosome integrity after cryopreservation. The levels of ACRBP in GFE and MFE were higher than

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PFE. The result of Vilagran et al. (2013) suggested that ACRBP might involve in acrosin pathway maturation, which GFEs presented a higher ability of acrosin maturation. According to Vilagran et al. (2013), low regulatory capacity of the acrosome reaction in PFE group was seen. They showed a decrease life span in freeze-thawed process because of the premature acrosome reaction.

Effect of breed on boar sperm freezability

The difference of sperm quality parameters between breeds has been demonstrated in many species including boars (Larsson and Einarsson, 1976; Waterhouse et al., 2006a; Kaeoket et al., 2010b). In the present study, the percentage of sperm motility varied among the breeds and there was higher percentage of motility in Landrace than Yorkshire and Duroc. The total motility of spermatozoa was significant higher in Landrace and Yorkshire than Duroc. In addition, the percentage of sperm viability was higher in Landrace and Yorkshire than Duroc. The percentage of acrosome integrity then was higher in Yorkshire than Duroc and Landrace. This finding concurred with results of Kaeoket et al. (2008), the total motility of spermatozoa was significantly higher in Landrace and Yorkshire. In addition, Landrace and Yorkshire spermatozoa had a higher percentage of acrosome integrity, compared to Duroc. The reason for individual variation among breed of boars in terms of sperm freezability is unclear. It seems to be related to the amount of long-chain polyunsaturated fatty acids (PUFA) in the sperm plasma membrane (Waterhouse et al., 2006b). In addition, the variations of population might be affected from genetic among individual boars (Holt et al., 2005).

For the amount of proteins, we found the higher TPI level and lower ACRBP level in Duroc. Moreover, Duroc had the lower FT- sperm quality parameters such as the percentage of total motility, progressive motility, sperm viability and plasma membrane integrity than Landrace and Yorkshire. It seemed to show that the difference among breeds might affect some sperm proteins and other FT boar sperm quality parameters. However, this aspect required further investigation. Zura Zaja et al. (2016) investigated that the influence of boar breeds (Swedish Landraces, German Landraces, Large Whites, Pietrains and Pig Improvement Company hybrids) on lipid,

protein concentrations and antioxidative system was varied in seminal plasma and spermatozoa which correlated with semen quality. The result showed the differences in seminal plasma and spermatozoa in terms of antioxidation, total protein and lipid variables obtained among breed of boars. This might be reasons for explaining the differences of FT sperm parameters investigated in our study.

Conclusions

In conclusion, seminal plasma proteins (FN1) and sperm proteins (TPI, ACRBP) of boars, significantly associated with the boar sperm freezability and the qualities of cryopreserved boar semen.FN1 and ACRBP were positively correlated with the motility of sperm whereas TPI was negatively correlated with motility of sperm. The ejaculates with higher level of FN1 had a higher freezability than those with a lower level of FN1. Duroc boar sperm had a higher TPI and lower ACRBP levels than Landrace and Yorkshire boar sperm. This might explain the breed difference concerning the qualities of boar semen after frozen-thawed. These results indicate the necessary of investigating some marker for indicating boar sperm freezability. This would have an advantage on the improvement of boar sperm cryopreservation innovation. Future works required to investigate on the mechanism behind the effect of these markers as well as the clinical application of these markers for boar semen cryopreservation. It could be implied that the presence of seminal plasma protein or composition in cooling or thawing process may improve frozen-thawed boar sperm motility. Therefore, the additional of these proteins during cooling and thawing process could be useful for improving the quality of FT- sperm qualities.

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APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
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Publications

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