ผลของไข่แดงในไข่ไก่ที่อุดมด้วยดีเอชเอและซีสเทอีนต่อคุณภาพของน้ำเชื้อสุกรแช่แข็ง

นางสาว พนิดา ชนาภิวัตน์

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EFFECT OF DHA-ENRICHED HEN EGG YOLK AND CYSTEINE ON QUALITY OF CRYOPRESERVED BOAR SEMEN

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Theriogenology

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พนิดา ชนาภิวัตน์: ผลของไข่แดงในไข่ไก่ที่อุดมด้วยดีเอชเอและซีสเทอีนต่อคุณภาพของน้ำเชื้อสุกรแช่แข็ง (EFFECT OF DHA-ENRICHED HEN EGG YOLK AND CYSTEINE ON QUALITY OF CRYOPRESERVED BOAR SEMEN) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.น.สพ. ดร. เผด็จ ธรรมรักษ์ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.น.สพ.ดร. กัมพล แก้วเกษ จำนวน, 48 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อเปรียบเทียบผลของการใช้สารละลายเจือจางน้ำเชื้อชนิดบีทีเอส และโมดีนาในระยะปรับสภาพต่อคุณภาพของน้ำเชื้อสุกรแช่แข็ง และศึกษาผลของการใช้ไข่แดงที่อุดมด้วยดีเอชเอและการเสริมซีสเทอีนในสารละลายน้ำเชื้อ ต่อคุณภาพของน้ำเชื้อสุกรแช่แข็ง การทดลองที่ 1 ทำการรีดเก็บน้ำเชื้อจำนวน 7 ครั้ง จากพ่อสุกรพันธุ์ดูรอค 7 ตัว น้ำเชื้อพ่อสุกรถูกแบ่งออกเป็น 2 ส่วน ส่วนที่ 1 เจือจางในสารละลายน้ำเชื้อบีทีเอส และส่วนที่ 2 เจือจางในสารละลายน้ำเชื้อโมดีนา เก็บไว้ที่อุณหภูมิ 15 °C นาน 120 นาที ก่อนนำไปทำการแช่แข็ง การทดลองที่ 2 รีดเก็บน้ำเชื้อจำนวน 15 ครั้ง จากพ่อสุกรพันธุ์เปียแตรง 5 ตัว น้ำเชื้อพ่อสุกรถูกแบ่งออกเป็น 4 กลุ่ม ได้แก่ กลุ่มที่ 1 ใช้สารละลายน้ำเชื้อที่มีไข่แดงจากไข่ไก่ธรรมดา กลุ่มที่ 2 ใช้สารละลายน้ำเชื้อที่มีไข่แดงจากไข่ไก่ที่อุดมด้วยดีเอชเอ กลุ่มที่ 3 ใช้สารละลายน้ำเชื้อที่มีไข่แดงจากไข่ไก่ธรรมดาร่วมกับการเสริมซีสเทอีน 5 มิลลิโมลาร์ และกลุ่มที่ 4 ใช้สารละลายน้ำเชื้อที่มีไข่แดงจากไข่ไก่ที่อุดมด้วยดีเอชเอร่วมกับการเสริมซีสเทอีน 5 มิลลิโมลาร์ น้ำเชื้อพ่อสุกรทุกกลุ่มถูกนำมาผ่านกระบวนการแช่แข็งโดยการใช้เครื่องควบคุมอุณหภูมิและทำละลายที่อุณหภูมิ 50°C นาน 12 วินาที ประเมินคุณภาพของน้ำเชื้อหลังกระบวนการแช่แข็งจากอัตราการเคลื่อนที่ไปข้างหน้า อัตราของตัวอสุจิมีชีวิต ความผิดปกติของอะโครโซม และการทำงานของพลาสมาเมมเบรน ผลการทดลองพบว่าน้ำเชื้อแช่แข็งที่ใช้สารละลายโมดีนาในระยะปรับสภาพมีอัตราการเคลื่อนที่ไปข้างหน้าและสัดส่วนของ normal apical ridge สูงกว่าบีทีเอสอย่างมีนัยสำคัญ ($P<0.01$) การเสริมซีสเทอีนในสารละลายน้ำเชื้อแช่แข็งช่วยเพิ่มอัตราการเคลื่อนที่ไปข้างหน้าอย่างมีนัยสำคัญ ($P<0.05$) คุณภาพของน้ำเชื้อสุกรแช่แข็งหลังการใช้ไข่แดงที่อุดมด้วยดีเอชเอไม่แตกต่างจากไข่แดงปกติ ($P>0.05$) การเสริมซีสเทอีนในสารละลายน้ำเชื้อแช่แข็งร่วมกับการใช้ไข่แดงที่อุดมด้วยดีเอชเอช่วยเพิ่มอัตราการเคลื่อนที่ไปข้างหน้าและสัดส่วนอสุจิที่มีอะโครโซมปกติอย่างมีนัยสำคัญ ($P<0.01$)

ภาควิชาสูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต.....พนิดา ชนาภิวัตน์.....
สาขาวิชาการสืบพันธุ์สัตว์ ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....
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PANIDA CHANAPIWAT: EFFECT OF DHA-ENRICHED HEN EGG YOLK AND CYSTEINE ON QUALITY OF CRYOPRESERVED BOAR SEMEN. THESIS PRINCIPAL ADVISOR: ASSOC. PROF. PADET TUMMARUK, THESIS CO-ADVISOR: ASSOC. PROF. KAMPON KAEOKET, 38 pp.

The present study was performed to investigate the influence of extenders used during equilibration (BTS® vs Modena™) and the effect of DHA-enriched hen egg yolks and/or cysteine supplementation in the freezing extenders on the quality of cryopreserved boar semen. In experiment I, seven ejaculates from seven Duroc boars were divided into two parts and each part was kept in BTS® or Modena™ at 15°C for 120 min before cryopreservation. In experiment II, 15 ejaculates from five Pietrain boars were divided into four groups according to the composition of the freezing extenders, i.e., normal hen egg yolk (group I), DHA-enriched hen egg yolk (group II), normal hen egg yolk with 5 mM of cysteine supplementation (group III) and DHA-enriched hen egg yolk with 5 mM of cysteine supplementation (group IV). The semen was cryopreserved by controlled rate freezer and was thawed at 50 °C for 12 sec. Progressive motility, sperm viability, acrosome integrity and functional integrity of sperm plasma membrane of the post-thawed semen were evaluated. The results revealed that the cryopreserved boar semen using Modena during equilibration yielded a better progressive motility and normal apical ridge than BTS ($P<0.01$). The supplementation of cysteine in the freezing extender improved progressive motility ($P<0.05$). The post-thawed semen qualities were not significantly different between DHA-enriched hen egg yolks and normal hen egg yolk ($P>0.05$). The combination of cysteine supplementation and DHA-enriched hen egg yolk increased progressive motility and acrosome integrity ($P<0.01$).

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ABBREVIATIONS

AI	artificial insemination
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
BTS	Beltsville thawing solution
CP	choline phosphoglycerides
DHA	docosahexaenoic acid
DMSO	dimethyl sulfoxide
DPA	docosapentaenoic acid
EDTA	ethylenediaminetetraacetic acid
EP	ethanolamine phosphoglycerides
EPA	eicosapentaenoic acid
ET	embryo transfer
EthD-1	ethidiumhomodimer-1
FITC-PNA	fluorescien isothiocyanate-labeled peanut agglutinin
FT	frozen-thawed
GPX/GRD	glutathione peroxidase/glutathione reductase
GSH	glutathione
H_2O_2	hydrogen peroxide
HO•	hydroxyl radical
IVF	in vitro fertilization
LN2	liquid nitrogen
LPO	lipid peroxidation
NAR	normal apical ridge
NO	nitric oxide
O_2^-	superoxide anion
$ONOO^-$	peroxynitrite anion
PUFAs	polyunsaturated fatty acids
R•	alkyl radical

ROO•	peroxyl radical
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
sHOST	short hypoosmotic swelling test
SOD	superoxide dismutase
TRIS	trishydroxymethylaminomethane

CHAPTER I
INTRODUCTION

Background and Rationale

The cryopreservation of boar semen needs to be developed for artificial insemination (AI) in the pig industry for a number of reasons including preserving a good genetic resource, increasing genetic improvement, distributing the genetic lines across countries and reducing boar transportation (Almlid and Hofmo, 1996; Johnson, 1998). In addition, frozen-thawed (FT) boar semen is also used for *in vitro* fertilization (IVF), embryo transfer (ET) and sex pre-selection (Gerrits et al., 2005). Unfortunately, the progress of sperm cryopreservation technology in pigs is slow, according to the pig producers who are satisfied with the liquid stored semen and also has limitations (low conception rate and litter size) of using FT boar semen have been reported (Eriksson et al., 2002). Under field conditions, low fertility is still obtained even when using FT boar semen with a sufficient motility (49-53 %) and a high number of spermatozoa for insemination (5×10^9 sperm/ml) (Johnson et al., 2000; Eriksson et al., 2002).

The use of FT boar semen was developed for AI in pigs a long time ago in Europe and North America (Polge, 1956; Larsson and Einarsson, 1976). In Thailand, on the other hand, few studies on boar semen cryopreservation have been established (Buranaamnuay et al., 2006 [a,b]). However, a great variation in the survival rate of post-thawed spermatozoa has been observed, due to the lack of biological background concerning the cryopreservation technique (Buranaamnuay et al., 2006 [a,b]). In recent years, many studies have focused on several ways of improving the boar semen cryopreservation technique, for instance, optimum freezing protocols (Eriksson and Rodrigrez-Martinez, 2000), types of freezing packages (Bwanga et al., 1991; Berger and Fischerleitner, 1992; Eriksson and Rodriguez-Martinez, 2000), centrifugation methods (Carvajal et al., 2004), thawing procedure (Eriksson and Rodrigrez-Martinez, 2000; Córdova-Izquierdo et al., 2006) and the supplement of some additives to the semen extender (Peña et al., 2003; Gadea et al., 2004; Roca et al., 2004, 2005).

Boar semen differs in several aspects from the semen of other domestic animals, for example, the semen is produced in large volumes and is highly sensitive to cold shock, the viability of the sperm cells being dramatically reduced when exposed to

temperatures below 15 °C (Gilmore et al., 1996). Therefore, the manipulation of boar semen requires special consideration during the cryopreservation process (Johnson et al., 2000). Factors that should be considered for boar semen cryopreservation are the composition of diluents, the type and concentration of cryoprotective agents, equilibration time, cooling rate and thawing procedure (Watson, 2000).

The relatively low fertility of FT boar semen is associated with many factors, including the highly sensitive plasma membrane of boar spermatozoa caused by the change in temperature during the cooling, freezing and thawing procedure (Holt, 2000[a]; Watson, 2000). The highly sensitive plasma membrane of boar spermatozoa is related to the lipid composition of the sperm plasma membrane. It has been demonstrated that spermatozoa that have a high resistance to cold shock and freezing contain high levels of polyunsaturated fatty acids (PUFAs) and a high cholesterol to phospholipids ratio (Darin-Bennett and White, 1977; Flesch and Gadella, 2000). The plasma membrane of boar spermatozoa contains a high level of PUFAs, i.e., docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and has a low cholesterol to phospholipids ratio (Parks and Lynch, 1992). DPA and DHA are dominant fatty acids in the plasma membrane of boar spermatozoa (Johnson et al., 1969).

During cryopreservation, PUFAs decrease dramatically due to lipid peroxidation (LPO) when the spermatozoa are attacked by reactive oxygen species (ROS) (de Lamirande and Gagnon, 1992; Sikka et al., 1995). In mammals, the major sources of ROS formation include leucocytes and defective/dead spermatozoa (Aitken et al., 1994; Silva, 2006). Excessive ROS formation has a negative effect on sperm motility, mid-piece abnormalities and sperm-oocyte fusion (Chatterjee et al., 2001; Agarwal et al., 2005).

The supplement of antioxidant compounds and some fatty acid to the semen extender have been reported to minimize ROS formation in many species and protect the plasma membrane function (Peña et al., 2003; Gadea et al., 2004; Roca et al., 2004, 2005; Maldjian et al., 2005). Rooke et al. (2001) found that DHA supplement in boar feed increases progressive motility and normal acrosome and decreases abnormal spermatozoa in fresh semen. For FT boar semen, it has been demonstrated that the proportion of DHA is significantly higher in semen diluted with an extender

supplemented with n-3 enriched hen egg yolks compared with semen diluted with normal hen egg yolks (Maldjian et al., 2005). Szczesniak-Fabianczyk et al. (2003) have shown that the addition of cysteine increases sperm survival time and reduces sperm chromatin damage. Recently, Kaeoket et al. (2008) reported that the supplement of DHA by adding fish oil improves the progressive motility, viability, plasma membrane integrity and acrosome integrity of FT boar semen.

It has been shown that the supplement of antioxidants e.g., Vitamin E, alpha-tocopherol, glutathione (GSH), taurine, cysteine, butylated hydroxytoluene (BHT), superoxide dismutase (SOD) and catalase in the semen extenders improves the freezing ability of spermatozoa in many species such as stallions (Aurich et al., 1997; Ball et al., 2001), bulls (Beconi et al., 1993; Bilodeau et al., 2001), rams (Uysal and Bucak, 2007; Bucak et al., 2007), avians (Donoghue and Donoghue, 1997), boars (Cerolini et al., 2000; Peña et al., 2003; Roca et al., 2004, 2005) and some wildlife (Leibo and Songsasen, 2002). A number of studies have shown that the supplement of alpha-tocopherol (Peña et al., 2003), BHT (Roca et al., 2004), SOD and catalase (Roca et al., 2005) in the semen extenders reduces the ROS formation and improves post-thawed sperm motility and viability. In addition, it has been found that the supplement of cysteine improves the viability and the functional status of chilled boar spermatozoa (Funahashi and Sano, 2005). The benefit of cysteine supplementation in the semen extender used for cryopreservation has never been investigated. Also no study has demonstrated clearly whether or not the supplement of DHA could improve the quality of boar spermatozoa after cryopreservation. The present study was performed to determine the effect of DHA-enriched hen egg yolks and/or cysteine supplementation in the semen extender on the quality of cryopreserved boar semen.

CHAPTER II
LITERATURE REVIEW

The cryopreservation of boar semen needs to be developed for AI in the pig industry for a number of reasons including the preservation of a good genetic resource, increasing genetic improvement, distributing of genetic lines across countries and reducing boar transportation (Almlid and Hofmo, 1996; Johnson, 1998). Widespread exchange of genetic material between breeding populations with liquid stored semen is difficult because of the short life span of the spermatozoa (Wagner and Thibier, 2000; Johnson et al., 2000). FT boar spermatozoa have been reported since 1956 (Polge, 1956). Unfortunately, FT spermatozoa have a very low fertilizing ability. In 1970, the first pregnancy was achieved with FT boar semen using a surgical insemination technique (Polge et al., 1970). In 1971, many studies reported pregnancies after intra-cervical insemination using FT boar semen in pigs (Crabo and Einarsson, 1971; Pursel and Johnson, 1971).

In general, there are many important factors in the process of producing FT boar semen that affect the post-thawed semen quality; for instance, the semen collection technique, equilibration time, type of semen extender, type and concentration of cryoprotectant, freezing package, freezing rate and thawing procedure (Johnson et al., 2000). Many types of freezing packages have been used for frozen boar semen, such as medium straws, maxi straws (Bwanga et al., 1991; Berger and Fisherleitner, 1992; Buranaamnuay et al., 2008), plastic bags (Bwanga et al., 1991) and FlatPack®/MiniFlatPack® (Eriksson and Rodriguez-Martinez, 2000). Most containers have been developed for storage suitability, ease of transportation, good post thawed semen quality and practical insemination.

The freezing and thawing procedures have a significant impact on the survival rate of sperm after cryopreservation (Johnson et al., 2000). However, optimal freezing and thawing rates vary depending on the type and concentration of the cryoprotectant (Mazur et al., 1970; Fiser et al., 1993). Currently, the optimal rates for boar sperm freezing are 30°C/min with 3% glycerol as cryoprotectant in 0.5 ml straws (Fiser and Fairfull, 1990) and 16°C/min with 3.3% glycerol in 5 ml straws (Pursel et al., 1972). For

both these methods the optimal thawing rate is 1200 °C/min (Westendorf et al., 1975; Fiser et al., 1993). Eriksson and Rodriguez-Martinez, (2000) found that the optimal freezing rate was 50 °C/min in 3% glycerol using a 900 °C/min thawing rate for a flattened plastic bag (FlatPack®) container. A variety of cryoprotectants is used in the freezing extenders of different species. Glycerol, egg yolk and sodium dodecyl sulphate (SDS) (Equex STM or Orvus ES paste) are commonly used as cryoprotectants for the cryopreservation of boar semen (Westendorf et al., 1975; Pursel et al., 1978; Holt, 2000[b]; Buranaamnuay et al., 2008). The optimal concentration of glycerol is approximately 3% in pig (Holt, 2000[a]). Egg yolk and SDS are non-permeable cryoprotectants used in freezing extender to have a protective effect of spermatozoa and improve post thawed sperm quality (Pursel et al., 1978). It has been suggested that SDS enhances the cryoprotective properties of the egg yolk protecting the sperm membrane from cryoinjuries (Pursel et al., 1978).

Cryoinjuries of boar spermatozoa during cryopreservation

The use of FT boar semen under field conditions results in a low conception rate and a reduced total number of piglets born per litter (Eriksson et al, 2002). These problems occur because of the poor post-thawed semen quality and the low survival rate of boar spermatozoa after cryopreservation (Hammerstedt et al., 1990; Curry, 2000). The detrimental effects of cooling, freezing and thawing cause an impairing of the the membrane integrity, structure and function of the spermatozoa and eventually its fertilizing ability (Hammerstedt et al., 1990; Guthrie and Welch, 2005).

It is well documented that the boar spermatozoa are highly susceptible to temperatures below 15 °C. The viability of spermatozoa is dramatically reduced within a few hours after exposure to cooling below 15 °C or so called 'cold shock' (Gilmore et al., 1996). Cold shock causes the damage to plasma membranes and an alteration in the metabolism of the spermatozoa. These caused by changes in the arrangement of plasma membrane composition especially phospholipids (reviewed by Medeiros et al., 2002). Sperm damage caused by cold shock is characterized by an irreversible loss of motility and a loss of sperm permeability. Boar spermatozoa seem to acquire cold shock resistance when semen is held at room temperature in seminal plasma for 1-5 hours

(Pursel et al., 1972). It has been found that the viability and fertilizing ability of boar spermatozoa is significantly improved when fresh semen is held at 15 °C for over 3 hours before cryopreservation (Almlid and Johnson, 1988; Eriksson et al., 2001).

During the freezing process, a decrease of temperature from -15 °C to -60 °C caused sperm damage (Mazur, 1970). This is because of the intracellular ice formation and cellular dehydration (osmotic stress). All subsequent physical events depend on the cooling rates. Intracellular ice formation occurs during rapid cooling when intracellular water does not leave the cell to maintain equilibration. If cooling is slow, the spermatozoa will lose water rapidly avoiding intracellular ice formation. However, if spermatozoa are cooled too slowly, they will be exposed to a high concentration of solutes which cause intracellular water to diffuse from the cell, dehydrating both the cell and plasma membrane (also known as solution effects) (Mazur, 1970; Parks and Graham, 1992). Gilmore et al. (1996) demonstrated that boar spermatozoa are sensitive to osmotic stress. Similar findings have also been found in dogs (Songsasen et al., 2002), cats (Pukazhenthi et al., 2000), rams (Curry and Watson, 1994), stallions (Ball and Vo, 2001) and bulls (Liu and Foote, 1998).

In addition to cold shock, intracellular ice formation and dehydration, oxidative stress is another important cause of sperm damage leading to abnormal sperm structure, function and subfertility. Currently, several studies have reported oxidative stress effecting the damage of sperm membrane, proteins and DNA in human (Agarwal et al., 2003), stallions (Baumber et al., 2000; 2003), bulls (Bilodeau et al., 2001) and boars (Roca et al., 2004; 2005).

Lipid composition of the sperm plasma membrane

The lipid composition of the plasma membranes of the mammalian spermatozoa are markedly different from that of somatic cells. In general, the sperm plasma membrane contains approximately 70% phospholipids, 25% neutral lipids, and 5 % glycolipids (Flesch and Gadella, 2000). All lipid components located in sperm membranes are responsible for the fluidity of the membrane lipid bilayers, the regulation of sperm maturation, spermatogenesis, capacitation, acrosome reaction and membrane

fusion (Parks and Hammerstedt, 1985; Martinez and Morros, 1996; Sanocka and Kurpisz, 2004).

The sperm plasma membrane is made up of a phospholipids bilayer, with the major phospholipids being choline phosphoglycerides (CP), ethanolamine phosphoglycerides (EP) and sphingomyelin with their proportions differing between species. These phospholipids contain a high proportion of long chain, polyunsaturated docosapentanoyl (22:5) and docosahexanoyl (22:6) groups of which both lipids represent approximately 50 to 60 % of the total phospholipids in boar and bull spermatozoa (Pursel and Graham, 1967; Johnson et al., 1969; Parks and Lynch, 1992). Cholesterol is the major sterol in the sperm lipids of all species. Cholesterol to phospholipid molar ratios are 0.26, 0.30, 0.36, and 0.45 for the sperm plasma membranes of the boars, roosters, stallions and bulls, respectively (Parks and Lynch, 1992). Glycolipids represent less than 10% of the total polar lipids in all species.

The variation among species in susceptibility to cold shock appears to correlate with the lipid composition of the sperm plasma membrane (Flesch and Gadella, 2000). The resistance of the mammalian spermatozoa to cold shock is high in species in which the cholesterol to phospholipids ratio and phospholipids saturation is high (Darin-Bennett and White, 1977). Avian spermatozoa have a high level of cold shock resistance and a higher level of saturated phospholipids compared to mammalian sperm (Parks and Lynch, 1992). The plasma membrane of boar spermatozoa is characterized by a high protein, low cholesterol and high proportion of EP compared to other species (Nikolopoulou et al., 1985; Parks and Lynch, 1992). In contrast, the protein content and EP proportion of the rooster sperm plasma membrane are low while the cholesterol content is intermediate (Parks and Lynch, 1992).

The sperm plasma membrane has very high amounts of PUFAs especially DPA and DHA (Johnson et al., 1969; Parks and Lynch, 1992). It has been suggested that the proportion of unsaturated fatty acid influences the properties of the sperm plasma membrane (Miller et al., 2005). High levels of long chain PUFAs, DPA and DHA, are associated with increased membrane fluidity (Quinn, 1985). During cryopreservation, the fluidity of the plasma membrane from boar spermatozoa is significantly decreased as compared to fresh spermatozoa which tend to restrict the post-thawed sperm quality

(Buhr et al., 1994). In humans, sperm with a high level of membrane fluidity have a higher post-thawed motility compared to sperm with a low level of membrane fluidity after cryopreservation (Giraud et al., 2000).

Docosahexaenoic acid (DHA)

Docosahexaenoic acid (commonly known as DHA; 22:6 (n-3)) is an omega-3 essential polyunsaturated fatty acid. DHA is most often found in cold water fatty fish (salmon, tuna) and in fish oil supplements, along with eicosapentaenoic acid (EPA). DHA is the main fatty acid composition of the spermatozoa as well as of the brain and the retina (Neuringer et al., 1988). In the sperm plasma membrane, DHA plays a major role in regulating the membrane fluidity in sperm and in the regulation of spermatogenesis (Ollero et al., 2000; Lin, et al., 2004). At puberty, spermatogenesis is accompanied by a tremendous proliferation of spermatogonia in which there are selective changes in the phospholipid molecules, particularly those containing DHA. Therefore, it suggested that the changes in the phospholipid composition of the testis may reflect the phospholipid composition of newly generated sperm cells. Additionally, DHA play a role in the maturation process of spermatozoa. (Lin, et al., 2004; Haidl and Opper, 1997). DHA content is significantly higher in immature spermatozoa than mature spermatozoa which indicates that there is a net decrease in DHA content during the process of sperm maturation (Haidl and Opper, 1997).

Studies have demonstrated that the supplement of PUFAs in the feed of boars improves the quality of boar spermatozoa (Paulenz et al., 1999; Rooke et al., 2001; Strezezek et al., 2004; Maldjian et al., 2005). In addition, Rooke et al. (2001) found that tuna oil supplemented in the boar diet increases viability, progressive motility and normal morphology. The supplementation of PUFAs also enhances the survival rate of post-thawed boar spermatozoa (Strezezek et al., 2004). DHA improves the reproductive performance of male turkeys (Blesbois et al., 2004). Maldjian et al. (2005) found that the use of DHA-enriched hen egg yolk in the semen extender and the supplement of 3% fish oil in boar feed increases the DHA content of boar spermatozoa post-thawed. However, the authors could not demonstrate an improvement of the quality of post-thawed spermatozoa.

Oxidative stress and sperm function

Oxidative stress is a condition associated with an increasing rate of cellular damage induced by oxygen and oxygen-derived oxidants, commonly known as ROS (Sikka et al., 1995). ROS are highly reactive oxidizing agents belonging to the class of free-radicals, which contains one or more unpaired electrons. Normally ROS include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\bullet}$) and the very reactive hydroxyl radicals ($OH^{\bullet}$). The nitrogen-derived free radical nitric oxide (NO) and peroxynitrite anion ($ONOO^-$) play an important role in fertilization. Two main resources of ROS in semen include leukocytes and immature or defective spermatozoa (Aitken et al., 1992; Silva, 2006).

It has been known for a longtime that ROS harm spermatozoa (Macleod, 1943). Recently, studies have shown that the cryopreservation of spermatozoa induces ROS formation and leads to lipid peroxidation as well as DNA oxidation. These events contribute to a decrease in sperm function and infertility (Alvarez and Storey, 1992; Agarwal, 2003). Nevertheless, spermatozoa normally produce small amounts of ROS which are needed for capacitation and acrosome reaction (de Lamirande and Gagnon, 1993; Agarwal et al., 2005).

Oxidative stress is the result of an imbalance between ROS generation and scavenging activities (Sikka et al., 1995; Sharma and Agarwal, 1996). Spermatozoa are sensitive to oxidative stress because of the low concentration of scavenging enzymes in the cytoplasm (de Lamirande and Gagnon, 1995; Saleh and Agarwal, 2002) and the plasma membranes containing high quantities of PUFAs (Alvarez and Storey, 1995). ROS act as triggers in a chain reaction of LPO (de Lamirande and Gagnon, 1992; Sikka et al., 1995). LPO of sperm plasma membrane is the key mechanism of ROS-induced sperm damage (Alvarez et al., 1987).

LPO of sperm membranes is an autocatalytic self-reaction composed of 3 steps (Nogushi and Niki, 1999). Firstly, the initiation step, is the formation of lipid radicals from unsaturated fatty acids by ROS. Secondly, in the propagation step, lipid radicals rapidly react with oxygen molecules to form lipid peroxyl radicals. Lipid peroxyl radicals attack other unsaturated fatty acids and abstract hydrogen atoms to produce lipid hydroperoxide (such as H_2O_2) with concomitant the formation of lipid radicals. The cycle

of propagation, which can continue indefinitely, is known as a chain reaction. Finally, in the termination step, the chain reaction is terminated when the lipid radical or lipid peroxyl radical is scavenged by antioxidants or stopped by a radical-radical reaction which produces a non-radical species (Sanocka and Kurpisz, 2004). LPO has been reported to affect that sperm dysfunction associated with decreased membrane fluidity, loss of membrane integrity and function of spermatozoa (Sanocka and Kurpisz, 2004). Furthermore, LPO also damage DNA and proteins resulting in an increase in the susceptibility to attack by the macrophage (Aitken et al., 1994).

Effect of antioxidants on oxidative stress and sperm function

Antioxidants are compounds that suppress the formation of ROS and protect spermatozoa against ROS (Sikka, 1996). Studies have demonstrated that seminal plasma contains a number of enzymatic antioxidants such as SOD (Alvarez et al., 1987), glutathione peroxidase/glutathione reductase (GPX/GRD) and catalase. These antioxidants protect the spermatozoa against LPO (Lenzi et al., 1996; Sikka et al., 1995; Saleh and Agarwal, 2002). SOD spontaneously dismutates (O_2^-) anions to form O_2 and H_2O_2. Catalase converts H_2O_2 to O_2 and H_2O. In addition, glutathione peroxidase, a selenium-containing antioxidant enzyme with GSH, is an electron donor that removes peroxyl (ROO^-) radicals from various peroxides including H_2O_2 (Sikka et al., 1995). In addition, seminal plasma contains a variety of non-enzymatic antioxidants such as ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), and reduced glutathione (Lenzi et al., 1994; Saleh and Agarwal, 2002; Silva, 2006).

Vitamin C is a major chain–breaking antioxidant present in extracellular fluid (Saleh and Agarwal, 2002). It neutralizes hydroxyl, superoxide and hydrogen peroxide radicals and prevents sperm agglutination (Agarwal et al., 2004). Vitamin E is a chain-breaking antioxidant in the cell membrane, and inhibits LPO by scavenging peroxyl and alkoxyl radicals ($RO^\bullet$). Glutathione is the most abundant antioxidant, and plays a role in protecting lipids, proteins and nucleic acids against oxidative stress.

Studies have shown that the supplementation of antioxidants in extenders, of both chilled and frozen-thawed semen such as alpha-tocopherol, BHT, SOD and catalase, cysteine or GSH have been reported to improve the semen quality in boars

(Pursel, 1979; Bamba and Cran, 1992; Brzezińska-ślebodzińska et al., 1995; Cerolini et al., 2000; Penã et al., 2003; Gadea et al., 2004, Roca et al., 2004, 2005; Funahashi and Sano, 2005; Breininger et al., 2005; Satorre et al., 2007), bulls (Bilodeau et al., 2001), turkeys (Donoghue and Donoghue, 1997), stallions (Aurich et al., 1997; Ball et al., 2001) and rams (Uysal and Bucak, 2007).

L-Cysteine

L-cysteine, an amino acid containing a sulphydryl group, is a precursor of intracellular GSH biosynthesis. *L*-cysteine plays a role in the intracellular protective mechanism against oxidative stress and as a membrane stabiliser and capacitation inhibitor (Johnson et al., 2000). GSH is the most common non-thiol protein in mammalian cells which protects plasma membranes from LPO, scavenges superoxide and minimized O_2^- formation. It has been demonstrated that the supplementation of cysteine in the semen extender prevents loss of sperm motility by minimizing H_2O_2 of FT semen in the bull (Bilodeau et al., 2001). Funahashi and Sano (2005) found that the supplement of cysteine at 5 mM improved the viability and functional status of boar spermatozoa during chilled storage.

Objectives

1. To compare the quality of FT boar semen after using normal hen egg yolk extender versus DHA-enriched hen egg yolk extender.
2. To study the influence of semen extender supplemented with cysteine on the quality of FT boar semen.
3. To investigate the effect of a combination of DHA-enriched hen egg yolk and cysteine supplementation on the quality of FT boar semen.

Expected output

1. DHA-enriched hen egg yolk and cysteine enhance the quality of FT boar semen.
2. Obtain more knowledge about boar semen cryopreservation techniques.
3. Preserve the genetic composition of superior boars in the pig industry.

CHAPTER III
MATERIALS AND METHODS

Animals

Semen from 7 Duroc boars (one ejaculate each) was used in experiment I and semen from 5 Pietrain boars (3 ejaculates each) was used in experiment II. The boars were aged between 1 and 3 years. The boars were housed in individual pens in an opened-house system. The boars were fed twice a day and water was available *ad libitum* . Semen was collected once a week. In experiment I, the boars were kept at large animal hospital chulalongkorn university. In experiment II, the boars were kept in a commercial swine herd in Nakornprathom province.

Semen collection

The semen was collected using the gloved-hand method. During collection the semen was filtered through gauze. The semen volume, pH, sperm motility, concentration, percentage of live and dead sperm and morphology were determined within 20 min of collection. Only ejaculates with a motility of $\geq$ 70 % and $\geq$ 80 % morphologically normal were used for cryopreservation. Afterthat, the semen was transported to the semen laboratory room at Faculty of Veterinary Science, Mahidol University for cryopreservation process.

Semen extender

Three types of semen extenders were used in the present study, extender I, II and III. In experiment I, two commercial semen extenders (Beltsville Thawing Solution; BTS®, Minitüb, Abfüll-und Labortechnik GmbH&Co. KG, Germany and Modena™, Swine Genetics International, Ltd., Iowa, USA) were compared. The compositions of the extender are presented in table 3.1. In experiment II, some compositions of extender II and III were modified (Table 3.2).

Table 3.1 Compositions of semen extender used for the cryopreservation of boar semen

Compositions (g/L)	Extender I	
	BTS®	Modena™
Glucose	37.00	25.00
Sodium citrate	6.00	6.90
EDTA	1.25	2.25
Sodium bicarbonate	1.25	1.00
Potassium chloride	0.75	-
Bovine serum albumin (BSA)	-	3.00
Trishydroxymethylaminomethane (TRIS)	-	5.65
Citric acid	-	2.00
Cysteine	-	0.05

Experimental design

Experiment I was performed to compare the quality of cryopreserved boar semen after using BTS® and Modena™ during equilibration time. The ejaculated semen was divided into 2 parts and each part was kept in BTS® or Modena™ for 120 min before further processing. In experiment II, the semen was divided into 4 groups according to the combination of extender II and III as demonstrated in Table 3.2.

Table 3.2 Classification of semen according to the combination of extenders II and III

Groups	Extender I	Extender II	Extender III
I	Modena™	Normal egg yolk	Normal
II	Modena™	DHA egg yolk[1]	DHA
III	Modena™	Normal egg yolk + *L*-cysteine[2] 5 mM	Normal
IV	Modena™	DHA egg yolk + *L*-cysteine 5 mM	DHA

[1] Dr. Henn®, Quality meat Co. Ltd., Thailand

[2] Fluka Chemie GmbH, Sigma-Aldrich, Switzerland

Evaluation of DHA-enriched hen egg yolk

The DHA hen eggs were produced by a commercial Layer farm (Dr. Henn®, Quality meat Co., Ltd, Thailand). Each egg contained DHA 200-250 mg. The DHA level, as well as the fatty acid composition in the egg yolk that was used for semen extender, was analyzed at the Institute of Nutrition, Mahidol University (AOAC, 2007). The results are shown in Table 3.3. The total number of DHA in extender I, II and III were calculated and demonstrated in Table 3.4.

Table 3.3 Fatty acid composition of normal and DHA-enriched hen egg yolks

Fatty acid	Normal hen eggs (mg/100g)	DHA-enriched hen eggs (mg/100g)
Palmitic acid (C16:0)	527	433
Palmitoleic acid (C16:1)	63	200
Stearic acid (C18:0)	183	146
Oleic acid (C18:1)	923	693
Linoleic acid (C18:2,n-6)	319	212
Linolenic acid (C18:2,n-3)	21	150
Arachidonic acid (C20:4,n-6)	0	0
Eicosapentaenoic acid (C20:5,n-3)	0	20
Docosahexaenoic acid (C22:6,n-3) (DHA)	280	450

Table 3.4 Concentrations of Docosahexaenoic acid (DHA) in extender I, II and III (mg/100ml)

Extenders	Groups			
	I (control)	II	III	IV
I (Modena™)	0	0	0	0
II	56.0	90.0	56.0	90.0
III	50.1	80.6	50.1	80.6

Semen freezing protocols

Shortly after collection, the semen was diluted (1:1 v/v) with extender I. The diluted semen was transferred to 50 ml centrifuge tubes, equilibrated at 15 ºC for 120 min and centrifuged at 800x g for 10 min. The supernatant was discarded and the sperm pellet was re-suspended (about 1-2:1) with extender II (80 ml of 11% lactose solution and 20 ml egg yolk) to a concentration of $1.5x10^9$ spermatozoa/ml. In experiment II, two types of extender II were prepared i.e., using normal hen egg yolk or DHA-enriched hen egg yolk (Table 3.2). Two parts of the semen were divided into two groups (with or without cysteine supplement) (Table 3.2). The diluted semen was cooled to 5 ºC for 90 min. Then, two parts of the semen were mixed with one part of extender III (89.5% extender II with 9% glycerol and 1.5% Equex-STM). The final concentration of semen was approximately $1.0x10^9$ spermatozoa/ml and contained 3% glycerol (modified after Westendorf et al., 1975 and Gadea et al., 2004). The processed semen was loaded into 0.5 ml straws. The straws were sealed with PVC powder before being placed in liquid nitrogen (LN_2) vapour at 3 cm above the level of LN_2 for 20 min and then plunged into LN_2 (Exp I) or placed in a controlled rate freezer (Icecube 14s, Sylab, Purkersdorf, Austria) (Exp II). The freezing rate was 3 ºC/min from + 5 to -5 ºC, 1 min of holding time, and thereafter 50 ºC /min from -5 to -140 ºC. Then the straws were plunged into liquid nitrogen (-196 ºC) for storage.

Thawing procedure

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 a Modena™ extender. The FT semen was incubated in a 37°C water-bath for 15 min before evaluating the post-thawed sperm quality.

Evaluation of semen quality

Sperm concentration was assessed by direct cell count using a Bürker haemocytometer (Boeco, Germany) (Beardon and Fuquay 1997). The visual motility of both fresh and FT sperm was evaluated at 38°C under a phase contrast microscope at

200x and 400x magnification. The motility was assessed by the same person (P. Chanapiwat), throughout the experiment.

The percentages of viable sperm and NAR (normal apical ridge) were determined separately by eosin-nigrosin staining as described by Dott and Foster (1972), this 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). Evaluation was undertaken using a bright-field microscope at 1000 x magnification with 200 sperm being examined for each smear.

The viability was evaluated with SYBR-14/Ethidiumhomodimer-1 (Fertilight®; Sperm Viability Kit, Molecular Probes Europe BV, The Netherlands). Ten µl of diluted semen were mixed with 2.7 µl of the user solution of SYBR-14 and 10 µl of EthD-1. 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, the spermatozoa were assessed (x1000) under fluorescent microscope. The nuclei of the spermatozoa with an intact plasma membrane were stained green with SYBR-14, while those with damaged membranes stained red with EthD-1. Spermatozoa were classified viable with an intact plasma membrane, viable with damaged plasma membrane and dead spermatozoa. The results are expressed as the percentage of viable spermatozoa with intact plasma membranes.

Acrosome integrity was evaluated using fluorescein isothiocyanate–labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining. Ten µl of the diluted semen was mixed with 10 µl of Ethidiumhomodimer-1 and incubated at 37 ºC for 15 min. Five µl of the mixture was smeared on a glass slide and fixed with 95 % ethanol for 30 second. Fifty µl Fit C-PNA (dilute Fit C-PNA with PBS 1:10 v/v) was spread over the slide and incubated in a moist chamber at 4 ºC for 30 min. After incubation, it was rinsed with cold PBS and air dried. Spermatozoa were assessed under fluorescent microscope at 1000x magnification and classified as intact acrosome, damaged acrosome and missing acrosome. The results are scored as the percentage of intact acrosome spermatozoa.

The functional integrity of the sperm plasma membrane was assessed using a short hypo-osmotic swelling test (sHOST) (Perez-Llano et al., 2001). Spermatozoa were incubated, at 38 °C for 30 min, with 75 mOsm/kg a hypo-osmotic solution that consisted

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. The coiled tail (sHOST positive) spermatozoa found following incubation were functional intact plasma membrane.

Lipophilic fluorescent dye C11-BODIPY$^{581/591}$ (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid, Molecular Probes, Leiden, The Netherlands) was used. Semen was washed over a discontinuous Percoll gradient. Sperm cells were resuspended to a concentration of 10 x 10^6 cells/ml and were incubated in sp-TALP containing C11-BODIPY$^{581/591}$ at a final concentration of 5 µM at 38°C for 30 min. Sperm cells were washed in sp-TALP to remove the excess probe (10 min × 700 g) (Ball and Vo, 2002; Brouwers et al., 2005). Sperm cells that expressed green fluorescence of C11-BODIPY$^{581/591}$ in the midpiece indicated lipid peroxidation whereas sperm cells without green fluorescence in the midpiece indicated unperoxidised. A total of 200 sperm cells per sample were recorded.

Statistical analysis

The statistical analysis was performed using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, Cary, N.C., USA). Descriptive statistics were used to describe semen quality before freezing and after thawing. The semen qualities before freezing 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 i.e., progressive motility, sperm viability, acrosome integrity and functional integrity of sperm plasma membrane. Normality of the data was evaluated using UNIVARIATE procedure option NORMAL PLOT. Skewness, kurtosis and Komorov-sminov D statistic were evaluated. Due to all sperm parameters were not normally distributed, arcsine transformation was applied. The analyses were performed based on arcsine transformed data. Data on progressive motility, sperm viability, acrosome integrity and functional integrity of sperm plasma membrane after thawing (arcsine transformation) were analyzed using the General Linear Mixed Model (MIXED) procedure of the SAS. The model included group of extender as a fixed effect and

included boar as random effects. The boars were classified as “good” and “poor” freezability according to post-thawed progressive motility. If post-thawed progressive motility greater than 30%, the boar was classified as “good”. If post-thawed progressive motility lesser than 30%, the boar was classified as “poor”. The statistical analysis was also performed by freezability of the boar semen. Least-square means were obtained from each class of the factors and were compared by least significant different (LSD) method. The differences of all sperm parameter among boars were analyzed using general linear model (GLM) procedure of SAS. The models included boar, group and interaction between boar and group. Least-squared means were compared using least significant different (LSD) method. Statistically significant difference was defined as $P<0.05$.

CHAPTER IV
RESULTS

In the present study, a total of 22 ejaculates (7 ejaculates in experimental I and 15 ejaculates in experimental II) were used for semen cryopreservation. Four ejaculates of fresh semen that were not qualified (individual motility below 70%) were excluded. Descriptive statistics (mean, standard deviation, range) on the sperm parameters of fresh semen used in experiment I and II are presented in Table 4.1 and 4.2, respectively.

The semen qualities before and after cryopreservation was evaluated using three sperm parameters including progressive motility, sperm viability and NAR. It was revealed that all of the sperm parameters evaluated were significantly decrease after cryopreservation process (Table 4.3, 4.4).

After cryopreservation three additional sperm parameters were evaluated i.e., acrosome integrity (Fig 4.1), PMI (Fig 4.2) and sHost. These parameters were used to compared differences between treatment groups (see below). After thawing, lipid peroxidation assayed was also performed using lipophilic fluorescent dye (C11-BODIPY$^{581/591}$). However, due to weak staining was observed, accurate results was hardly to achieve. The results of this assay were therefore excluded.

Table 4.1 Descriptive statistics for sperm parameters measurements of fresh semen (n=7) (Experiment I)

Sperm parameters	Mean±SD	Range
Concentration (x10^6 spz/ml)	291.2 ± 109.2	150-527
Progressive motility (%)	78.1 ± 4.6	70-85
Sperm viability (%)	80.0 ± 6.7	67-88
NAR (%)[1]	83.9 ± 9.4	66-92

[1]NAR= normal apical ridge

Table 4.2 Descriptive statistics for sperm parameters measurements of fresh semen (n=15) (Experiment II)

Sperm parameters	Mean±SD	Range
Concentration (x10^6 spz/ml)	354.5 ± 39.2	302-413
Progressive motility (%)	86.0 ± 3.4	80-90
Sperm viability (%)	85.9 ± 5.4	73-91
Acrosome integrity (%)	83.7 ± 7.3	70-93
sHost (%)[1]	74.8 ± 8.2	63-91

[1]sHost = functional integrity of sperm plasma membrane

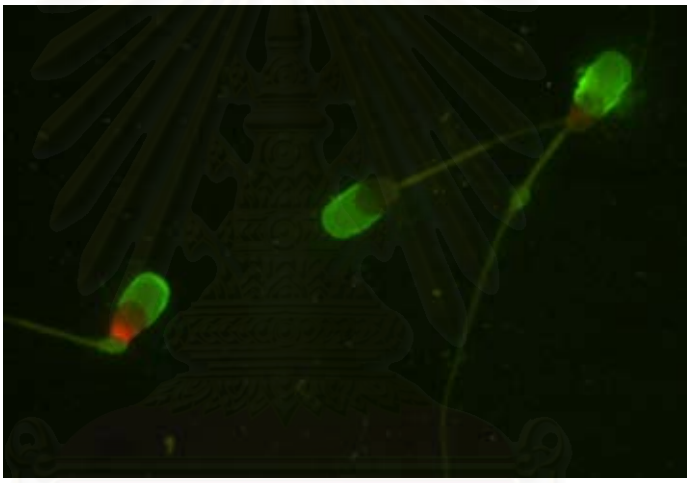

Fig 4.1 Imaging of boar spermatozoa stained with FITC-PNA/Ethidiumhomodimer-1, the spermatozoa with intact acrosome are stained green with FITC-PNA, while dead spermatozoa are stained red with EthD-1.

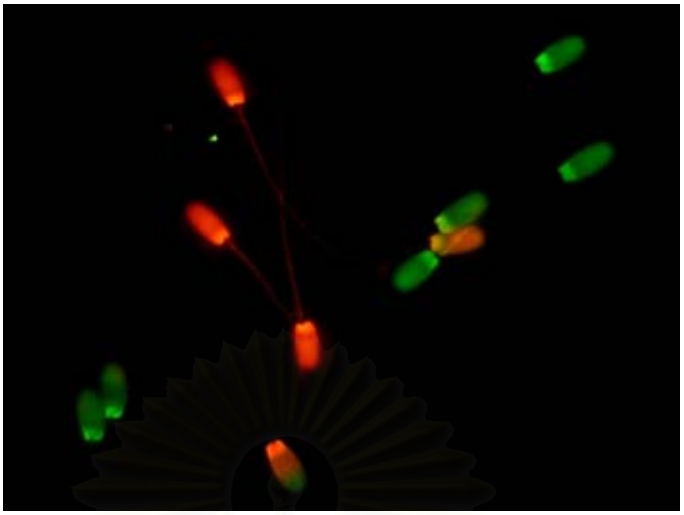

Fig 4.2 Imaging of boar spermatozoa stained with SYBR-14/Ethidiumhomodimer-1, the nuclei of spermatozoa with intact plasma membrane are stained green with SYBR-14, while those with damaged membranes are stained red with EthD-1

Experiment I

The semen quality i.e., progressive motility, sperm viability, NAR, sHost, acrosome integrity (Fig 4.1) and PMI (Fig 4.2), of fresh and frozen-thawed semen between BTS® and Modena™ groups is presented in Table 4.3 and 4.4. All of the sperms parameters were significantly decreased after cryopreservation ($P<0.001$) (Table 4.3 and 4.4). The progressive motility and NAR of FT spermatozoa were significantly higher in the Modena™ than the BTS® groups ($P<0.05$). The progressive motility was correlated with sperm viability (r=0.84; $P<0.001$) and plasma membrane integrity (r=0.68; $P<0.05$)

Table 4.3 Comparisons of semen qualities before and after frozen-thawed after holding in BTS® before cryopreservation (Mean±SD) (n=7 ejaculates)

Sperm parameters	Before	After	Difference [2]	*P*-value [3]
Progressive motility (%)	78.1 ± 4.6	19.3 ± 10.5	-58.8	<0.001
Sperm viability (%)	80.0 ± 6.7	31.7 ± 9.2	-48.3	<0.001
NAR (%) [1]	83.9 ± 9.4	17.7 ± 5.6	-66.2	<0.001

[1]NAR= normal apical ridge, [2] the average of reduction from each ejaculation, [3] Paired *t*-test

Table 4.4 Comparisons of semen quality before and after frozen-thawed after holding in Modena™ before cryopreservation (Mean±SD) (n=7 ejaculates)

Sperm parameters	Before	After	Difference [2]	*P*-value [3]
Progressive motility (%)	78.1 ± 4.6	24.7 ± 15.1	-53.4	<0.001
Sperm viability (%)	80.0 ± 6.7	31.9 ± 7.2	-48.1	<0.001
NAR (%) 1	83.9 ± 9.4	20.9 ± 5.7	-63.0	<0.001

[1]NAR= normal apical ridge, [2] the average of reduction from each ejaculation, [3] Paired *t*-test

Table 4.5 showed that progressive motility and NAR of FT semen were significantly higher in Modena™ than BTS® groups. Others sperm parameters including sperm viability, PMI, acrosome integrity and sHost were not significantly difference between Modena™ and BTS® groups ($P>0.05$).

Table 4.5 Mean±standard deviation (SD) and range of sperm parameters of frozen-thawed semen between BTS® and Modena™ extenders (n=7 ejaculates per group)

Sperm parameters	BTS®		Modena™	
	Mean±SD	Range	Mean±SD	Range
Concentration (x10^6 spz/ml)	1,040 ± 67[a]	955-1,125	1,067 ± 67[a]	1,015-1,175
Progressive motility (%)	19.3 ± 10.5[a]	10.0-40.0	24.7 ± 15.1[b]	10.0-50.0
Sperm viability (%)	31.7 ± 9.2[a]	17.5-47.0	31.9 ± 7.2[a]	21.5-44.5
NAR (%)[1]	17.7 ± 5.6[a]	9.0-24.0	20.9 ± 5.7[b]	12.0-27.0
PMI (%)[2]	32.4 ± 12.0[a]	15.0-50.5	32.1 ± 11.1[a]	19.5-55.5
Acrosome integrity (%)	39.3 ± 11.1[a]	25.5-60.0	39.5 ± 9.7[a]	27.0-57.0
sHost (%)[3]	25.3 ± 7.8[a]	15.0-38.0	24.6 ± 9.9[a]	14.0-41.0

[1]NAR= normal apical ridge, [2]PMI = plasma membrane integrity, [3]sHost = functional integrity of sperm plasma membrane

[a,b] values with different superscripts within row differ significantly (P<0.01)

Experiment II

The quality of FT semen is presented in Table 4.6. On average, all of the sperm parameters were in group II, III and IV was higher than group I (Table 4.6). All of the sperm parameters were most superior in group IV compared to other groups (Table 4.6). In group IV, progressive motility and acrosome integrity (Fig 4.1) were significantly higher than group I (P<0.01). The progressive motility in group III was significantly higher than group I (P<0.05). Acrosome integrity and progressive motility in group II were slightly improved compared to group I, but there was no significant difference from either group I or group IV (Table 4.6).

The sperm viability in group IV was 7% higher than group I *(P=0.12)*. The functional integrity of the sperm plasma membrane (Fig 4.2) in group IV was 4% higher than in the group I *(P=0.13)*.

Table 4.6 Means ± standard deviation of progressive motility (%), sperm viability (%), acrosome integrity (%) and sHost (%) of frozen-thawed boar semen between 4 groups (n=15 ejaculates per group)

Sperm parameters	Frozen-thawed semen			
	Group I	Group II	Group III	Group IV
Progressive motility (%)	23.7 ± 7.7[a]	27.3 ± 9.2[ab]	35.3 ± 11.7[b]	36.3 ± 10.6[b]
Sperm viability (%)	46.4 ± 13.3[a]	49.0 ± 14.0[a]	51.6 ± 12.8[a]	53.7 ± 12.4[a]
Acrosome integrity (%)	31.9 ± 12.1[a]	37.0 ± 12.4[ab]	41.8 ± 15.6[ab]	49.1 ± 12.6[b]
sHost (%)[1]	17.4 ± 9.5[a]	18.5 ± 10.1[a]	19.5 ± 8.2[a]	21.8 ± 10.7[a]

[1]sHost = functional integrity of sperm plasma membrane

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

Effect of individual boar on post-thawed semen quality

In the present study, post thawed semen quality i.e., progressive motility, sperm viability, acrosome integrity and functional integrity of sperm plasma membrane of each baor are presented in Fig 4.3, 4.4, 4.5 and 4.6. On average, boar B, C and D yielded a better post-thawed semen quality than boar A and E in all groups ($P<0.05$).

Effect of DHA and cysteine on post-thawed semen quality in good and poor freezability spermatozoa

The post-thawed semen qualities i.e., progressive motility, sperm viability, acrosome integrity and functional integrity of sperm plasma membrane between good and poor freezability of boar spermatozoa within group were presented in Fig 4.7. All of the sperm parameters were significantly higher in good than poor freezability spermatozoa regardless to the treatments groups.

For between groups comparison, the sperm parameters of good and poor freezability spermatozoa are presented in Table 4.7 and 4.8, respectively. On average, the improvement of sperm parameters after supplementation of DHA and/or cysteine were more pronounced in good rather than poor freezability spermatozoa (Table 4.7 and 4.8). Progressive motility and acrosome integrity in group IV were significantly higher

than group I (6.2%, *P*<0.001; 11.7%, *P*=0.03, respectively). In addition, the supplement of cysteine (group III) significantly improved progressive motility (6.7%, *P*<0.001) and acrosome integrity (9.8%, *P*=0.06).

Table 4.7 Means ± standard deviation of progressive motility (%), sperm viability (%), acrosome integrity (%) and sHost (%) of frozen-thawed boar semen (good boar) between 4 groups

Sperm parameters	Frozen-thawed semen			
	Group I	Group II	Group III	Group IV
Progressive motility (%)	33.8 ± 4.8[a]	35.7 ± 6.1[a]	40.5 ± 9.1[b]	40.0 ± 8.3[b]
Sperm viability (%)	49.1 ± 7.8[a]	58.2 ± 9.1[a]	56.9 ± 7.2[a]	57.6 ± 8.9[a]
Acrosome integrity (%)	38.3 ± 9.6[a]	47.3 ± 8.4[ab]	48.1 ± 12.4[ab]	50.0 ± 11.8[b]
sHost (%)[1]	26.3 ± 4.1[a]	26.8 ± 7.9[a]	22.1 ± 6.9[a]	24.8 ± 9.8[a]

[1]sHost = functional integrity of sperm plasma membrane

[a,b] values with different superscripts within row differ significantly (*P*<0.05)

Table 4.8 Means ± standard deviation of progressive motility, sperm viability, acrosome integrity and sHost of frozen-thawed boar semen (poor boar) among 4 groups

Sperm parameters	Frozen-thawed semen			
	Group I	Group II	Group III	Group IV
Progressive motility (%)	20.0 ± 4.5[a]	21.3 ± 2.5[a]	20.0 ± 2.7[a]	21.7 ± 2.9[a]
Sperm viability (%)	45.4 ± 15.0[a]	34.6 ± 14.0[a]	40.3 ± 13.1[a]	36.5 ± 12.2[a]
Acrosome integrity (%)	30.5 ± 11.9[a]	36.7 ± 12.9[a]	28.1 ± 7.1[a]	36.7 ± 12.9[a]
sHost (%)[1]	14.2 ± 8.9[a]	12.3 ± 7.4[a]	11.3 ± 4.6[a]	10.1 ± 4.3[a]

[1]sHost = functional integrity of sperm plasma membrane

[a,b] values with different superscripts within row differ significantly (*P*<0.05)

Correlations among sperm parameters after frozen-thawed

All sperm parameters i.e., progressive motility, sperm viability, acrosome integrity and sHost after FT were significantly correlated (Table 4.9). The progressive

motility of FT spermatozoa was significantly correlated with sperm viability (r=0.63; *P*<0.001), acrosome integrity (r=0.71; *P*<0.001) and sHost (r=0.69; *P*<0.001).

Table 4.9 Pearson's correlation coefficient (r) and significance levels among progressive motility, sperm viability, acrosome integrity and sHost after frozen-thawed

	Viability (%)	Acrosome integrity (%)	sHost (%)
Progressive motility (%)	0.63***	0.71***	0.69***
Sperm viability (%)		0.68***	0.72***
Acrosome integrity (%)			0.67***

* = <0.01<*P*<0.05, ** = 0.001<*P*<0.01, *** = *P*<0.001, ns = *P*>0.05, sHost = functional integrity of sperm plasma membrane

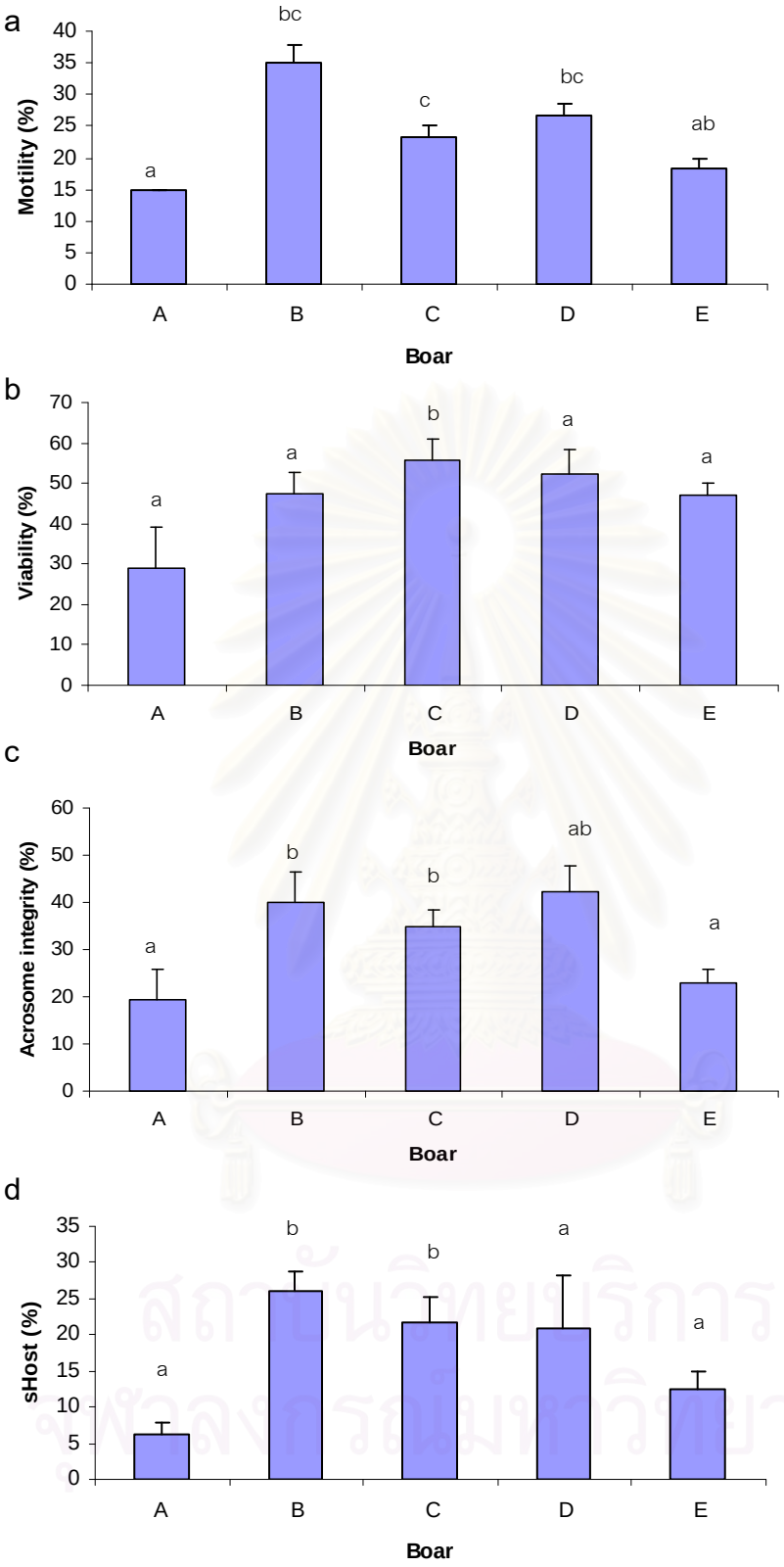

Fig. 4.3 Post-thawed semen quality of group I (control group) in each individual boars, progressive motility (a), sperm viability (b), acrosome integrity (c) and sHost-functional integrity of sperm plasma membrane (d). Values are means±SEM, Means with different superscripts (a,b,c) differ significantly ($P<0.05$)

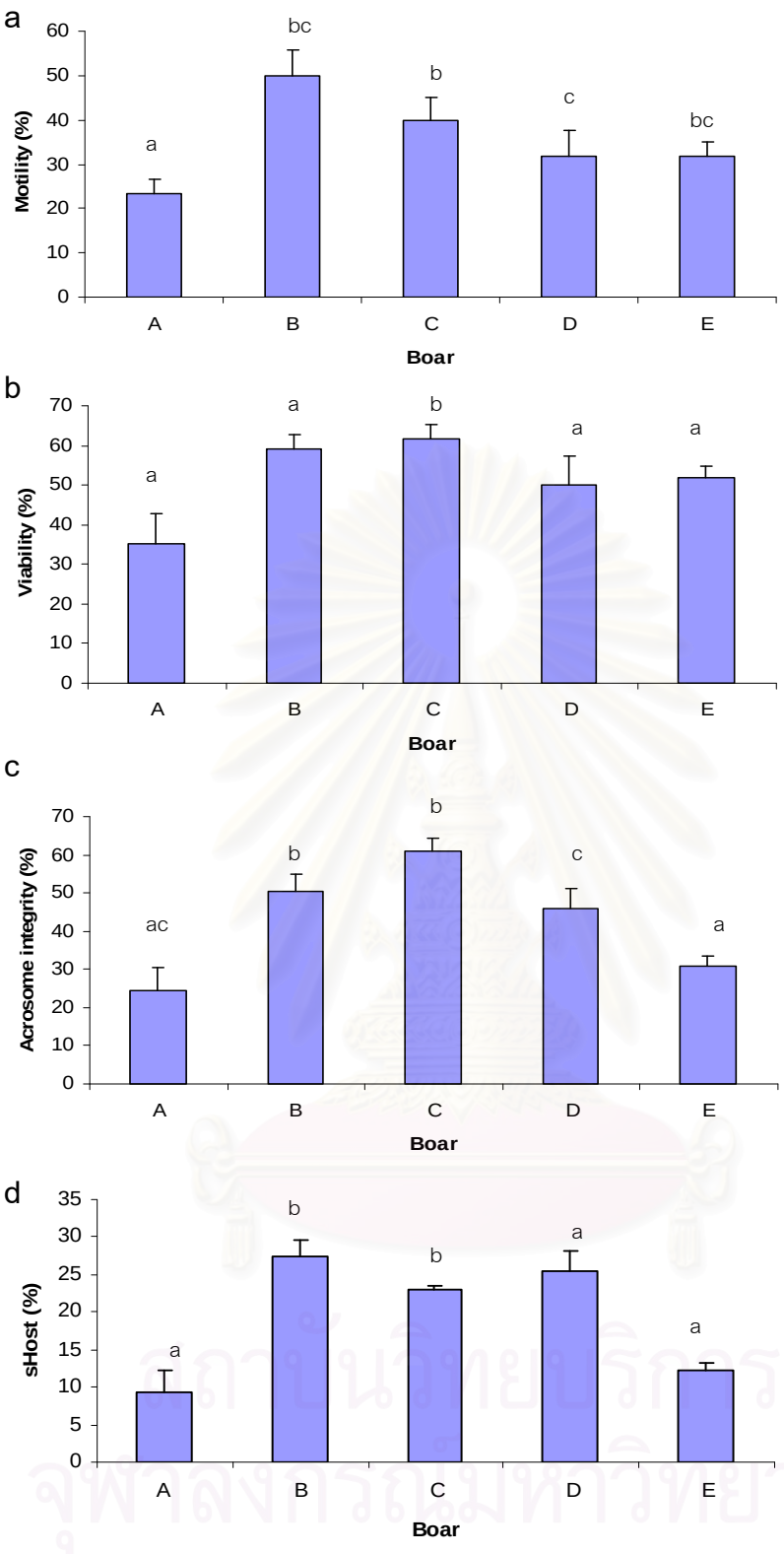

Fig. 4.4 Post-thawed semen quality of group II in each individual boar, progressive motility (a), sperm viability (b), acrosome integrity (c) and sHost-functional integrity of sperm plasma membrane (d). Values are means±SEM, Means with different superscripts (a,b,c) differ significantly ($P<0.05$)

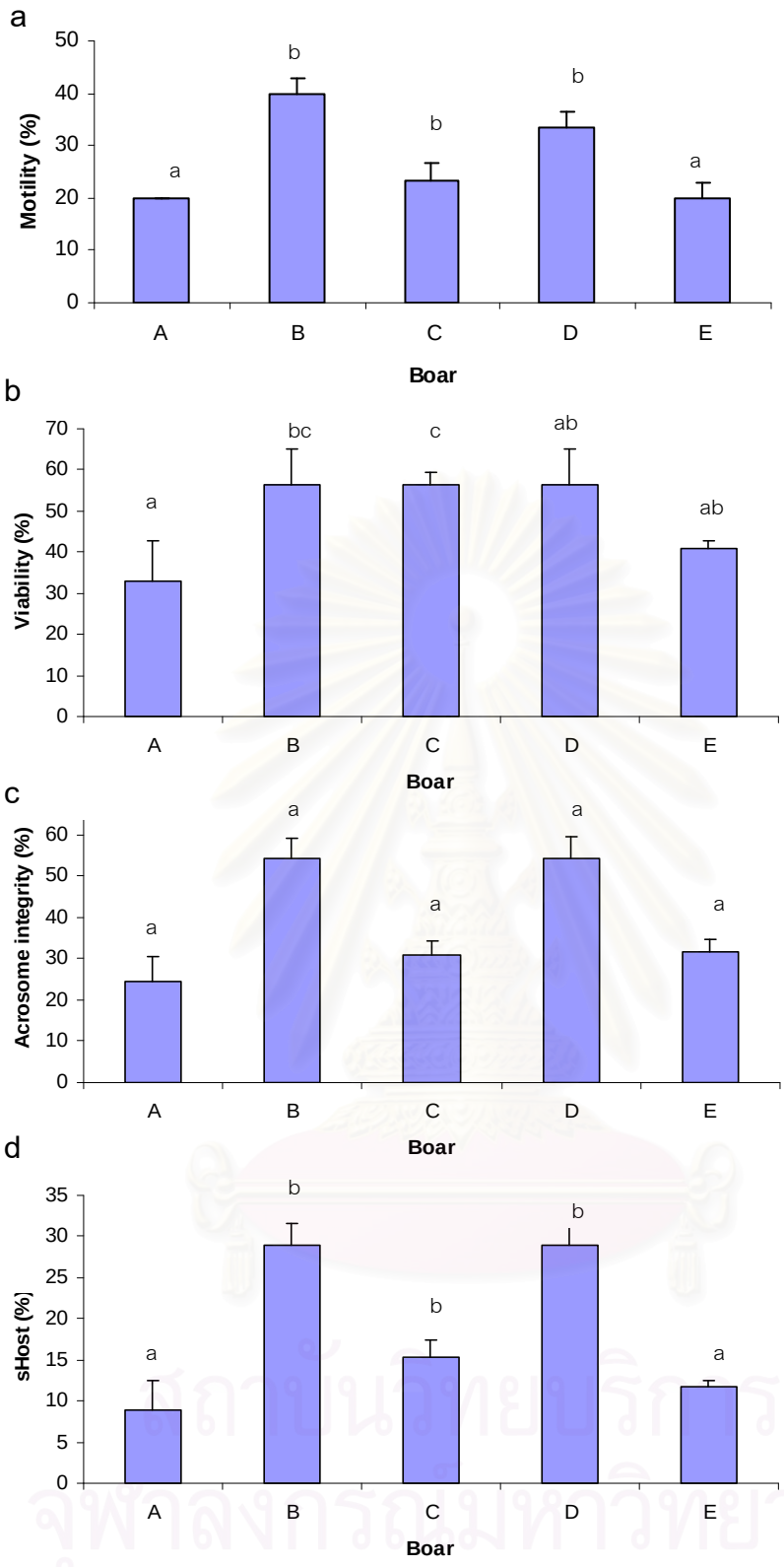

Fig. 4.5 Post-thawed semen quality of group III in each individual boars, progressive motility (a), sperm viability (b), acrosome integrity (c) and sHost-functional integrity of sperm plasma membrane (d). Values are means±SEM, Means with different superscripts (a,b,c) differ significantly ($P<0.05$)

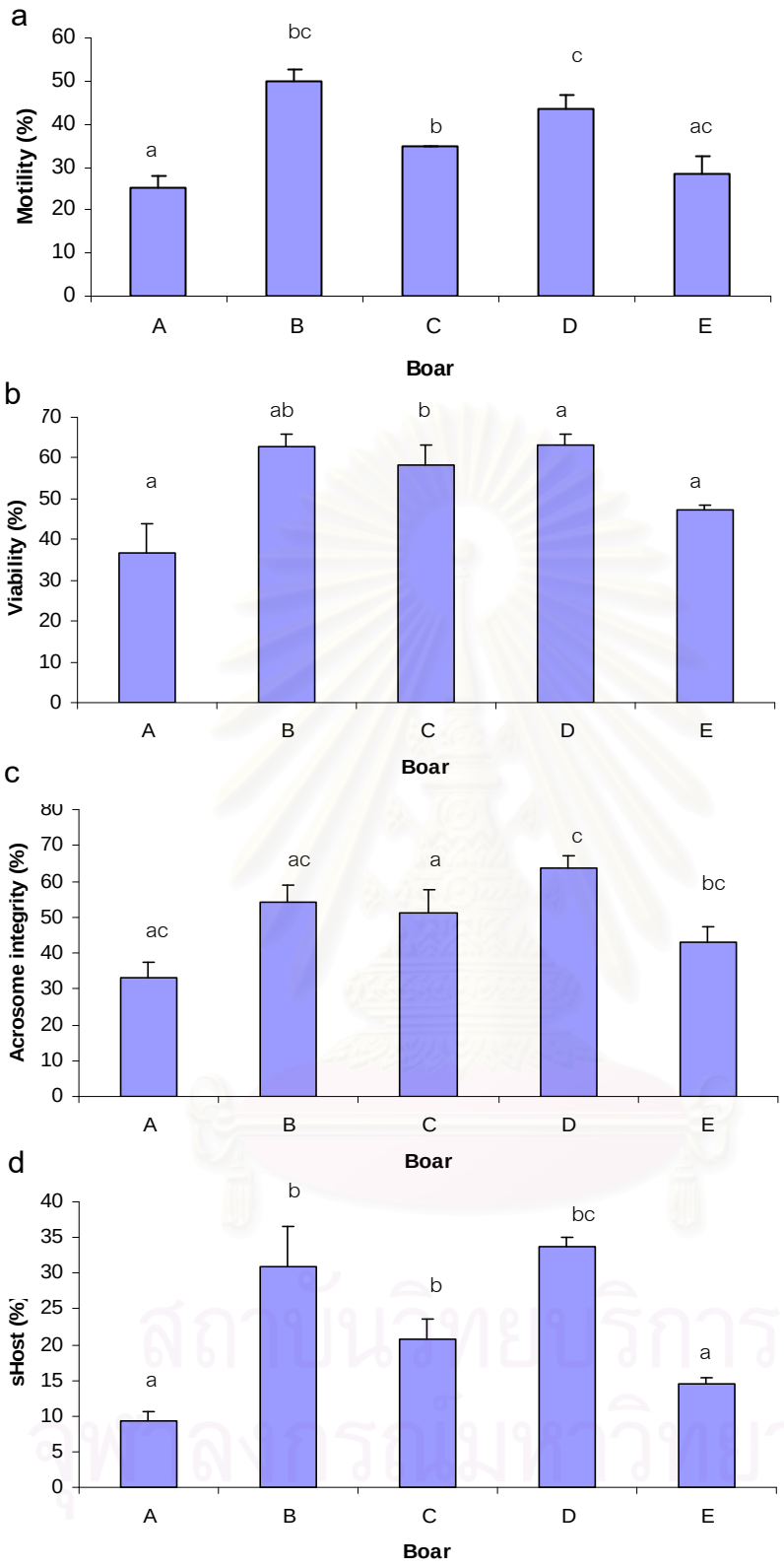

Fig. 4.6 Post-thawed semen quality of group IV in each individual boars, progressive motility (a), sperm viability (b), acrosome integrity (c) and sHost-functional integrity of sperm plasma membrane (d). Values are means±SEM, Means with different superscripts (a,b,c) differ significantly ($P<0.05$).

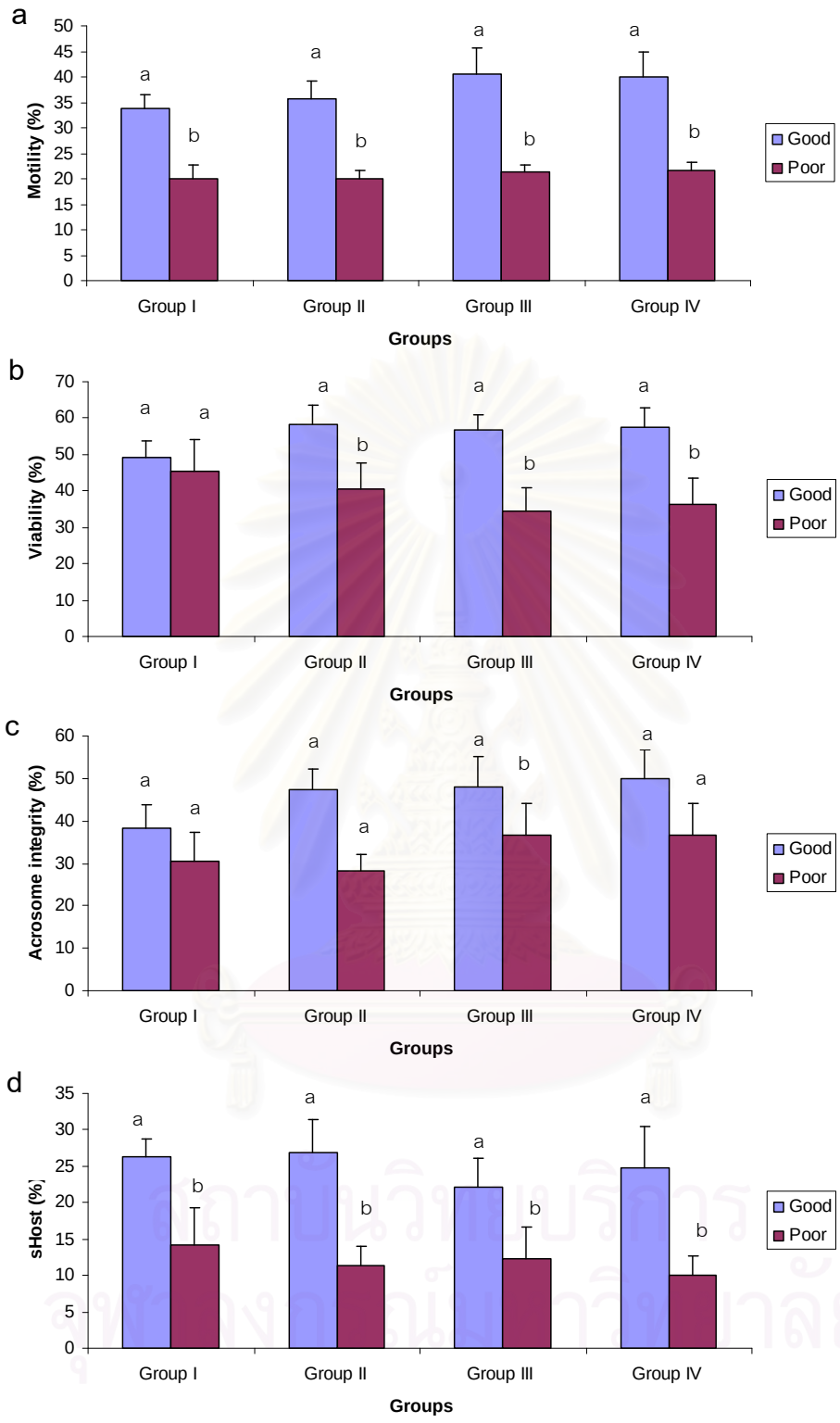

Fig. 4.7 Effect of DHA and cysteine on post-thawed semen quality in good and poor freezability of boar spermatozoa, progressive motility (a), sperm viability (b), acrosome integrity (c) and sHost-functional integrity of sperm plasma membrane (d). Values are Means±SEM, Means with different superscripts (a,b) differ (P<0.05) within group.

CHAPTER V
DISCUSSION

Effect of extenders during equilibration on post-thawed semen quality

In general, BTS® is normally used during equilibration to cool the boar semen to 15 °C for 2-3 h in the first step for cryopreservation (Westendorf et al., 1975; Buranaamnuay et al., 2008). Guthrie and Welch (2005) demonstrated that the storage of semen in different types of extenders prior to cryopreservation had a significant impact on the plasma membrane function and progressive motility of the boar spermatozoa. It was demonstrated that an increasing the holding time from 3 to 24 h in BTS had no significant effect on plasma membrane integrity (PMI), while increase holding time from 3 to 24 h in Androhep Plus decreased PMI of boar spermatozoa both before and after cryopreservation (Guthrie and Welch, 2005). In the present study, the equilibration time was fixed at 2 h and it was found that the boar semen equilibrated in Modena™ yielded a better progressive motility and NAR after thawing than the semen equilibrated in BTS®. This finding has never been reported before. The reason might be related with the difference of composition in the extenders. The composition of Modena™ that increases the post-thawed progressive motility and NAR of boar semen may include TRIS, BSA, EDTA and cysteine. TRIS is a pH regulator. BSA protects sperm against cold shock (de Ambrogi et al., 2006) and strengthens the sperm plasma membrane by reducing lipid peroxidation (Dixon and Kreider, 1981). EDTA is a chelating agent that blocks the action of calcium as a mediator of sperm capacitation and the acrosome reaction. An increase of intracellular calcium levels following cryopreservation reduces the motility and fertilizing ability of spermatozoa (McLaughlin and Ford, 1994). EDTA also counteracts toxicity by binding some metal ions in the semen. Finally, cysteine acts as an antioxidant to stabilize sperm plasma membrane and inhibit sperm capacitation (Johnson et al., 2000). The influence of cysteine on the freezing ability of boar spermatozoa is discussed below. However, it is suggested that additional studies on the physiological changes in spermatozoa should be performed to prove whether the favourable effect of Modena™ occurs during liquid storage or during the cryopreservation process.

Effect of cysteine on post-thawed semen quality

In the present study, the supplementation of cysteine in the freezing extender improved the motility of boar spermatozoa after cryopreservation. This finding is in accordance with Bilodeau et al. (2001) who found that the supplementation of cysteine in combination with GSH increase the motility of the FT bull spermatozoa. In addition, it has been shown that the supplement of cysteine in extended fresh semen significantly increases sperm viability and acrosome integrity (Szczesniak-Fabianczyk et al., 2003; Funahashi and Sano, 2005;). Recently, cysteine has also been shown to improve the sperm viability and acrosome integrity of FT ram's semen (Uysal and Bucak, 2007). The positive effects of cysteine on many sperm parameters might be due to several reasons. For instance, cysteine is the precursor of intracellular GSH biosynthesis, which can reactivate ROS and catalyzes the detoxification of H_2O_2 as well as other superoxides (Meister, 1992). However, the susceptibility of spermatozoa to LPO differs among species. The doses of antioxidant used for different species may be different.

Effect of DHA–enriched hen egg yolk on post-thawed semen quality

Differences in the lipid composition of the sperm plasma membrane have been suggested as a major factor influencing the freezability of the spermatozoa. The proportion of PUFAs, especially DHA and DPA, in the plasma membrane of spermatozoa is affected by diluents and the freezing process (Maldjian et al., 2005). High levels of PUFAs in the membrane phospholipids increase membrane fluidity and flexibility (Lenzi et al., 1996), consequently improve the freezability of the spermatozoa. Maldjian et al. (2005) found that the proportion of DHA in phospholipids of spermatozoa could be increased by using DHA-enriched hen egg yolk instead of normal hen egg yolk. However, the use of DHA-enriched hen egg yolk failed to improve any of the sperm parameters following cryopreservation. In the present study, the supplement of DHA-enriched egg yolk also failed to improve the post-thawed semen quality, which is in agreement with the previous study (Maldjian et al., 2005). On the other hand, Kaeoket et al. (2008) found that the supplement of DHA by adding fish oil to the freezing extender successfully improved the progressive motility, viability, plasma membrane integrity and

acrosome integrity of FT boar spermatozoa. This might be because the sources and the concentration of DHA differed from the present study. Furthermore, the ability of DHA uptake of spermatozoa might differ among individual boars and breeds (Vazquez and Roldan, 1997). In the animal model, it was found that the supplement of DHA in boar feed significantly increased the progressive motility and intact acrosome of fresh semen (Rooke et al., 2001; Strezezek et al., 2004). However, the supplement of DHA-enriched cod liver oil in boar diet failed to improve the freezability of boar semen (Paulenz, 1999). The influence of DHA on the freezing ability of boar spermatozoa is still contradictory and remains to be further investigated. Sources and concentrations of DHA as well as breed and the individual boar effect on DHA uptake should also be taken into consideration.

Effect of DHA-enriched hen egg yolk and cysteine combinations on post-thawed semen quality

It is known that both DHA and cysteine play a major role in protecting sperm structure and function. In the present study, the combination of DHA-enriched hen egg yolk and cysteine significantly increased progressive motility and intact acrosome. Acrosome integrity is a parameter used to evaluate the functional membrane status of the spermatozoa (Garner and Johnson, 1995). Viable spermatozoa with intact acrosome are able to undergo the acrosome reaction, penetrate the zona pellucida and fuse with the oocyte. DHA and cysteine combination may protect acrosome by enhancing fluidity and increasing ROS scavenging. However, the use of DHA and cysteine combinations and the use of cysteine alone are not significantly different. This might be due to variation in concentration of DHA. In order to improve the post-thawed boar semen quality, the effect of optimal concentrations of both DHA content and cysteine supplementation should be considered.

Effect of individual boar on post-thawed semen quality

In the present study, the post-thawed semen qualities differed among individual boars. The present finding is in accordance with a number of earlier studies (reviewed by Johnson et al. 2000). It is well established that successful boar semen cryopreservation depends on internal and external factors. The internal factors included the inherent characteristic of spermatozoa, and difference between boar and ejaculates, while the external factors included 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 (reviewed by Johnson et al. 2000). The present study demonstrated a significant impact of individual boars on the post thawed semen qualities. The individual boars influenced the sperm susceptibility to cryoinjury as shown that the post thawed sperm quality differed significantly among boars within the same breed. This is in agreement with previous findings (Larsson and Einarsson, 1976; Holt et al., 2005). The reason for individual boar in cryotolerance of spermatozoa is unknown at present, it may have genetic difference among individual boars (Holt et al., 2005). Furthermore, it has been suggested that cold shock resistance of spermatozoa may relate to the lipid composition within the membrane bilayer of the sperm plasma membrane (Flesch and Gadella, 2000). Boar spermatozoa that have a high sensitivity to cold shock contain high protein, low cholesterol and high proportion of EP compared to other species (Nikolopoulou et al., 1985; Parks and Lynch, 1992). Therefore, these physiological characteristic of the spermatozoa from each boar may contribute to the individual variation.

Effect of DHA and cysteine on post-thawed semen quality in good and poor freezability spermatozoa

In the present study neither DHA nor cysteine supplementation improved the FT sperm parameters in poor freezability spermatozoa. This indicated that both DHA cysteine did not enhance internal factors of the boar spermatozoa. On the other hands, boars that were classified as a good freezability (boars B, C and D), the supplement of both DHA and cysteine does improve the FT semen qualities. This might be due to the

difference of lipid composition in sperm plasma membrane and the ability of DHA and cysteine uptake of spermatozoa differs among boars (Vazquez and Roldan, 1997). It has been reported that cryopreservation process significantly reduced intracellular GSH levels of boar spermatozoa (Gadea et al., 2004). Therefore, supplementation with cysteine, a precursor of intracellular GSH biosynthesis, has a beneficial effect while intracellular GSH levels are decreased.

Conclusions

- The use of Modena™ during equilibration yielded a better post-thawed progressive motility and normal apical ridge than BTS®.

- The combination of cysteine and DHA-enriched hen egg yolk significantly improved the progressive motility and acrosome integrity of FT boar spermatozoa.

- The favorable effects of both DHA and cysteine on FT semen qualities were more pronounced in good freezability spermatozoa than poor freezability spermatozoa.

Additional comments

Further studies may be needed to evaluate the optimal concentration of cysteine and DHA in order to improve post-thawed semen quality and minimize individual variation. Additionally, in vitro fertility tests such as zona pellucida binding assay and IVF should be assessed before implications to field conditions.

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