องค์ประกอบกรดไขมันในน้ำเชื้อสุกรและผลของสารเสริมอาหารต่อคุณภาพน้ำเชื้อ

นายนัทธี อ่ำอินทร์

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FATTY ACID COMPOSITION OF BOAR SEMEN AND EFFECTS OF FEED SUPPLEMENTATION ON SEMEN QUALITY

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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นัทชี อ่ำอินทร์ : องค์ประกอบกรดไขมันในน้ำเชื้อสุกรและผลของสารเสริมอาหารต่อคุณภาพน้ำเชื้อ (FATTY ACID COMPOSITION OF BOAR SEMEN AND EFFECTS OF FEED SUPPLEMENTATION ON SEMEN QUALITY) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.น.สพ.ดร. วิชัย ทันตศุภารักษ์,อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.น.สพ.ดร. มงคล เตชะกำพุ, Assoc.Prof. Roy N Kirkwood, D.V.M., Ph.D., 76 หน้า

การทดลองที่ 1 ศึกษาเพื่อหาความแตกต่างของไขมันและกรดไขมันไม่อิ่มตัวในองค์ประกอบของตัวอสุจิและน้ำเลี้ยงอสุจิในพ่อสุกรที่มีการเคลื่อนไหวของตัวอสุจิที่ต่างกัน รีดเก็บน้ำเชื้อจากพ่อสุกรที่มีอัตราการเคลื่อนไหวของตัวอสุจิ >60% (53 ตัว) และอัตราการเคลื่อนไหวของตัวอสุจิ <60% (53 ตัว) แยกอสุจิออกจากน้ำเลี้ยงอสุจิ และเก็บที่อุณหภูมิ -20 องศาเซลเซียส จนกระทั่งนำไปตรวจวิเคราะห์องค์ประกอบไขมันโดยวิธีก๊าซโครมาโตกราฟี ตรวจหาสารต้านอนุมูลอิสระของน้ำเลี้ยงเชื้อโดยใช้ชุดตรวจสำเร็จรูป ผลการศึกษาพบว่ามีความแตกต่างของปริมาณไขมันรวม คอเลสเตอรอล กรดไขมันอิ่มตัว กรดไขมันอิ่มตัวชนิด โอเมก้า 3 ดีเอชเอและอัตราส่วนระหว่างกรดไขมันอิ่มตัวชนิด โอเมก้า 6 ต่อ 3 ระหว่างน้ำเชื้อที่มีอัตราการเคลื่อนไหวของตัวอสุจิ>60%และ<60% (P<0.05) พบความสัมพันธ์เชิงบวกระหว่างปริมาณไขมันรวม คอเลสเตอรอล กรดไขมันอิ่มตัว ดีเอชเอ กรดไขมันอิ่มตัวชนิด โอเมก้า 3 ในตัวอสุจิและสารต้านอนุมูลอิสระของน้ำเลี้ยงเชื้อกับอัตราการเคลื่อนไหว (n=159; P<0.05; r=0.34, 0.63, 0.76, 0.59, 0.43, 0.58 ตามลำดับ) การมีชีวิต (n=159; P<0.05; r=0.34, 0.63, 0.75, 0.57, 0.41, 0.54 ตามลำดับ) ลักษณะปกติ (n=159; P<0.05; r=0.16, 0.28, 0.38 , 0.33, 0.24, 0.28 ตามลำดับ) และความสมบูรณ์ของเยื่อหุ้มเซลล์ของตัวอสุจิ (n=159; P<0.05; r=0.53, 0.60, 0.73, 0.57, 0.42, 0.56 ตามลำดับ) ผลการทดลองบ่งชี้ว่าองค์ประกอบไขมันในตัวอสุจิอาจมีผลต่ออัตราการเคลื่อนไหวของตัวอสุจิ การมีชีวิต ลักษณะปกติและความสมบูรณ์ของเยื่อหุ้มเซลล์ของตัวอสุจิ

การทดลองที่ 2 ศึกษาเปรียบเทียบการมีชีวิต ไลปิดเปอร์ออกซิเดชั่น องค์ประกอบไขมันในตัวอสุจิสุกรและสารต้านอนุมูลอิสระระหว่างกลุ่มที่มีการเคลื่อนไหวของตัวอสุจิ >50% (10 ตัว) และ <50% (10 ตัว) เมื่อเก็บรักษาด้วยการแช่เย็น 24 ชั่วโมง น้ำเชื้อถูกรีดเก็บจากพ่อสุกรทุกตัว 1 ครั้งต่อสัปดาห์เป็นเวลา 3 สัปดาห์ ตรวจอัตราการเคลื่อนไหว ความเข้มข้น จำนวนตัวอสุจิทั้งหมด ความปกติ อัตราการมีชีวิตและความสมบูรณ์ของเยื่อหุ้มเซลล์ของตัวอสุจิ ตัวอสุจิถูกสกัดและวิเคราะห์เพื่อหาองค์ประกอบไขมัน และตรวจหาปริมาณไลปิดเปอร์ออกซิเดชั่นในตัวอสุจิและสารต้านอนุมูลอิสระในสารละลายน้ำเชื้อจากน้ำเชื้อที่แช่เย็น 0 และ 24 ชั่วโมง ผลการทดลองพบว่าการลดลงของปริมาณไขมันรวม อัตราส่วนคอเลสเตอรอลในตัวอสุจิ สารต้านอนุมูลอิสระในสารละลายน้ำเชื้อ และการเพิ่มขึ้นของไลปิดเปอร์ออกซิเดชั่นในตัวอสุจิ เป็นสาเหตุให้เกิดการลดลงของอัตราเคลื่อนไหว การมีชีวิต ความสมบูรณ์ของเยื่อหุ้มเซลล์และคุณสมบัติการเก็บรักษา

การทดลองที่ 3 การศึกษาผลของน้ำมันปลา วิตามินและซีลีเนียมต่อองค์ประกอบไขมันของตัวอสุจิและคุณภาพน้ำเชื้อ ใช้พ่อสุกรจำนวน 21 ตัวในการศึกษานี้ สุกรถูกแบ่งเป็น 3 กลุ่มแบบสุ่ม กลุ่มที่ 1 ให้อาหารเสริมเป็นเวลา 8 สัปดาห์ (7ตัว) กลุ่มที่ 2 ให้อาหารเสริมเป็นเวลา 16 สัปดาห์ (7ตัว) และกลุ่มที่ 3 กลุ่มควบคุม (7ตัว) อาหารเสริมประกอบด้วยน้ำมันปลา 40 มิลลิลิตร วิตามินอี 480 ไอยู วิตามินซี 2,400 มิลลิกรัม และซีลีเนียม 300 มิลลิกรัม และให้ 1 ครั้งต่อวัน เก็บน้ำเชื้อจากพ่อสุกรทุกตัว สัปดาห์ละหนึ่งครั้งโดยใช้ถุงมือรีดเก็บเริ่มต้นตั้งแต่ 7 สัปดาห์ก่อนให้อาหารเสริมจนกระทั่งสิ้นสุดการทดลองรวม 23 ครั้งต่อตัว ตรวจคุณภาพน้ำเชื้อ แยกตัวอสุจิจากน้ำเลี้ยงเชื้อด้วยเครื่องปั่นเหวี่ยงและเก็บที่อุณหภูมิ -20 องศาเซลเซียสจนกระทั่งวิเคราะห์หาองค์ประกอบไขมันและกรดไขมันอิ่มตัวในตัวอสุจิและตรวจหาสารต้านอนุมูลอิสระ กลูตาไทโอนเปอร์ออกซิเดสโดยใช้ชุดตรวจสำเร็จรูป จากการศึกษาเปรียบเทียบระหว่างกลุ่มควบคุมและกลุ่มที่ได้รับอาหารเสริม 8 และ 16 สัปดาห์ ผลการทดลองพบว่าจำนวนตัวอสุจิทั้งหมด (70.84 เปรียบเทียบกับ 71.69 และ 71.35 พันล้านตัว ตามลำดับ; P<0.05) ปริมาตรน้ำเชื้อ (290.14 เปรียบเทียบกับ 341.60 และ 326.24 มิลลิลิตร ตามลำดับ; P<0.05) อัตราส่วนดีเอชเอ (14.91 เปรียบเทียบกับ 16.21 และ 16.16% ตามลำดับ; P<0.05) และกรดไขมันไม่อิ่มตัวชนิดโอเมก้า 3 (14.12 เปรียบเทียบกับ 16.82 และ 16.95% ตามลำดับ; P<0.05) ในตัวอสุจิเพิ่มขึ้น ระยะเวลาที่หลั่งน้ำเชื้อยาวนานขึ้น (374.82 เปรียบเทียบกับ 439.01 และ 419.30 วินาที ตามลำดับ; P<0.05) และปริมาณกลูตาไทโอนเปอร์ออกซิเดสในน้ำเลี้ยงเชื้อเพิ่มขึ้น (1.22 เปรียบเทียบกับ 1.45 และ 1.51 มิลลิโมลต่อมิลลิลิตร ตามลำดับ; P<0.05) ในพ่อสุกรที่ได้รับอาหารเสริมเปรียบเทียบกับกลุ่มควบคุม

ภาควิชา สูติศาสตร์,เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อสิต.....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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NUTTHEE AM-IN: FATTY ACID COMPOSITION OF BOAR SEMEN AND EFFECTS OF FEED SUPPLEMENTATION ON SEMEN QUALITY. THESIS ADVISOR: ASSOC. PROF. WICHAI TANTASUPARUK, D.V.M., Ph.D, THESIS CO-ADVISOR : PROF. MONGKOL TECHAKUMPHU, D.V.M., Doctorate de 3e cycle, Assoc.Prof. Roy N Kirkwood, D.V.M., Ph.D.76 pp

EXP I was conducted to determine the differences in lipid and fatty acid (FA) composition of boar sperm and seminal plasma in the ejaculates of boars having different sperm motilities. Semen was collected from two groups of boars having >60% (n=53) and <60% (n=53) sperm motility and separated the sperm from the seminal plasma. Both fractions were kept in -20°C until analyzed for lipid content and FA profile by gas liquid chromatography. Total antioxidant status (TAS) of seminal plasma was determined using a commercial kit. The results demonstrated that there were differences in sperm total lipids, cholesterol, saturated fatty acids (SFA), phospholipids, *n-3* polyunsaturated fatty acids (PUFA), docosahexaenoic acid (DHA) and the ratio of *n-6:n-3* PUFA between normal and low sperm motility boars ($P<0.05$), and there were positive correlations among total lipids, cholesterol, phospholipids, PUFA, DHA, *n-3* PUFA and TAS of seminal plasma with sperm motility (n=159; $P<0.05$; r=0.53, 0.60, 0.73, 0.57, 0.42, 0.56 respectively), viability (n=159; $P<0.05$; r=0.16, 0.28, 0.38 , 0.33, 0.24, 0.28 respectively), normal morphology (n=159; $P<0.05$; r=0.34, 0.63, 0.75, 0.57, 0.41, 0.54 respectively) and normal plasma membrane (n=159; $P<0.05$; r=0.34, 0.63, 0.76, 0.59, 0.43, 0.58 respectively). Therefore, these results indicated that lipid composition of boar sperm can influence sperm motility, viability, normal morphology and plasma membrane integrity.

EXP II was conducted to compare viability, lipid peroxidation, fatty acid composition of boar spermatozoa and antioxidant capacity of seminal plasma between >50% (n=10) and <50% (n=10) of sperm motility groups at 24 h of cool storage. Semen was collected from all boars once a week for 3 wks and the semen evaluated for motility, sperm concentration, and total number of sperm, percentage of normal morphology and membrane permeability, and viability. Sperm was extracted and analysed for lipid profiles, lipid peroxide and total antioxidant was determine in the extender at 0 and 24 h. The results demonstrated the decreasing of total lipid, proportion of cholesterol in sperm membranes, TAS and showed that increasing of LPO level have a cause in reduction in motility, viability, membrane permeability and hence, storability of sperm

EXP III was conducted to determine the effect of fish oil, vitamins and selenium on-top feed supplemented on boar spermatozoa lipid composition and semen quality. Twenty one boars were assigned for this experiment. All boars were randomly divided into three experimental groups: 1) supplemented diet for 8 weeks (n=7); 2) supplemented diet for 16 weeks (n=7) and 3) control (n=7). Fish oil (40 ml), vitamin E (480 iu), vitamin C (2,400 mg) and selenium (0.3 mg) were given once a day by on-top feeding. The semen was collected from all boars using the glove-hand method once a week starting from 7 weeks prior to the supplementation and continues for a total of 23 collections per boar. Semen was evaluated and centrifuged to separate the sperm from seminal plasma and kept at -20°C until analyzed sperm pellet for lipid content, FA profile. The seminal plasma was analyzed for total antioxidant status and glutathione peroxidase (GPX) using a commercial kit. The results indicated that, when compared among the control group and groups supplemented with fish oil for 8 and 16 weeks, the number of total sperm (70.84 vs. 71.69 and 71.35 $x10^9$ sperm respectively; $P<0.05$), semen volume (290.14 vs. 341.60 and 326.24 ml respectively; $P<0.05$), Proportion of DHA (14.91 vs. 16.21 and 16.16% respectively; $P<0.05$), and total *n-3* (14.12 vs. 16.82 and 16.95% respectively; $P<0.05$) in sperm composition were increased in boar fed a supplemented diet. Duration of ejaculation (374.82 vs. 439.01 and 419.30 sec respectively; $P<0.05$) was longer and glutathione peroxidase in seminal plasma (1.22 vs. 1.45 and 1.51 mmol/ml respectively; $P<0.05$) was improved, in the boars fed a diet supplemented diet comparing with the control group.

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

Field of Study: Theriogenology Advisor's Signature.....

Academic Year: 2010 Co-Advisor's Signature.....

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CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.3 Introduction

The improvements of reproductive performances in boars having the good genetic are focused on semen preservation for intensification of boar utilization and reduction of the cost. Thus, any methods for preserving sperm should be developed. Methods, which improve fluidity and stability of plasma membrane, are ones of all. The sperm plasma membrane has been demonstrated to contain a high content of 20 (C20) and 22 (C22) carbon atoms of polyunsaturated fatty acids (PUFAs). Phospholipids of mammalian sperm cell membranes characteristically contain very high proportions of long-chain (C22) polyunsaturated fatty acids, particularly *n-3* series. In most mammals, docosahexaenoic acid (DHA, 22:6*n-3*) is the dominant polyunsaturated fatty acid, although, in several species docosapentaenoic acid (DPA, 22:5*n-6*) is also a major component of the sperm cell membranes (Alvarez and Storey, 1992; Cerolini et al., 2000; Mazur et al., 2000; O'Flaherty et al., 1997; Park and Graham, 1992). The importance of C22 polyunsaturates in relation to male fertility has been illustrated by studies in humans demonstrating that the amount of DHA in spermatozoa is positively correlated with sperm motility (Conquer et al., 1999; Nissen and Kreysel, 1983; Zalata et al., 1998). Moreover, PUFAs which are concentrated in the head and tail membrane regions of spermatozoa have been shown to play an important role in both sperm capacitation and the interaction between spermatozoa and uterine surface environment (Conquer et al., 1999; Zalata et al., 1998). However, the sperm plasma membranes are susceptible to lipid peroxidation because of the high content of PUFA (Miller et al., 1993; O'Flaherty et al., 1997; Sprecher, 1989). In order to prevent the weakness on low sperm motility and the damage from lipid peroxidation process, and to enhance the fertilizing capacity, the methods of increasing the level of PUFAs in the plasma membrane of spermatozoa and decreasing the lipid peroxidation of plasma membrane were developed. One of those methods is the diet supplemented with DHA to increase -

number and lifespan of spermatozoa (Estienne et al., 2008; Strzezek et al., 2004). Vitamin E, C and selenium have been added to the diet to reduce the lipid peroxidation (Audet et al., 2004). Nonetheless, a number of literatures reported no effect (Rooke et al., 2001), while some diet supplement products for increasing the fertility of boars are commercialized. Enhancing the sperm production and viability after long-term storage is contributed to the development of artificial insemination technology. Thus, effect of PUFAs and DHA, together with antioxidant in the diet, on sperm motility and storability of boar semen should be investigated. This thesis consisted of three studies. First study, the semen was collected from the boars having normal motility >60% and low motility <60% to compare lipid composition in the semen, which were used as basic information in boars. Second study, the differences of lipids, lipid peroxide and total antioxidants status in semen after cool storage at 18ºC for 24 h were investigated the alteration of lipids and antioxidants in semen during cooling period. Third study, fish oil, vitamins and selenium were supplemented on boar feed to investigate the alteration of boar sperm lipid composition and semen quality.

1.4 Literature Review

1.2.1 Structure of plasma membrane lipid composition

The plasma membrane consists of three classes of amphipathic lipids: glycolipids, phospholipids and cholesterols. The amount of each depends upon the type of cell, but in the main of cases phospholipids are the most abundant. Polyunsaturated fatty acids (PUFA) in phospholipids and glycolipids usually contain an even number of carbon atoms, typically between 16 and 20. The 16- and 18-carbon fatty acids are the most common. Fatty acids may be saturated or PUFA, with the configuration of the double bonds nearly always *cis*. The length and the degree of PUFA chains have a profound effect on membrane fluidity and flexibility. The ability of some organisms to regulate the permeability of their cell membranes by altering lipid composition is called homeoviscous adaptation. The whole membrane is held together via non-covalent interaction of hydrophobic tails; however, the structure is quite fluid and not fixed rigidly

in place. Under physiological conditions, phospholipid molecules in the plasma membrane are in the liquid crystalline state. It means the lipid molecules are free to diffuse and exhibit rapid lateral diffusion along the layer where they are demonstrated. However, the exchange of phospholipid molecules between intracellular and extracellular of the bilayer is a very slow process. Lipid rafts and caveolae are examples of cholesterol-enriched microdomains in the cell membrane. In animal cells cholesterol is normally found dispersed in varying degrees throughout cell membranes, in the irregular spaces between the hydrophobic tails of the membrane lipids, where it confers stiffening and strengthening effect on the membrane (Alberts et al., 2010).

1.2.2 Incorporation of polyunsaturated fatty acids in spermatogenesis and maturation

The seminiferous tubules consist of many different cell types with different phospholipid classes and fatty acid compositions. Rat germ cells contained mostly phosphatidylcholine (PC) and phosphatidylethanolamine (PE), whereas Sertoli cells had a more evenly distributed phospholipid pattern. A greater proportion of the phospholipid of rat Sertoli cells than of germ cells was composed of phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin and lysophosphatides. The fatty acid profiles of phospholipids of rat Sertoli cells contained less 16:0 and 22:5*n-6* and more 18:1, 20:4*n-6*, and 22:4*n-6* than did the corresponding phospholipids of the germ cells (Beckman and Coniglio, 1979). These findings were also consistent with data from studies of the lipid composition of separated rat testicular germ cell types, which showed that PE and PS contained a high concentration of 22:5*n-6* (Beckman et al., 1978). Also, the fatty acid patterns of the total lipids of separated cell fractions enriched in rat Sertoli cells or germ cells have previously been studied. The fractions enriched in rat Sertoli cells contained less 22:5*n-6* and 22:6*n-3* than did the germ cell fractions. A 24-carbon PUFA were only detected in the triacylglycerols, but not in the phospholipids (Beckman and Coniglio, 1979; Beckman et al., 1978), and were present in higher concentrations in triacylglycerols in germ cells than in Sertoli cells (Beckman and Coniglio, 1979). *In vitro* studies have shown that after incubation of ^{14}C-labeled 22:4*n-6*

or 22:5n- 3 with isolated human testicular cells, higher amounts of these fatty acids were esterified into triacylglycerols than in phospholipids. With 20-carbon *n-3* and *n-6* as the substrates more 20:5*n-3* and 20:4*n-6* was esterified into phospholipids than in triacylglycerols (Retterstol et al., 2001). In the phospholipid fraction more fatty acid substrates were esterified into PC than in PE, PS or PI. In cultures of cell preparations enriched in rat Sertoli cells most of the incubated 18:2*n-6*, 18:3*n-3*, 20:3n-9, 20:4*n-6*, and 20:5*n-3* were incorporated into PC and PE. The metabolites, 22:5*n-6* and 22:6*n-3*, were esterified into triacylglycerols, cholesterol esters, and phospholipid classes PC and PE (Retterstol et al., 1998). After incubation of Sertoli cells with 20- and 22-carbon *n-3* and *n-6* PUFA, more *n-6* than *n-3* fatty acids were esterified into phospholipids, and fewer *n-6* PUFA than *n-3* PUFA were esterified into triacylglycerols. With high substrate concentrations, high amounts of 20- and 22-carbon *n-3* and *n-6* PUFA were esterified into PC, and small amounts of these PUFA were esterified into PE, PI, or PS (Retterstol et al., 2000).

The spermatozoa have to undergo a complex process of maturation in the seminiferous epithelium and the epididymis (Amann et al., 1993; Jones, 1989). This process involves a broad range of events, including the remodeling of membrane components (Jones, 1989) leading to the acquisition of motility (Esponda, 1991; Hegde, 1996) and the ability to undergo the acrosome reaction (Alvarez and Storey, 1995; Toshimori, 1998; Yeung et al., 1996), that require an overall increase in membrane fluidity (Flechon, 1985; Myles et al., 1987). Therefore, polyunsaturated fatty acids, and especially docosahexaenoic acid (DHA), are thought to play a key role in regulating membrane fluidity and sperm function. The process of spermatogenesis takes place in the seminiferous epithelium of the testis and is closely regulated and coordinated by the Sertoli cell (de Kretser et al., 1998; Griswold, 1998; Griswold et al., 1988; Kerr, 1995). This paracrine process is synergistically regulated by testosterone and follicle stimulating hormone (FSH) (McLachlan et al., 2002; Tesarik et al., 1998; Weinbauer and Nieschlag, 1997). Regulation of spermatogenesis involves genetically programmed changes in chromatin structure and gene expression in the developing germ cells

(Escalier, 2001; Grootegoed et al., 1995; Grootegoed et al., 2000; Wang et al., 1997). The disruption of this double regulation system could lead to alterations in the development of sperm cell function. Spermatogenesis in the seminiferous epithelium is conventionally divided into stages and steps. Each stage contains a specific set of cell types (e.g., spermatogonia, primary and secondary spermatocytes and round spermatids). The number of these stages varies significantly in a species-specific manner. For example, spermatogenesis in the rat involves 14 stages, whereas 6 stages are involved in the human. The process of differentiation of a round spermatid into a differentiated spermatozoon, known as spermiogenesis, involves 19 steps in the rat, whereas the same process is completed in the human in only 8 steps. In the mouse and Rhesus monkey, spermiogenesis spans a total of 16 steps, whereas in dog, stallion, and bovine sperm it spans 12 steps (Clermont, 1963). The remodeling of the sperm plasma membrane, which occurs during sperm passage through the epididymis, is believed to be crucial in the acquisition of motility and the ability to penetrate and fertilize the egg (Esponda, 1991; Jones and Orcutt, 1998). This remodeling process includes the uptake of secreted epididymal glycoproteins, removal or utilization of specific phospholipids from the inner leaflet of the bilayer, processing of existing or acquired glycoproteins by endoproteolysis, and repositioning of both protein and lipid molecules to different membranes (James et al., 1999; Jones and Orcutt, 1998). These modifications are carefully coordinated at different zones of the epididymis and indirectly affect intracellular membranes, organelles and even nuclear components (Jones and Orcutt, 1998). Lipid composition of the plasma membrane may be a key in the interaction of spermatozoa with the epididymal environment. Secretion by epididymal cells of lipid-binding proteins may mediate changes in sterol content of particular membrane domains. The contact of sperm cells with membranous vesicles and merocrine secretions may be crucial in the development of physiological changes (Cooper, 1998; Kirchhoff et al., 1997).

Since DHA is essential in maintaining membrane fluidity leading to the acquisition of motility and the zona-induced acrosome reaction, mammalian sperm have

been forced during evolution to develop a powerful enzymatic antioxidant defense system, involving superoxide dismutase, glutathione peroxidase, and phospholipase A2 (Alvarez et al., 1987). Most of the studies dealing with the analysis of fatty acid content in sperm and germ cells have been performed on experimental models, especially in rodents. It is worth noting that there are interspecific differences in the relative importance of polyunsaturated fatty acids, and the identity of the most abundant polyunsaturates. DHA, or 22:6*n-3*, is the predominant polyunsaturated fatty acid in sperm from humans and ruminants, whereas rodent and rabbit sperm contain mostly the *n-6* docosapentaenoic acid (DPA, 22:5*n-6*) (Poulos et al., 1973). In an early study performed in the mouse, Grogan *et al.* (1983) isolated different populations of germ cells by light scattering sorting and assessed their lipid content. They found that 22:5*n-6* (the most abundant polyunsaturate) increased progressively from 2 to 20% of total fatty acid in the progression of germinal cell differentiation from preleptotene spermatocyte to condensing spermatid but decreased in mature sperm. Its precursor, arachidonic acid (20:4*n-6*), showed a roughly reciprocal relationship, whereas 22:6*n-3* showed no significant correlation with cell type. 22:5*n-6* was found highest in triglycerides at later stages of differentiation, whereas 20:4*n-6* and 22:6*n-3* were found primarily in phospholipids from all cell fractions. The same authors showed that the *n-6* polyunsaturated fatty acids of the germinal cells labeled with radioactive acetate contained levels of radioactivity in each lipid class that were consistent with the importance of long-chain polyenoic fatty acid metabolism in germ cell function. Cells at later stages of maturation incorporated much higher levels of radioactivity into fatty acids derived from 20:4*n-6* by elongation desaturation pathways than did less mature cells or whole testis *in vitro* (Grogan and Huth, 1983). Therefore, germ cells reach the epididymis containing the biochemical tools necessary for polyunsaturated fatty acid biosynthesis. However, the changes that sperm undergo in their membrane components during epididymal transit seem to be mostly due to redistribution rather than to acquisition of new components. Thus, immature spermatozoa of the caput epididimys show higher ability to incorporate labeled fatty acids compared with mature

spermatozoa from the cauda (Srivastava and Olson, 1996). Nevertheless, a change of the typical asymmetric pattern of membrane phospholipids has been attributed to postgonadal maturation of sperm. A marked decrease in the hosphatidylethanolamine/phosphatidylcholine ratio in the inner membrane has been reported (Rana et al., 1993), as well as profound changes in the distribution of saturated and unsaturated phospholipid-bound fatty acids. Also, depletion of components has been reported. Aveldano *et al.* (1992) described a decrease in phosphatidylcholine and phosphatidylethanolamine in rat epididymis, leading to a relative increase in plasmenylcholine, which becomes the main phospholipid. Oleate and linoleate are decreased, whereas the proportion of longer chain and more unsaturated fatty acids is increased. The most relevant change corresponds to the relative increase in n-9 polyenes, accounting for more than half of the acyl chains present in plasmenylcholine of cauda spermatozoa (Aveldano, 1992; Aveldano et al., 1992). In an early study, Adams *et al.* (1977) showed how spermatozoa from the caput epididymis had a significantly greater content of phospholipid, cholesterol, cholesterol ester, and free fatty acid than those from the cauda epididymias. Spermatozoa from the corpus epididymis had a significantly greater content of monoglyceride than those from the caput epididymis and a greater content of phospholipid, cholesterol, free fatty acids, and monoglyceride than those from the cauda epididymis (Adams and Johnson, 1977). In the ram and in the boar, the species containing the highest proportion of polyunsaturated fatty acids in mature spermatozoa (Poulos et al., 1973), a net loss in phospholipid content and phospholipid-bound fatty acids has been described (Evans and Setchell, 1979). This decrease in phospholipid content seems to be due to phosphatidylethanolamine, resulting in an increase in the hosphatidylcholine/phosphatidylethanolamine ratio, as well as in the cholesterol/phospholipid ratio, whereas desmosterol becomes negligible after maturation. The relative predominant fatty acids switch from palmitic in the less mature to DHA on the more mature spermatozoa (Parks and Hammerstedt, 1985). In a more recent study performed in the epididymal sperm of human patients of prostate

carcinoma, Haidl and Opper (1997) found that the ratio between phosphatidylcholine and phosphatidylserine plus phosphatidylethanolamine plus sphingomyelin was significantly higher in spermatozoa from the cauda compared to those from the caput and corpus. This was attributed to both an increase of phosphatidylcholine and a decrease of other phospholipids. With regard to fatty acids, those with saturated chains predominated in caput spermatozoa, whereas the highest concentration of unsaturated longchain fatty acids was found in cauda spermatozoa. In another study using rat epididymal sperm, 56% of the phospholipid consisted of choline and ethanolamine phosphoglycerides; the remainder consisted of sphingomyelin, phosphatidylserine, and diphosphatidylglycerol. The mole percent of phosphatidylethanolamine increased in sperm proceeding from the caput to the corpus epididymis and then declined from the corpus to the cauda epididymis. The phospholipid-bound fatty acids consisted primarily of palmitate and stearate (saturated), with a significant increase in the mole percent 22:5*n-6* in cauda sperm (Hall et al., 1991). More recently there were analysis of the fatty acid content of cells isolated from the seminiferous tubules and the epididymis of adult mice, and they found that the content in both 22:5*n-6* and 22:6*n-3* is higher in cells from seminiferous tubules than from epididymis. The total fatty acid content was found to be 6-fold higher in cells from the seminiferous tubules. Since the decrease in fatty acid content per cell in epididymal sperm, compared to cells from the seminiferous tubules, was much more pronounced than the decrease in 22:6*n-3* content, when DHA was expressed as the percent of total fatty acid, there appeared to be an apparent increase in DHA in epididymal sperm (Ollero et al., 2000).

1.2.3 Effect of Reactive Oxygen Species (ROS) on semen quality

Boar spermatozoa are sensitive to peroxidative damage due to the high content of unsaturated fatty acids in the phospholipids of the plasma membrane (Cerolini et al., 2000; Park and Graham, 1992) and the relative low antioxidant capacity of boar seminal plasma (Brezezinska-Slebodzinska et al., 1995). It has been demonstrated in human (Alvarez and Storey, 1992), bull (O'Flaherty et al., 1997) and mouse spermatozoa (Mazur et al., 2000) that cryopreservation is associated with oxidative stress. Moreover, freezing

and thawing of bovine spermatozoa increase the generation of reactive oxygen species (ROS) (Chatterjee and Gagnon, 2001), DNA damage (Lopes et al., 1998), cytoskeleton alterations (Hinshaw et al., 1986), inhibition of the sperm–oocyte fusion (Aitken et al., 1989) and can affect the sperm axoneme that is associated with the loss of motility (de Lamirande and Gagnon, 1992[a]).

The oxidation of membrane phospholipid bound docosahexaenoic acid (DHA) has been shown to be one of the major factors that limit the motile lifespan of sperm *in vitro*. Sperm samples show high cell-to-cell variability in lifespan and, consequently, in susceptibility toward lipid peroxidation. Therefore, it is postulated that there is also cell-to-cell variability in DHA content in human spermatozoa and that the content of the main substrate of lipid peroxidation (DHA) is critical and highly regulated during the sperm maturation process. Several studies have been performed to analyze the fatty acid content of germ cells and sperm at different stages of maturation, including *in vivo* studies in animal models, and *in vitro* approaches in human spermatozoa. One of the consequences of defective sperm maturation in the seminiferous epithelium is the retention of residual cytoplasm. This residual cytoplasm, which is attached to the midpiece and retronuclear area of the sperm head, has been shown to produce high levels of reactive oxygen species (ROS) (Aitken et al., 1994; Gil-Guzman et al., 2001; Gomez et al., 1996). In addition, the membranes enclosing the residual cytoplasm are enriched in polyunsaturated fatty acids such as DHA (Huszar and Vigue, 1993; Ollero et al., 2000). The combination of high polyunsaturated fatty acid content and high ROS production in these immature sperm has been shown to lead to increased lipid peroxidation and subsequent loss of sperm function (Aitken et al., 1994; Gil-Guzman et al., 2001). ROS-mediated damage to human spermatozoa was characterized in the early 1980s (Alvarez et al., 1984; Alvarez and Storey, 1982, 1984[a,b], 1995; Alvarez et al., 1987) and has been shown by many authors to be an important factor in the pathogenesis of male infertility (Aitken and Fisher, 1994; Aitken et al., 1994; de Lamirande and Gagnon, 1994; Sharma and Agarwal, 1996). To a first approximation, the process of lipid peroxidation involves the initial abstraction of a hydrogen atom from the bis-allylic

methylene groups of polyunsaturated fatty acids, mainly DHA, by molecular oxygen. This leads to molecular rearrangement to a conjugated diene and addition of oxygen, resulting in the production of lipid peroxide radical. This peroxyradical can now abstract a new hydrogen atom from an adjacent DHA molecule leading to a chain reaction that ultimately results in lipid fragmentation and the production of malonaldehyde and toxic shortchain alkanes (*e.g.,* propane). These propagation reactions are mediated by oxygen radicals. DHA is the major polyunsaturated fatty acid in sperm from a number of mammalian species, including the human (Alvarez and Storey, 1995; Poulos et al., 1973; Zalata et al., 1998), accounting in this species for up to 30% of phospholipid-bound fatty acid and up to 73% of polyunsaturated fatty acids (Alvarez and Storey, 1995). At the same time, DHA is the main substrate of lipid peroxidation, accounting for 90% of the overall rate of lipid peroxidation in human spermatozoa (Alvarez and Storey, 1995). Lipid peroxidation has profound consequences in biological membranes. The generation of the polar lipid peroxides ultimately results in the disruption of the membrane hydrophobic packing, inactivation of glycolytic enzymes, damage of axonemal proteins (loss of motility), acrosomal membrane damage, and DNA alterations (Alvarez et al., 2002; Fraga et al., 1991). Oxidation of phospholipid-bound DHA has been shown to be the major factor that determines the motile lifespan of sperm *in vitro* (Aitken et al., 1993[a]; Alvarez and Storey, 1995; Jones et al., 1979). Three basic factors determine the overall rate of lipid peroxidation of sperm *in vitro*: oxygen concentration and temperature in the medium (OXIDANT), the presence of antioxidant defenses (ANTIOXIDANT), and the content of membrane-bound DHA (SUBSTRATE). Thus, the higher the temperature and the concentration of oxygen in solution, the higher the rate of lipid peroxidation as measured by malonaldehyde production (Alvarez et al., 1987). The balance between these key factors determines the overall rate of peroxidation *in vitro*. In this system, the substrate seems to play a key role. The main substrates for lipid peroxidation are polyunsaturated fatty acids, especially docosahexaenoic acid.

1.2.4 Effect of vitamins and selenium on semen quality

Selenium is an essential component of the enzyme glutathione peroxidase. Glutathione peroxidase is a major intracellular antioxidant that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to nontoxic compounds. Vitamin E (alpha-tocopherol) is the major antioxidant in cell membranes. Numerous experiments have demonstrated an antioxidant synergism between glutathione peroxidase (selenium) and vitamin E. The effects of added selenium on semen characteristics were more pronounced than the effects of added vitamin E, and selenium supplementation resulted in greater fertilization rates when gilts were bred with semen from the experimental boars (Marin-Guzman et al., 1997). However, Audet and co-workers (2004) concluded that supplementation of boar diets with high levels of vitamin C; fat soluble vitamins or water soluble vitamins had no appreciable effects on semen or libido characteristics in boars. It should be noted that current U.S. Food and Drug Administration (FDA) regulations allow up to 0.136 mg of added selenium/pound of diet for all pigs. The immune system is one example of where this synergism is most easily observed. Wuryastuti and co-workers (1993) found that vitamin E deficient sows had a significantly low immune response to these mitogens. However, when both selenium and vitamin E were deficient, impairment of mitogenic stimulation occurred even earlier, compared to those sows that were only deficient in vitamin E. These data suggested that optimum immune response required adequate levels of both selenium and vitamin E. In this same review, it was reported that selenium and vitamin E independently increased antibody titers in sheep exposed to the para-influenza3 virus. Oxidative stress may be the link that explains the interactions between animals stress, nutritional deficiencies and decreasing of disease resistance, which often observed in the field. In cattle, the production of reactive oxygen metabolites can be increased by the consumption of Maillard reaction products, mycotoxins, and endophyte-infected tall fescue. Unbound or “ill-place” iron can increase the production of reactive oxygen metabolites. Miller and co-workers (1993) showed that inflammation, infections and environmental stresses might encourage the formation of ill-placed iron. Under these conditions, the need for

antioxidants is increased. If animals are marginally deficient in selenium and/or vitamin E, the responsiveness of their immune system is impaired. These animals are more likely to become sick and/or dead, while animals with a strong immune system may not show any symptoms. In the past few years, there has been an effort to increase the energy density of animal diets. With calorie-dense diets animal performance is maximized, feed efficiency is improved and less animal waste is generated. Fats additions are a common means of increasing caloric density. However, recent data suggested that adding certain types of fat can increase the requirements for antioxidants. Beck and co-workers (1994) conducted a study in mice that the diets were supplemented with lard (saturated fat) or menhaden fish oil (unsaturated oil). The diets were supplemented with 0.2 ppm of selenium and 38.4 mg/kg of d-alpha tocopheryl actate. These levels of selenium and vitamin E are considered to adequate for mice, but they are not supernutritional levels. Diets deficient in vitamin E, selenium, or both were also fed. After being fed the test diets for four weeks, the mice were exposed to a virus that caused lesions in the heart. Both selenium and vitamin E deficiency increased the cardiac muscle damage. However, the most severe lesions were seen in mice fed the menhaden fish oil diets deficient in both selenium and vitamin E. No lesions were observed in mice fed diets that were adequate in selenium and vitamin E. Unsaturated fatty acids like those in fish oil increase the need for antioxidants in the diet. When animals are in a “marginal antioxidant status”, the type of fat source added to the diet may affect an animal’s susceptibility to disease. Large-scale animal production units are constantly seeking ways to minimize stress and enhance immune function. Antibiotic feeding has been used in many production settings to control subclinical diseases. Although this has been effective in the past, the real dangers of continued use are still controversial. It is likely that this practice were increasingly regulated in the future. Consequently, maximizing the immune status of the animal is critical to optimum health and longevity. Antioxidant concentrations in the diet, especially selenium and vitamin E, were increasing emphasized. Feeding a trace mineralized salt and vitamin premix that have been properly fortified with selenium and vitamin E respectively; is essential to maintaining animal health and productivity

Ascorbic acid (vitamin C), a major water-soluble antioxidant, acts as scavenger for a wide range of ROS. Ascorbic acid is a powerful electron donor that reacts with superoxide, peroxide and hydroxyl radicals to form dehydroascorbic acid. It is also present at approximately 10-fold higher concentration in seminal plasma compare with blood plasma in human (Fraga et al., 1991; Lewis et al., 1997) suggesting a physiological role in seminal plasma. Further, ascorbic acid concentration in seminal plasma are positively related to the percentage of morphologically normal spermatozoa (Thiele et al., 1995)

1.2.5 Effect of polyunsaturated fatty acids supplementation on semen quality

Linoleic acid (an omega-6 fatty acid) is the only fatty acid for which NRC has established requirements for sexually active boars (0.1% of diet). However, the effect of dietary supplementation of various fatty acids, particularly the omega-3 fatty acids, on semen and libido characteristics in boars has increasing interest by swine researchers. The omega-3 fatty acids are linolenic, eicosapentaenoic (EPA) and docosahexaenoic (DHA). The boar diets commonly contain large amounts of cereals, with most supplementary protein being added in the form of oilseed meals such as soya-bean. Thus, dietary fatty acids have a (*n-6*):(*n-3*) ratio of greater than 6:1 and do not contain long chain polyunsaturated (*n-3*) fatty acids. If 22:6(*n-3*) is essential for optimal fertility in pig spermatozoa, as it is in human spermatozoa (Conquer et al., 1999; Nissen and Kreysel, 1983; Zalata et al., 1998), then it is possible that boar diets provide a suboptimal supply of 22:6(*n-3*) for spermatogenesis. This suboptimal supply may arise from either a deficit of (*n-3*) fatty acids per se or a reduced synthesis of 22:6(*n-3*) from 18:3(*n-3*) owing to competition between (*n-6*) and (*n-3*) fatty acids for desaturase enzymes (Sprecher, 1989). Supplementing the diet of the boars with tuna oil increased the proportion of viable sperm cells and the percentages of sperm cells with progressive motility, normal acrosome morphology, and normal morphology (Rooke et al., 2001). Furthermore, when pregnant sows were offered diets containing either 18:3(*n-3*) or 22:6(*n-3*), feeding 18:3(*n-3*) did not result in increases of 22:6(*n-3*) in sow adipose tissue or piglet tissues at birth, indicating a limited biosynthetic capacity of 22:6(*n-3*) to 18:3(*n-*

3). To date, studies in which fish oil has been fed to boars (Paulenz et al., 1995) have not improved fertility, although these studies were carried out against a background of good fertility. PROSPERM® (Minitube America, Inc., Minneapolis, MN) is a commercially available product that contains DHA, vitamin E and selenium. In a commercial field trial thirty-five boars were fed diets with or without PROSPERM for 16 weeks. Sperm concentration (502 million for control, 584 million for supplemented), number of sperm/ejaculate (74.1 billion for control, 83.4 billion for supplemented), and sperm motility score (3.9 for control, 4.5 for supplemented) were increased by PROSPERM. Four hundred, seventy-eight gilts were mated via artificial insemination with boars that received the supplement or those that did not. Significant improvements were demonstrated for conception rate (83% for control, 90% for supplemented) and number of pigs born alive (10.2 for control, 10.6 for supplemented). Remaining to be determined is the relative contribution of each of the components of PROSPERM (DHA, vitamin E and selenium) toward the overall positive effect on reproduction (Strzezek et al., 2004). In many experiments, 8-week period was used as the control period because spermatogenesis in boars requires 34–39 d and epididymal transport involves another 9–12 d (Swierstra, 1968). It is not surprising that a 7–8 week period may be necessary after dietary supplementation (Estienne et al., 2008; Strzezek et al., 2004).

1.3 Objective of the thesis

1. To investigate proportion of fatty acid (FA) composition of spermatozoa from the boars which have normal or low sperm motility.
2. To evaluate the relationship between conventional sperm parameters and FA composition in these boars.
3. To investigate the level of lipid composition and lipid peroxide in spermatozoa and seminal plasma at 0 and 24 hours after cooling.
4. To investigate the total antioxidants in seminal plasma at 0 and 24 hours after cooling.

5. To compare the total antioxidants in seminal plasma at 0 and 24 hours cooling between the semen which has normal storability and low storability.
6. To investigate the effect of fish oil supplemented on lipid composition of boar spermatozoa.
7. To investigate the effect of vitamins and selenium supplemented on the total antioxidant status in seminal plasma.
8. To investigate the effect of fish oil, vitamins and selenium supplemented on semen quality.

1.4 Hypothesis

1. The boar with ejaculated normal motility sperm has higher level of polyunsaturated fatty acids than boar with low motility sperm.
2. There are the differences of lipids, lipid peroxide and total antioxidants status in semen after cooling.
3. Fish oil, vitamins and selenium on-top feed supplemented can change the ratio boar spermatozoa lipid composition and improve semen quality.

1.5 Keywords: sperm, plasma membrane, polyunsaturated fatty acids, feed supplementation, boars

1.6 Research benefits

1. The information of FA composition of spermatozoa from the boars which have >60% and <60% sperm motility.
2. Knowledge about changes of the level of lipids, lipid peroxide and antioxidants in semen after preservation.
3. Knowledge about the effect of fish oil supplementation on lipid composition of boar spermatozoa.
4. Knowledge about the effect of fish oil, vitamins and selenium supplementation on semen quality.

5. Knowledge about the effect of vitamins and selenium supplemented on the antioxidant status in seminal plasma.

CHAPTER II

RRELATION BETWEEN LEVEL OF POLYUNSATURATED FATTY ACIDS AND SEMEN QUALITY IN BOAR

2.1 Abstract

Sperm plasma membrane lipids have an important role in membrane fluidity and sperm motility. The objective of the present study was to determine whether there are differences in lipid and fatty acid (FA) composition of boar sperm and seminal plasma in the ejaculates of boars having different sperm motilities. Semen was collected from normal (>60%; n=53) and low (<60%; n=53) motility boars and evaluated for motility, morphology and vitality. Semen was then centrifuged to separate sperm from seminal plasma and both kept at -20°C until analyzed for lipid content and FA profile by gas chromatography. Total antioxidant status (TAS) of seminal plasma was determined using a commercial kit. Comparing normal and low sperm motility boars, there were differences ($P<0.05$) in sperm total lipids, cholesterol, saturated fatty acids (SFA), phospholipids, *n-3* polyunsaturated fatty acids (PUFA), docosahexaenoic acid (DHA) and the ratio of *n-6*:*n-3* PUFA. There were positive correlations ($P<0.05$) between total lipids, cholesterol, phospholipids, PUFA, DHA and *n-3* PUFA with sperm motility, viability, normal morphology and normal plasma membrane. In contrast, SFA and ratio of *n-6*: *n-3* PUFA were negatively correlated ($P<0.05$) with sperm motility, viability, normal morphology and normal plasma membrane. The TAS of seminal plasma from normal motility boars was higher ($P<0.05$) than that of low motility boars and TAS was positively correlated ($P=0.0001$) with sperm motility, viability, normal morphology and normal plasma membrane. In summary, differences in sperm motility were related to *n-3* PUFA content in the sperm plasma membrane and extracellular antioxidants in seminal plasma, which protect sperm plasma membranes from lipid peroxidation during periods of oxidative stress.

2.2 Introduction

In sperm, lipid composition of the plasma membrane plays an important role in sperm membrane fluidity as well as sperm motility and viability (Ahluwalia and Holman, 1969; Miller et al., 2004). Boar sperm plasma membranes have a higher percent of *n-6* than of *n-3* polyunsaturated fatty acids (PUFA) (Ahluwalia and Holman, 1969; Rooke et al., 2001). The main fatty acid in boar sperm plasma membranes is docosapentaenoic acid (DPA) (Rooke et al., 2001) and levels did not change during storage and were not related to decreasing sperm motility. In contrast, boar sperm membrane docosahexaenoic acid (DHA) was decreased after storage and was positively correlated with sperm motility (Cerolini et al., 2000). Further, human sperm from asthenozoospermic, oligozoospermic and oligoasthenozoospermic men had a lower level of DHA and PUFA than those from normozoospermic men (Aksoy et al., 2006) and a high DHA content in both seminal plasma and sperm has been positively associated with sperm motility in men (Nissen and Kreysel, 1983; Zalata et al., 1998). DHA may contribute to membrane fluidity necessary for motility of sperm tails (Connor et al., 1998). Sperm total lipid content was positively correlated with sperm motility and viability while saturated fatty acids (SFA) (Aksoy et al., 2006) and *n-6* PUFA (Safarinejad et al., 2010) were negatively correlated with sperm motility and viability.

Plasma membranes of boar sperm are sensitive to lipid peroxidation (LPO) damage caused by reactive oxygen species (ROS) due to the high content of unsaturated fatty acids in the phospholipids (Alvarez and Storey, 1992; Cerolini et al., 2000; Parks and Lynch, 1992) and relatively low antioxidant capacity of boar seminal plasma (Brezezinska-Slebodzinska et al., 1995). The major source of ROS in seminal plasma is sperm metabolism (Cerolini et al., 2000) and these ROS may be associated with the time-dependant decreased motility, viability and membrane permeability observed during storage. In particular, an aromatic amino acid oxidase enzyme system activated following death of sperm has been identified as major source of ROS production in semen (Upreti et al., 1998) and ROS such as hydrogen peroxide are known to arrest sperm motility (Wales et al., 1959). It is therefore plausible that during

storage, an increasing level of ROS causes increased sperm membrane LPO and so decreased sperm motility. We hypothesize that relatively poor sperm quality is associated with an altered fatty acid composition of sperm and seminal plasma. The aim of the present study was to compare lipid content and fatty acid composition of boar sperm and seminal plasma derived from boars expressing normal and low sperm motility.

2.3 Materials and methods

2.3.1 Animals

The animal use and care committee of Chulalongkorn University, Thailand approved this study. The study employed 106 healthy boars (Duroc, n=78, Yorkshire, n=10 and Landrace n=18) of 1 to 3 yrs of age on 16 commercial pig farms in Nakhonpathom, Ratchaburi and Burirum provinces, Thailand. Immediately prior to the study, five ejaculates from each boar were evaluated for sperm motility immediately after collection and the boars then allocated to groups having normal motility (>60%; n=53) and low motility (<60%; n=53). Criteria for dividing the boars were 60% sperm motility which is acceptable motility of sperm used in pig farm for fresh semen.

2.3.2 Semen collection and evaluation

For all boars, semen was collected into pre-warmed (37°C) containers using the glove-hand method once a week for three weeks consecutively. Immediately after collection, semen was diluted 1:1 in BTS and motility determined twice under light microscopy at magnification ×100 on a prewarmed (37°C) slide and sperm concentration determined using a hemocytometer. Total sperm number was calculated using sperm concentration and ejaculate volume. Sperm morphology was evaluated using the William's staining method for head abnormality and the formal saline method for tail abnormality (Buranaamnuay et al., 2009). The minimum number of sperm counted per slide was 500 for head and 200 for tail assessments. Sperm viability was evaluated by eosin-nigrosin staining of a minimum of 300 sperm. Plasma membrane

permeability was evaluated by the HOS test (Jeyendran et al., 1984). At least 200 sperm were examined using a phase contrast microscope at magnification ×400 for evidence of tail swelling. Immediately following sperm evaluations, 30 ml of the semen was transported to the laboratory at 37°C within 2 hours. The sperm was separated from the seminal plasma by centrifugation at 700 ×g for 20 min at 4°C. The sperm was re-suspended in the same volume of normal saline and re-centrifuged. This process was repeated twice and the washed sperm and the seminal plasma were stored at –20°C until needed for analysis (Rooke et al., 2001).

2.3.3 Sperm lipid extraction and analysis

Total lipids were extracted from 3×10^9 sperm, and from seminal plasma, after homogenization in chloroform–methanol (2:1, v/v) (Christie, 1982). The lipids were classified into their major classes (phospholipid, free cholesterol, triacylglycerol, free fatty acids and cholesterol ester) by thin layer chromatography on silica gel G using a solvent system of hexane:diethyl ether:formic acid (80:20:1, v/v). After visualization under UV light following spraying with 0.1% (w/v) 2,7-dichlorfluorescein in methanol, the separated bands were scraped from the plates. Phospholipid was eluted from the plates by washing three times with 2 ml methanol, and the other lipid classes were eluted with diethyl ether. Free cholesterol was determined by a colorimetric assay. The fatty acid extract was saponified in 1.0 ml of methanolic 1 M NaOH for 45 min at 70°C and the fatty acids were then methylated by addition of 1.0 ml methanolic boron trifluoride (15 min at 70°C). The samples were allowed to cool to room temperature and 3.0 ml water added. Methylated fatty acids were extracted with diethyl ether, the ether removed under nitrogen and methyl esters dissolved in *n*-pentane for further analysis by gas-liquid chromatography (SP-2330 capillary column [SIS, Ringoes, NJ, USA], 120 m at 120 to 210°C, 4°C/min, He 1.0 ml/min) (Christie et al., 1970; Rooke et al., 2001). Fatty acids were identified according to their retention time compared to those of standards analyzed under the same conditions. Deuterated Chlorobenzene was used as internal

standard. Total lipids are expressed as μg/10^9 sperm and μg/ml seminal plasma. All lipid analysis was done at TCUG, Bangpli, Samut Prakan.

2.3.4 Total antioxidants in seminal plasma assay

An aliquot of 10 ml of freshly thawed seminal plasma was analyzed for total antioxidant status (TAS) with the use of the TAS Randox® kit (Cat No. NX2332, Randox laboratories, Ltd, Crumlin, UK) using a Sunrise Absorbance Reader (TECAN®, Austria). The linearity of calibration extended from to 0.025 to 2.5 mmol/l of Trolox. Inter and intra-assay coefficients of variation were 6.4% and 5.3%, respectively.

2.3.5 Statistical analysis

Statistical analysis was performed with SAS 9.2 (The SAS Institute Inc., Cary, NC, USA). Normal distribution of residuals from the statistical models was tested using the UNIVARIATE procedure option NORMAL. Since the sperm per ejaculate, normal plasma membrane and total lipid in sperm and seminal plasma did not follow a normal distribution, they were arcsine-transformed before inclusion in the model. Sperm parameters, lipid composition in both sperm and seminal plasma, and antioxidant capacity in seminal plasma, are presented as mean±SD. All parameters were subjected to analysis of variance, which was performed using the MIXED-procedure according to a statistical model including the fixed effect of breed (3), collection number (3) and the interaction between breed and collection number to compare differences between groups. Boars were used as random effect in the model. Correlations between variables were determined by using the Pearson correlation coefficient in case of normal distribution and by Spearman's rank correlation coefficient in case of non-normal distribution. The limit of significance was set at $P<0.05$.

2.4 Results

Boar age was 1 to 3 yrs and was not different between sperm motility groups ($P>0.05$). Total sperm produced per ejaculate was similar for each motility group ($P>0.05$) but when compared the low motility sperm group with the normal sperm

motility group. The latter group had higher (P<0.05) percentages of motility, viability, and normal membrane permeability as well as higher (P<0.05) percent normal morphology (Table 1).

Normal motility sperm had higher (P<0.05) total lipid content, percent phospholipids (P<0.05) and percent cholesterol (P<0.05) than those from low motility boars (Table 2). Regarding fatty acid compositions, normal motility sperm had higher (P<0.05) percentages of DHA and total *n-3* fatty acids (P<0.05) than those from low motility boars (Table 2). In contrast, normal motility sperm had lower percent saturated fatty acids, total *n-6*: *n-3* ratio and DPA: DHA ratio (P<0.05 for each; Table 2)

Table 1 Characteristics of normal and low motility boar sperm (Means ± SD).

Characteristics	Normal motility	Low motility
Sperm per ejaculate ($\times 10^9$)	88.6±41.7[a]	76.9±36.2[a]
Sperm motility, %	82.6±5.2[a]	30.6±12.8[b]
Sperm viability, %	86.7±5.8[a]	31.5±14.9[b]
Normal morphology, %	96.2±1.9[a]	85.1±4.9[b]
Normal plasma membrane, %	83.3±7.4[a]	15.7±7.5[b]

Rows with different superscripts (a,b) differ P<0.05

Table 2 Lipid and fatty acid profiles of spermatozoa from normal and low motility boar sperm (Means ± SD).

	Normal motility	Low motility
Total lipid, μg /10^9 sperm	166.21±18.35[a]	154.13±19.66[b]
Cholesterol, %	33.85±1.97[a]	30.20±2.48[b]
Cholesterol ester, %	1.44±0.19[a]	1.45±0.46[a]
Phospholipids, %	64.20±2.01[a]	60.78±2.48[b]
Phospholipid fatty acids (%)		
Saturated	26.66±1.94[a]	32.93±1.77[b]
Monounsaturated	12.11±1.53[a]	12.13±1.65[a]

18:2(*n-6*)	9.68±0.64 [a]	9.45±0.62 [a]
20:2(*n-6*)	2.16±1.31 [a]	2.14±1.54 [a]
20:3(*n-6*)	2.17±1.09 [a]	2.16±0.81 [a]
20:3(*n-9*)	1.08±0.89 [a]	1.02±0.81 [a]
20:4(*n-6*)	7.16±0.75 [a]	7.25±0.61 [a]
20:5(*n-3*) EPA	4.29±1.21 [a]	4.52±2.24 [a]
22:4(*n-6*)	6.33±0.66 [a]	6.25±0.65 [a]
22:5(*n-6*) DPA	13.59±1.80 [a]	12.76±2.17 [b]
22:6(*n-3*) DHA	15.46±4.06 [a]	10.23±3.63 [b]
Total (*n-6*)	41.10±2.86 [a]	40.01±3.16 [a]
Total (*n-3*)	19.75±4.11 [a]	14.75±3.67 [b]
Total (*n-6*):(*n-3*)	2.21±0.69 [a]	2.95±1.07 [b]
DPA:DHA	0.97±0.41 [a]	1.53±0.97 [b]

Rows with different superscripts (a,b) differ P<0.05

The lipid, fatty acid profiles and total antioxidant status of seminal plasma are presented in Table 3. Total lipid content and percent cholesterol were not different between motility groups. However, seminal plasma from normal motility boars had higher percentages of phospholipids, DHA and total *n-3* fatty acids (P<0.05), and lower ratios for total *n-6*: *n-3* and DPA: DHA (P<0.05) than seminal plasma from low motility boars. Additionally, seminal plasma from normal motility boars had a higher antioxidant total status than that from low motility boars (P<0.05)

Table 3 Lipid, fatty acid profiles and total antioxidant status (TAS) of seminal plasma from normal and low motility boar sperm (Means ± SD).

	Normal motility	Low motility
Total lipid, µg /ml	15.53±1.66 [a]	15.57±2.09 [a]
Cholesterol, %	44.21±3.27 [a]	44.06±4.19 [a]
Cholesterol ester, %	9.60±3.36 [a]	10.09±3.75 [a]

Phospholipids, %	39.91±5.64 [a]	34.28±5.97 [b]
Phospholipid fatty acids (%)		
Saturated	24.91±2.37 [a]	27.11±2.09 [a]
Monounsaturated	4.13±1.40 [a]	3.83±0.99 [a]
18:2(*n-6*)	1.83±0.80 [a]	2.11±1.44 [a]
20:2(*n-6*)	n/a	n/a
20:3(*n-6*)	n/a	n/a
20:3(*n-9*)	6.7±2.83 [a]	9.36±4.21 [a]
20:4(*n-6*)	2.21±1.31 [a]	2.26±1.24 [a]
20:5(*n-3*) EPA	1.42±1.98 [a]	1.53±2.84 [a]
22:4(*n-6*)	1.96±1.09 [a]	1.75±0.83 [a]
22:5(*n-6*) DPA	25.11±1.80 [a]	24.36±2.21 [a]
22:6(*n-3*) DHA	31.62±3.92 [a]	27.06±3.62 [b]
Total (*n-6*)	31.11±2.20 [a]	30.93±2.71 [a]
Total (*n-3*)	33.05±3.97 [a]	28.59±3.69 [b]
Total (*n-6*):(*n-3*)	0.96±0.15 [a]	1.11±0.20 [b]
DPA:DHA	0.81±0.13 [a]	0.92±0.19 [b]
TAS	1.54±0.35 [a]	0.80±0.56 [b]

Rows with different superscripts (a,b) differ P<0.05

Significant positive correlations (P=0.02 to 0.0001) were detected between measures of sperm quality (percentages of motility, viability, normal morphology, and normal plasma membrane permeability) and percentages of sperm total lipids, cholesterol, phospholipids, PUFA, DHA, the DPA: DHA ratio, total *n-3* PUFA (Table 4). Significantly negative correlations (P=0.009 to 0.0001) were evidences between the measures of sperm quality and SFA and ratio of total *n-6*: *n-3* PUFA (Table 4). Total antioxidant capacity of seminal plasma was positively correlated (P=0.003 to 0.0001) with measures of sperm quality (Table 4).

Table 4 Correlations between sperm parameter and lipid content from sperm and antioxidant capacity from seminal plasma

	Motility		Viability		Morphology (% normal)		Normal plasma membrane	
	r	P	r	P	r	P	r	P
Total lipids	0.34	0.0004	0.34	0.0004	0.16	0.1	0.53	0.0005
Cholesterol	0.63	0.0001	0.63	0.0001	0.28	0.004	0.60	0.0001
Phospholipids	0.64	0.0001	0.64	0.0001	0.34	0.0004	0.61	0.0001
SFA	-0.84	0.0001	-0.83	0.0001	0.41	0.0001	-0.81	0.0001
PUFA	0.76	0.0001	0.75	0.0001	0.38	0.0001	0.73	0.0001
DPA	0.14	0.2	0.15	0.1	0.03	0.8	0.12	0.2
DHA	0.59	0.0001	0.57	0.0001	0.33	0.0006	0.57	0.0001
DPA:DHA	0.56	0.0001	0.54	0.0001	0.30	0.0002	0.54	0.0001
Total (*n-6*)	0.10	0.06	0.12	0.2	0.04	0.7	0.09	0.4
Total (*n-3*)	0.43	0.0001	0.41	0.0001	0.24	0.02	0.42	0.0001
Total (*n-6*):(*n-3*)	-0.42	0.0001	-0.39	0.0001	-0.29	0.009	-0.41	0.0001
TAS	0.58	0.0001	0.54	0.0001	0.28	0.003	0.56	0.0001

2.5 Discussion

We were successful in our attempt to create populations of boars having relatively normal and poor measures of sperm quality, specifically percentages of motility, viability, morphologically normal sperm and normal sperm plasma membrane permeability. Although direct measures were not available, it is reasonable to suggest that fertility expectations would be lower for the low motility boars. Producers keep these low motility boars because they have been imported and are of superior genetic merit and so are perceived to have great value to their owners who, therefore, are very reluctant to cull them. The present results also clearly indicate an association between the conventional measures of sperm quality we employed and lipid composition of the washed sperm and their seminal plasma. In common with recent data from fertile and

infertile men (Safarinejad et al., 2010), we noted that measures of sperm quality were positively correlated with their *n-3* PUFA content and negatively correlated with their ratio total *n-6*; *n-3* PUFA content. Lipid content of sperm plasma membranes is known to correlate with sperm motility, viability and a functional plasma membrane (Ahluwalia and Holman, 1969; Aksoy et al., 2006; Conquer et al., 2000; Parks and Lynch, 1992; Rooke et al., 2001; Zalata et al., 1998), which is consistent with the positive correlation of sperm total lipid content with sperm motility, viability and normal plasma membrane noted in this study.

Cholesterol is an important component of the lipid fraction in the sperm plasma membrane, playing an important role in promoting sperm membrane permeability and fluidity in bulls (Beer-Ljubic et al., 2009; Moraes et al., 2009), stallions (Glazar et al., 2009; Oliveira et al., 2009) and rams (Moce et al., 2009). Fluidity and permeability of sperm plasma membranes contributes to the maintenance of their motility and viability (Beer-Ljubic et al., 2009; Glazar et al., 2009; Moce et al., 2009), which is consistent with our results showing the low motility sperm having a lower cholesterol content. It has been reported that boar sperm lose up to half of the cholesterol from their plasma membranes after cryopreservation (Cerolini et al., 2001). This is likely one of the causes of a premature capacitation-like effect seen in cryopreserved sperm cells. It is known that a loss of cholesterol from the plasma membrane is one of the first events in capacitation which decreases the stability of the membrane (Tulsiani et al., 1997). This premature capacitation-like state of is thought to be one reason why cryopreserved cells do not remain viable in the female reproductive tract as long as fresh sperm. Further, when boars were fed a diet supplemented with tuna oil, the cholesterol content of sperm was increased as was sperm motility, viability, and normal acrosomes (Rooke et al., 2001). In contrast, some study found no differences in sperm motility after fed the boars which have a good motility of sperm by omega-3 fatty acid. Perhaps, increasing of sperm motility depends on percentage of sperm motility before feed by supplementation (Estienne et al., 2008). The increment of antioxidants requirement after adding fat to the diet must be considered (Beck et al., 1994). Interestingly, with human sperm there was a

negative correlation of cholesterol content with sperm motility, viability, permeability and normal morphology (Zalata et al., 2010).

Sperm concentration and motility have been shown to decrease with age in bulls and this change in sperm quality was associated with reduced *n-3* PUFA, likely due to reduced activity of desaturase enzymes necessary for synthesis of long chain PUFA (Kelso et al., 1997). This is consistent with the non-age related lower *n-3* PUFA content of sperm and seminal plasma from the low motility boars in the present study. However, whether there is an age-independent causal relationship between lower enzyme activity, PUFA content, and sperm quality remains to be determined.

The phospholipids in the plasma membrane affect sperm function. In particular, DHA and other *n-3* PUFA are important for sperm development, motility and viability (Kelso et al., 1997; Safarinejad et al., 2010). DHA was decreased in sperm membrane phospholipids of asthenospermic men (Aksoy et al., 2006; Zalata et al., 1998) and this was related to oxidative damage (Comhaire et al., 2000). In the present study, phospholipids, PUFA and the *n-3* PUFA including DHA correlate positively with sperm motility, viability, normal morphology and normal plasma membrane. However, *n-6* PUFA are the main PUFA of sperm plasma membranes of boar (Rooke et al., 2001), bull (Moraes et al., 2009), poultry (Douard et al., 2000) and human (Aksoy et al., 2006; Zalata et al., 1998), but as shown in the current study and those of previous workers (Aksoy et al., 2006; Cerolini et al., 2000; Conquer et al., 1999; Douard et al., 2000; Rooke et al., 2001; Wathes et al., 2007; Zalata et al., 1998), *n-6* PUFA do not correlate with our measures of sperm quality. The present study did demonstrate that the ratio of *n-6*: *n-3* PUFA in phospholipids is negatively correlated with sperm quality, which agrees with data from human sperm (Aksoy et al., 2006).

The high PUFA content in sperm plasma membranes make them vulnerable to ROS-induced lipid peroxidation (Aitken et al., 1989; Aitken et al., 1993[a,b]; Alvarez and Storey, 1995; Awda et al., 2009). Sperm can be protected from lipid peroxidation by extra-cellular antioxidants in seminal plasma and, consistent with this, a low antioxidant capacity has been associated with human male infertility (de Lamirande and Gagnon,

1992[a]; Fraga et al., 1991; Khosrowbeygi and Zarghami, 2007; Lewis et al., 1997; Murawski et al., 2007). Previous reports showed that high level of antioxidants presented in boar seminal plasma are derived from low molecular weight fractions (Strzezek, 1999; Strzezek et al., 2002). Thus, a deficiency of these fractions can affect the overall protection of sperm from oxidative damage, which can have a negative effect on sperm motility and fertilization (Strzezek et al., 2004). The present data conform to this in that we note a lower total antioxidant status in seminal plasma derived from the low motility boars (Table 3).

In summary, differences in sperm motility were associated with the *n-3* PUFA content in sperm plasma membranes, which play an important role in sperm motility, viability and fluidity. Also, extra-cellular antioxidants in seminal plasma protect sperm plasma membrane from lipid peroxidation during oxidative stress. It is possible that in the event of lower sperm motility and fertility, supplementing semen with antioxidants or adding a source of *n-3* PUFA to the boar diet may alleviate the problem.

CHAPTER III

THE CHANGES OF THE LEVELOF LIPIDS, LIPIDPEROXIDE AND ANTIOXIDANTS IN BOAR SEMEN AFTER COOL STORAGE

3.1 Abstract

Lipids have an important role in membrane fluidity and sperm motility. Imbalance of antioxidants and Reactive Oxygen Species (ROS) can make lipid peroxidation (LPO) of the sperm membrane which plays an important role in damage and loss of function associated with sperm storage. The aims of this study were to investigate the changes in viability, lipid peroxidation, fatty acid composition in boar spermatozoa and antioxidant capacity comparing between normal (>50%) or low (<50%) sperm motility at 24 h. For the present study, 20 boars were pre-selected based on normal (>50%) or low (<50%) sperm motility at 24 h after collection and each normal boar paired by age with a low motility boar. All boars were housed under the same conditions. Semen was collected from each boar once a week for 3 wks and the semen evaluated for motility, sperm concentration, and total number of sperm, percentage of normal morphology and membrane permeability, and viability. Additionally, sperm motility, membrane permeability and vitality were assessed following storage in Beltsville Thawing Solution (BTS) for 24 h at 18°C. Lipids were extracted and analysed for lipid profiles, and lipid peroxide products quantified directly using a commercially available LPO kit using sperm separated from BTS extender after 0 and 24 h. Total antioxidant in extender at 0 and after 24 h were quantified using TAS Randox® kit. Effects of storability group were observed after 24 h storage for motility, viability and membrane permeability (66.7±4.8 vs. 37.3±6.4, 66.2±7.4 vs. 32.3±3.7 and 68.2±5.3 vs. 41.3±7.1, for normal and low storability groups respectively; $P<0.05$). After storage for 24 h, LPO levels of normal motility sperm were lower ($P<0.05$) than of the low motility sperm (11.53±1.42 vs. 19.14±1.12). Contrastingly, total lipid, proportion of cholesterol and TAS of normal motility sperm were higher ($P<0.05$) than of the low motility sperm (161.58±21.36 vs 155.12±12.31, 33.41±1.82m vs 25.91±1.23, 1.18±0.35 vs0.97±0.36, respectively). We

concluded from the present data that LPO level, total lipid, proportion of cholesterol in sperm membranes and TAS have an involvement in reductions in motility, viability, membrane permeability and hence, storability of sperm.

3.2 Introduction

Fertility of liquid boar semen progressively decreases with duration of storage (Bennemann et al., 2005; Waberski et al., 1994) and is associated with compromised sperm quality (Kumaresan et al., 2009) and a reduced ability of sperm to bind to oviductal epithelium (Waberski et al., 2006). These changes in fresh boar sperm, as well as reduced fertility of cryopreserved boar sperm, likely involve changes in the sperm plasma membrane. Boar sperm are sensitive to peroxidative damage due to the high content of unsaturated fatty acids in the phospholipids of their plasma membranes (Alvarez and Storey, 1992; Cerolini et al., 2000; Park and Graham, 1992) and the relatively low antioxidant capacity of boar seminal plasma (Brezezinska-Slebodzinska et al., 1995). It has been demonstrated in human (Alvarez and Storey, 1992, 1995; O'Flaherty et al., 1997) and mouse sperm (Mazur et al., 2000) that cryopreservation is associated with oxidative stress with consequent inhibition of motility (de Lamirande and Gagnon, 1992[b]) and sperm-oocyte fusion (Aitken et al., 1989). Sperm are efficient producers of reactive oxygen species (Cerolini et al., 2000) and these ROS may be one factor involved in the decreased motility, viability and membrane permeability associated with storage of sperm. The ROS were produced from several potential sources during cryopreservation of sperm (Bailey et al., 2000; Wang et al., 1997). In particular, an activated aromatic amino acid oxidase enzyme system following death of sperm has been identified as major source of ROS production in semen (Upreti et al., 1998) and ROS such as hydrogen peroxide are known to arrest sperm motility (Wales et al., 1959). Total antioxidant capacity is reduced during storage of boar semen at 18°C (Brezezinska-Slebodzinska et al., 1995). It is therefore plausible that an increasing level of ROS causes increased sperm membrane LPO and strongly decreased sperm motility during storage (White, 1993). The responsibilities for the sperm deterioration during in

vitro storage are not clear. Evidence is accumulating that changes in the sperm membrane structure and thus in membrane properties are responsible for the decreased the ability of stored spermatozoa. For example, the ability of the molecular organization of the sperm membrane to respond to cooling may be impaired through an inability to change fluidity (Buhr et al., 1989). Fluidity is related to the integrity of the membrane lipids (Stubbs and Smith, 1984), and changes in the lipid composition of the plasma membrane may therefore be associated with the cooling and storage effects. Sperm cells are characterized by a high level of polyunsaturated fatty acids; lipid peroxidation may be one of the mechanisms responsible for the negative biochemical and physiological changes during sperm storage. However, data on the changes in sperm lipid composition and their susceptibility to peroxidation during storage are limited. The aim of this study was to investigation of changes in viability, lipid peroxidation, fatty acid composition in boar spermatozoa and antioxidant capacity comparing between normal (>50%) and low (<50%) sperm motility at 24 h.

3.3 Materials and methods

3.8.1 Animals

The animal use and care committee of Chulalongkorn University approved this study, which employed 20 mature (22 m) boars housed at a commercial farm in Burirum province, Thailand. Boars were pre-selected on the basis of normal (>50%) or low (<50%) sperm motility at 24 h after collection and each of 10 normal boars were paired by age with a low motility boar. Criteria for dividing the boars were 50% sperm motility which is acceptable motility of sperm used in pig farm after 24 h of cool storage. Each sperm motility group comprised 4 Duroc, 3 Yorkshire, and 3 Landrace boars and all boars were kept under the same conditions. Each boar was housed individually and allowed 2.5 kg/d of commercial gestating sow ration. Water was available ad libitum.

3.8.2 Semen collection and evaluation

For all boars, semen was collected and evaluation as description in chapter II

3.8.3 Sperm lipid extraction and analysis

Total lipids were extracted from 3×10^9 sperm from 0 and 24 h in BTS extender and analyzed for lipids and fatty acid profiles as description in chapter II

3.8.4 Lipid peroxide assay

Sperm pellets at 0 and 24 h in BTS extender were thawed in a 37°C water bath for 5 min. Lipids were extracted and analyzed lipid peroxide as description in chapter II

3.8.5 Total antioxidants assay

An aliquot of 10 ml of supernatant separated from semen at 0 and 24h were analyzed for total antioxidant status (TAS) with the use of the TAS Randox® kit (Cat No. NX2332, Randox laboratories, Ltd, Crumlin, UK) using a Sunrise Absorbance Reader (TECAN®, Austria). The linearity of calibration extended from to 0.025 to 2.5 mmol/l of Trolox. Inter and intra-assay coefficients of variation were 6.4% and 5.3%, respectively.

3.8.6 Statistical analysis

All data were analyzed using SAS 9.1 (The SAS Institute Inc., Cary, NC, USA). Differences within motility group were examined using a paired t-test and between motility groups examined using t-test. All of the sperm parameters are presented as mean±SD.

3.4 Results

There was no difference between motility groups for any parameter at the time of collection (fresh semen). After 24 h storage in BTS, sperm motility, membrane permeability and vitality had decreased in both sperm motility groups ($P<0.001$; Table 1). Further, compared to the normal motility group, at 24-h the low motility group had

lower (P<0.05) percent motility (66.7±4.8 vs. 37.3±6.4), percent viability (66.2±7.4 vs. 32.0±3.7) and percent membrane permeability (68.3±5.3 vs. 41.3±7.1) (Table 5).

Table 5 Comparison of sperm parameters from normal and low sperm motility groups (means ± SD)

Variables	Normal motility group at 24 h	Low motility group at 24 h
Semen volume (ml)	239.2±97.3	259.7±90.1
Sperm ($\times 10^6$/ ml)	240.1±47.0	250.7±24.3
Head normality (%)	97.0±3.6	97.8±2.5
Tail normality (%)	96.8±1.9	93.6±7.6
Fresh semen membrane permeability (%)	76.1±7.9 [c]	84.6±6.0 [c]
Membrane permeability at 0 h (%)	75.3±7.2 [c]	81.4±5.3 [c]
Membrane permeability at 24 h (%)	68.2±5.3 [ad]	41.3±7.1 [bd]
Fresh semen motility (%)	79.7±4.1 [c]	80.7±4.5 [c]
Motility at 0 h (%)	78.4±5.3 [c]	79.4±3.5 [c]
Motility after 24 h (%)	66.7±4.8 [ad]	37.3±6.4 [bd]
Fresh sperm viability (%)	82.6±5.1 [c]	83.4±5.4 [c]
Viability at 0 h (%)	77.6±7.1 [c]	81.1±4.2 [c]
Viability after 24 h (%)	66.2±7.4 [ad]	32.3±3.7 [bd]

[a,b] within row, means followed by different letters differ, *P*<0.05.

[c,d] within column block, means followed by different letters differ, *P*<0.05.

Table 6 Comparison of lipids, fatty acid profiles, lipid peroxide of sperm and total antioxidant status (TAS) at 0 and 24 h of normal and low motility boar sperm at 24 h (Means ± SD).

	Normal sperm motility at 24 h		Low sperm motility at 24 h	
	0h	24h	0h	24h
Total lipid, μg /10^9 sperm	165.42±16.33[a]	161.58±21.36[a]	162.14±18.46[a]	155.12±12.31[b]
Cholesterol, %	34.91±1.34[a]	33.41±1.82[a]	32.23±2.39[a]	25.91±1.23[b]
Cholesterol ester, %	1.33±0.28[a]	1.31±0.12[a]	1.36±0.41[a]	1.34±0.58[a]
Phospholipids, %	63.12±3.11[a]	61.13±4.21[a]	62.73±2.37[a]	59.12±2.83[a]
Phospholipid fatty acids (%)				
Saturated	25.11±4.33[a]	25.32±4.23[a]	26.32±3.23[a]	25.31±2.83[a]
Monounsaturated	13.09±1.23[a]	12.35±1.65[a]	11.87±1.98[a]	11.49±1.69[a]
18:2(*n-6*)	10.09±1.23[a]	10.13±1.45[a]	9.13±1.11[a]	10.14±2.43[a]
20:2(*n-6*)	2.33±1.21[a]	3.22±1.83[a]	2.18±1.76[a]	2.65±1.77[a]
20:3(*n-6*)	3.26±2.58[a]	3.64±1.58[a]	2.29±1.52[a]	2.78±1.56[a]
20:3(*n-9*)	1.18±0.35[a]	2.29±1.51[a]	1.63±1.05[a]	1.63±1.43[a]
20:4(*n-6*)	6.97±2.31[a]	6.43±3.51[a]	7.09±1.87[a]	7.98±2.67[a]
20:5(*n-3*) EPA	2.44±1.55[a]	2.07±1.43[a]	2.44±1.32[a]	1.67±1.43[a]
22:4(*n-6*)	6.53±1.56[a]	6.86±1.34[a]	6.89±1.78[a]	6.43±1.32[a]
22:5(*n-6*) DPA	12.98±2.35[a]	11.08±4.25[a]	13.55±2.56[a]	13.18±3.45[a]
22:6(*n-3*) DHA	16.01±1.85[a]	16.91±1.29[a]	15.54±1.76[a]	16.74±1.35[a]
Total (*n-6*)	42.16±6.75[a]	41.36±7.45[a]	41.13±4.56[a]	43.16±2.45[a]
Total (*n-3*)	18.45±2.33[a]	18.98±2.28[a]	18.98±3.43[a]	18.41±2.21[a]
Total (*n-6*):(*n-3*)	2.28±0.98[a]	2.45±0.75[a]	2.18±0.76[a]	2.48±0.92[a]
DPA:DHA	0.81±0.21[a]	0.75±0.43[a]	0.87±0.43[a]	0.79±0.41[a]
TAS, mmol/L	1.34±0.72[a]	1.18±0.35[a]	1.44±0.72[a]	0.97±0.36[b]
Lipid peroxide, mmol /10^9 sperm	8.12±0.74[a]	11.53±1.42[a]	8.32±1.81[a]	19.14±1.12[b]

[a,b] within row, means followed by different letters differ, $P<0.05$.

After storage for 24 h, LPO levels in sperm of normal motility sperm were lower ($P<0.05$) than of the low motility sperm as determined (11.53±1.42 vs 19.14±1.12; Table 6). Contrastingly, total lipid, proportion of cholesterol and TAS of normal motility sperm were higher ($P<0.05$) than of the low motility sperm (161.58±21.36vs 155.12±12.31, 33.41±1.82vs 25.91±1.23, 1.18±0.35vs0.97±0.36, respectively). There was no difference of phospholipid fatty acid between normal and low sperm motility at 24h ($P>0.05$).

3.5 Discussion

The present findings confirm reduced sperm motility, membrane permeability and vitality after storage of sperm for 24 h in BTS. Further, the magnitudes of these reductions were markedly larger for the low motility group. Similarly, the amount of LPO was increased after 24-h storage with the increase being much larger for the low motility group. One factor influencing sperm motility and viability is the production of ROS (Cummins et al., 1994) which are known to induce lipid peroxidation of sperm membranes, inhibit sperm metabolism, deplete ATP, and cause leakage of intracellular enzymes (Brezezinska-Slebodzinska et al., 1995; Cerolini et al., 2000; Chatterjee and Gagnon, 2001). The increased LPO noted in the present study agrees with previous reports where increased LPO was documented after storage of liquid boar semen (Kumaresan et al., 2009) and frozen-thawed boar sperm (Roca et al., 2004). Similar effects have also been observed for cryopreserved buffalo (Kumaresan et al., 2006) and bull sperm (O'Flaherty et al., 1997).

ROS are a normal byproduct of cellular metabolism but become injurious when produced in excess. ROS were produced from several potential sources during cryopreservation of sperm (Bailey et al., 2000; Wang et al., 1997). In particular, an aromatic amino acid oxidase enzyme system activated following sperm death has been identified as a major source of ROS production in semen (Upreti et al., 1998), which is consistent with the higher sperm mortality and LPO content with storage in the present study, especially in the low motility group. Although speculative, it is plausible that the sperm in the low motility group were more sensitive to ROS and associated LPO

production resulting in increased sperm mortality. This, in turn, would increase ROS production via the aromatic amino acid oxidase enzyme system and so accentuate the LPO effect, reducing motility and membrane permeability resulting in further sperm mortality. This would be further accentuated by a lower antioxidant capacity of seminal plasma from the low motility group. The etiology of defects of sperm function are likely multifactorial, although a major involvement of LPO seems likely (Aitken et al., 1989; Alvarez and Storey, 1992; Audet et al., 2009; Chatterjee and Gagnon, 2001; de Lamirande and Gagnon, 1992[a]; Kumaresan et al., 2009).

In particular, DHA and other *n-3* PUFA are important for motility and viability (Kelso et al., 1997; Safarinejad et al., 2010). DHA was decreased in sperm membrane phospholipids of asthenospermic men (Aksoy et al., 2006; Zalata et al., 1998) and this was related to oxidative damage (Comhaire et al., 2000). In the present study, the *n-3* PUFA including DHA have no positive results on sperm motility, viability. However, *n-6* PUFA are the main PUFA of sperm plasma membranes of boar (Rooke et al., 2001), bull (Moraes et al., 2009), poultry (Douard et al., 2000) and human (Aksoy et al., 2006; Zalata et al., 1998), but as shown in the current study and those of previous workers (Aksoy et al., 2006; Cerolini et al., 2000; Conquer et al., 1999; Douard et al., 2000; Rooke et al., 2001; Wathes et al., 2007; Zalata et al., 1998), *n-6* PUFA do not have positive effects on sperm quality.

Cholesterol is an important component of the lipid fraction in the sperm plasma membrane, playing an important role in promoting sperm membrane permeability and fluidity in bulls (Beer-Ljubic et al., 2009; Moraes et al., 2009), stallions (Glazar et al., 2009; Oliveira et al., 2009) and rams (Moce et al., 2009). Fluidity and permeability of sperm plasma membranes contributes to the maintenance of their motility and viability (Beer-Ljubic et al., 2009; Glazar et al., 2009; Moce et al., 2009), which is consistent with our results showing the low motility sperm to have a lower cholesterol content. It has been reported that boar sperm lose up to half of the cholesterol from their plasma membranes after cryopreservation (Cerolini et al., 2001). This is likely one of the causes of a premature capacitation-like effect seen in cryopreserved sperm cells. It is known

that a loss of cholesterol from the plasma membrane is one of the first events in capacitation which decreases the stability of the membrane (Tulsiani et al., 1997).

We concluded from the present data that LPO level, total lipid, proportion of cholesterol in sperm membranes and TAS have an involvement in reductions in motility, viability, membrane permeability and hence, storability of sperm.

CHAPTER IV

THE EFFECT OF FISH OIL, VITAMINS AND SELENIUM ON-TOP FEED SUPPLEMENT ON LIPID COMPOSITION OF THE BOAR SPERMATOZOA AND EXTEND SEMEN QUALITY

4.1 Abstract

DHA can improve sperm plasma membrane lipids which have an important role in membrane fluidity and sperm motility. The objective of the present study was to determine the effect of fish oil, vitamins and selenium on-top feed supplemented on boar spermatozoa lipid composition and semen quality. Twenty one boars were assigned to this experiment. All boars were assigned randomly to one of the three experimental groups: 1) supplemented diet for 8 weeks (n=7); 2) supplemented diet for 16 weeks (n=7) and 3) no supplemented diet (control) (n=7). Fish oil (40 ml), vitamin E (480 iu), vitamin C (2,400 mg) and selenium (0.3 mg) were given once a day by on-top feeding assigned as presented in 3 groups. The semen was collected from all boars (n=21) using the glove-hand method once a week starting from 7 week prior to the supplementation and continues for a total of 23 collections per boar. Semen evaluation was performed using conventional methods. Semen was then centrifuged to separate sperm from seminal plasma and both kept at -20°C until analyzed sperm pellet for lipid content, FA profile by gas chromatography and seminal plasma for total antioxidant status (TAS) and glutathione peroxidase (GPX) using a commercial kit. Total sperm per ejaculate in Group I increased from $69.21\pm3.21\times10^9$ during Control Period and $70.53\pm3.21\times10^9$ during Period I to $72.35\pm3.21\times10^9$ during Period II, also in Group II increased the total sperm (P=0.04) from $69.65\pm2.87\times10^9$ during Control Period and $70.98\pm2.87\times10^9$ during Period I to $72.49\pm2.87\times10^9$ during Period II. In Control Group, the total sperm was 71.23×10^9 during Control Period, 70.24×10^9 during Period I and 70.45×10^9 during Period II (S.E.M= 3.21, 2.87 comparing with Group I and II respectively). Semen volume on Group I increased (P=0.01) from 300.24±27.23 ml during Control Period and 318.37±27.23 ml during Period I to 400.21±27.23 ml during Period II and also in Group II, the volume increased (P=0.04) from 295.69±31.43 ml

during Control Period and 309.54±31.43 ml during Period I to 348±31.43 ml during Period II. In Control Group, the volume was 278.65 ml during Control Period, 288.36 ml during Period I and 295.23×10^9 during Period II (S.E.M= 27.23, 31.43 comparing with Group I and II respectively). Proportion of DHA and total *n-3* in sperm composition were increased Group I and II comparing with Control ($P<0.05$). Glutathione peroxidase in seminal plasma in Group I was increased ($P=0.02$) from 1.28±0.14 mmol/ml during Control Period and 1.31±0.22 mmol/ml during Period I to 1.49±0.29 mmol/ml during Period II, and Group II was increased ($P=0.009$) from 1.32±0.31 mmol/ml during Control Period and 1.34±0.34 mmol/ml during Period I to 1.55±0.24 mmol/ml during Period II (Table 14). In Control Group, glutathione peroxidase in seminal plasma was 1.18 mmol/ml during Control Period, 1.21 mmol/ml during Period I and 1.22 mmol/ml during Period II (S.E.M= 0.29, 0.31 comparing with Group I and II respectively). The duration of ejaculation was increased on Group I ($P<0.001$) and II ($P<0.04$) comparing with Control Group. In conclusion, the number of total sperm, semen volume, Proportion of DHA, and total *n-3* in sperm composition was increased, duration of ejaculation was altered and glutathione peroxidase in seminal plasma were improved, in the boars fed a diet supplemented with combination of fish oil, vitamin and selenium.

4.2 Introduction

Mammalian spermatozoa consist of a particularly high proportion of long chain polyunsaturated fatty acids (PUFA) with species-specific variability in fatty acid contents (Wathes et al., 2007). Lipid composition of the plasma membrane of spermatozoa has plays an important role in sperm membrane fluidity as well as sperm motility and viability (Ahluwalia and Holman, 1969; Miller et al., 2004) and susceptibility to lipid peroxidation (Aitken, 1995; Stubbs and Smith, 1984). Several studies have been conducted to evaluate the effect of dietary omega-3 (*n-3*) PUFA supplementation on semen parameters and try to make the correlations among plasma membrane lipid composition, fluidity and sperm quality (Cerolini et al., 2000; Kelso et al., 1997; Nissen and Kreysel, 1983; Rooke et al., 2001). There were many studies with fowl (Blesbois et al., 2004; Surai et al., 2000) and with boars (Estienne et al., 2008; Mitre et al., 2004;

Rooke et al., 2001; Strzezek et al., 2004) which indicated benefits of *n-3* fatty acids on sperm quality.

Dietary lipid supplements have been used to improve sperm quality. In boars, tuna oil improved progressive motility after 5 weeks of feeding (Rooke et al., 2001), feeding shark oil increased velocity and motility of sperm (Mitre et al., 2004), and top-dressing boar diet with *n-3* fatty acids increased the number of sperm per ejaculation after 7 weeks of supplementation (Estienne et al., 2008). We hypothesize that relatively poor sperm quality is associated with an altered fatty acid composition of sperm and seminal plasma. However, other researches in humans (Conquer et al., 2000), turkeys (Zaniboni et al., 2006), rabbits (Gliozzi et al., 2009) and boars (Paulenz et al., 1995) did not demonstrate any effect of *n-3* PUFA supplementation on semen quality. Due to these conflicting results, likely related to large variation in type and quantity of dietary fats especially for *n-3* PUFA, additional research had to be conducted. Thus, the objective of the present study was to determine the effect of fish oil, vitamins and selenium on-top feed supplemented on boar spermatozoa lipid composition and semen quality

4.3 Materials and Methods

4.3.1 Animals

Twenty one boars (9 Duroc, 6 Yorkshire, 6 Landrace) were assigned to this experiment. All boars were distributed, according to age and breed, to three groups of 7 animals each. Within each group, the animals were assigned randomly to one of the three experimental groups: 1) supplemented diet for 8 weeks (n=7); 2) supplemented diet for 16 weeks (n=7) and 3) no supplemented diet (control) (n=7) (figure 2). They are housed individually in 2x3 m pens on semi-slatted floors and were given 2.5 kg of gestation sow formula feed. Water was available ad libitum.

4.3.2 Diet supplementation

Fish oil (40 ml), vitamin E (480 iu), vitamin C (2,400 mg) and selenium (0.3 mg) were given once a day by on-top feeding assigned as presented in 3 groups. Three

samples of basal diet and supplemented diet (at start, mid and late of experimental period) were analyzed for lipid composition profile by gas liquid chromatography (Table 7).

Table 7 Lipid and fatty acid profiles of basal diet and supplemented diet

	Basal diet	Supplemented diet
Total lipid, g/kg dry matter	184.38±3.52	196.83±2.54
Cholesterol, %	12.43±2.51	17.45±1.34
Cholesterol ester, %	1.05±0.45	1.23±0.87
Phospholipids, %	65.51	63.58
Phospholipid fatty acids (%)		
Saturated	83.17±2.81	35.28±3.25
Monounsaturated	4.32±1.18	8.86±1.43
18:1(*n-9*)	3.32±0.89	9.63±2.45
18:3(*n-3*)	8.54±0.05	1.30±2.25
20:4(*n-6*)	ND	2.08±0.34
20:5(*n-3*) EPA	ND	2.79±1.65
22:5(*n-6*) DPA	ND	6.62±3.5
22:6(*n-3*) DHA	ND	33.18±3.52

4.3.3 Semen collection and evaluation

Semen collection was performed for each boar. The semen was collected from all boars (n=21) using the glove-hand method once a week starting from 7 week prior to the supplementation and continues for a total of 23 collections per boar. Semen was evaluation and prepared to analyse their compositions as description in chapter II.

4.3.4 Sperm lipid extraction and analysis

Every week, total lipids from sperm of all boars were extracted from 3×10^9 sperm and analyzed for lipids and fatty acid profiles as description in chapter II.

4.3.5 Lipid peroxide assay

Sperm pellets were thawed in a 37°C water bath for 5 min. Lipids were extracted and analyzed lipid peroxide as description in chapter II. This assay was processed on semen which were collected in week -7, 0, 7 and 15 respectively.

4.3.6 Total antioxidants in seminal plasma assay

An aliquot of 10 ml of freshly thawed seminal plasma was analyzed for total antioxidant status (TAS) as description in chapter II. This assay was processed on semen which were collected at week -7, 0, 7 and 15 respectively.

4.3.7 Glutathione peroxidase (GPX) in seminal plasma assay

Ten milliliters of freshly thawed seminal plasma were analyzed with the use of the RANSEL Randox® kit (Cat No. RS505, Randox laboratories, Ltd, Crumlin, UK) on Sapphire120® Auto analyzer (Audit Diagnostics, Ireland). Measurements in duplicate were used to calculate intra-assay variability. This assay was processed on semen which were respectively.

4.3.8 Sexual behaviour test

Two measurements were recorded: T1, the time between entrance in the collection area and onset of ejaculation, and T2, the duration of ejaculation (Audet et al., 2004; Louis et al., 1994)

4.3.9 Statistical analysis

All of the sperm parameters were presented as mean±SD. Spermatozoa FA composition were reported as μg/10^9 spermatozoa. The ratios of *n-6*/*n-3* FA and the SFA, MUFA and PUFA were calculated. The lipid peroxide was reported as mmol lipid peroxides/10^9 spermatozoa. Total antioxidants in seminal plasma were reported as mmol/l. Glutathione peroxidase in seminal plasma were reported as mmol/l. Data between groups were analyzed by repeated measurements ANOVA. The model

included group, boar within group, week, and group×week as possible source of variation.

For subsequent analysis, means for semen volume, total sperm, behaviour characteristic, lipid peroxide, total antioxidants, and glutathione peroxidase in each boar were calculated for Control Period (7-week prior to study), Period 1 (Weeks 0-7) and Period 2 (Weeks 8-15). Data were analysed as above, using a model included group, boar within group, period, and group×period as possible sources of variation. Individual means were compared using the PDIFF option of GLM procedure of SAS.

Correlations between *n-3* PUFA in sperm composition (DHA and total *n-3*) and semen volume, total sperm and the duration of ejaculation were determined by using the Pearson correlation coefficient in case of normal distribution and by Spearman's rank correlation coefficient in case of non-normal distribution.

4.4 Results

There was no significant difference of all semen parameter and lipid profiles, which affected by groups and week of collection, among Control, Group I and Group II (Table 8,9,10; P>0.1). Semen volume and total sperm have a tendency for an effect of group×week; comparing between Control and Group I (Table 8; 0.1>P>0.05), comparing between Control and Group II (Table 9; 0.1>P>0.05). We found some effect of group×week on DHA and total *n-3* in sperm composition from Group I (table 11) and II (Table 12) comparing with Control (P<0.05). Other semen parameters were not affected by group×week (P>0.05).

Table 8 Semen characteristics of boars fed a control diet or a diet supplementation for 7 weeks and collected semen weekly for 23 weeks

Characteristics	Control	7-week of supplement	S.E.M.	P-values		
				group	week	group×week
Volume (ml)	290.14	341.60	25.87	0.85	0.06	0.05
Sperm ($\times 10^9$)	70.84	71.69	4.84	0.26	0.21	0.05
Sperm motility, %	84.53	84.66	3.21	0.15	0.15	0.38

Sperm viability, %	89.10	89.55	4.25	0.21	0.21	0.45
Normal morphology, %	94.58	95.28	2.35	0.18	0.41	0.21
Normal plasma membrane, %	88.34	87.83	3.26	0.30	0.32	0.17

Table 9 Semen characteristics of boars fed a control diet or a diet supplementation for 14 weeks and collected semen weekly for 23 weeks

Parameter	Control	14-week of supplement	S.E.M.	P-values		
				group	week	group×week
Volume (ml)	290.14	326.27	28.32	0.84	0.06	0.05
Sperm ($\times 10^9$)	70.84	71.35	3.12	0.12	0.14	0.06
Sperm motility, %	84.53	84.74	4.25	0.32	0.32	0.32
Sperm viability, %	89.10	89.33	2.38	0.56	0.54	0.19
Normal morphology, %	94.58	94.92	3.32	0.45	0.12	0.42
Normal plasma membrane, %	88.34	88.15	2.15	0.41	0.33	0.14

Table 10 Semen characteristics of boars fed a diet supplementation for 7 weeks or a diet supplementation for 14 weeks and collected semen weekly for 23 weeks

Parameter	7-week of supplement	14-week of supplement	S.E.M.	P-values		
				group	week	group×week
Volume (ml)	341.60	326.27	19.56	0.21	0.32	0.21
Sperm ($\times 10^9$)	71.69	71.35	3.28	0.35	0.12	0.54
Sperm motility, %	84.66	84.74	5.21	0.24	0.45	0.25
Sperm viability, %	89.55	89.33	4.12	0.62	0.12	0.14
Normal morphology, %	95.28	94.92	3.36	0.23	0.36	0.31
Normal plasma membrane, %	87.83	88.15	4.12	0.18	0.41	0.12

Table 11 Lipid and fatty acid profiles of spermatozoa from boars fed a control diet or a diet supplementation for 7 weeks and collected semen weekly for 23 weeks

	Control	7-week of supplement	S.E.M.	P-values		
				group	week	group×week
Total lipids, μg /10^9 sperm	153.07	154.73	9.56	0.21	0.12	0.21
Cholesterol, %	33.10	33.08	1.25	0.63	0.23	0.89
Cholesterol ester, %	1.24	1.22	0.21	0.36	0.25	0.63
Phospholipids, %	62.57	65.51	2.85	0.74	0.14	0.31
Phospholipid fatty acids (%)						
Saturated	25.97	25.26	1.25	0.25	0.23	0.25
Monounsaturated	11.92	12.11	2.36	0.56	0.29	0.65
18:2(*n-6*)	9.05	9.19	2.30	0.25	0.36	0.36
20:2(*n-6*)	1.05	1.32	1.12	0.55	0.32	0.21
20:3(*n-6*)	0.75	0.88	0.32	0.76	0.12	0.27
20:3(*n-9*)	5.71	5.05	3.08	0.22	0.89	0.12
20:4(*n-6*)	7.03	7.20	1.56	0.364	0.14	0.09
20:5(*n-3*) EPA	3.19	3.35	1.12	0.56	0.76	0.55
22:4(*n-6*)	7.41	7.20	2.25	0.29	0.35	0.12
22:5(*n-6*) DPA	12.97	13.46	1.28	0.39	0.09	0.09
22:6(*n-3*) DHA	14.91	16.21	1.31	0.09	0.81	0.04
Total (*n-6*)	38.26	37.64	2.69	0.07	0.25	0.08
Total (*n-3*)	18.12	20.82	2.87	0.09	0.84	0.04
Total (*n-6*):(*n-3*)	2.71	2.24	0.25	0.12	0.14	0.07
DPA:DHA	0.87	0.83	0.14	0.14	0.36	0.09

Table 12 Lipid and fatty acid profiles of spermatozoa from boars fed a control diet or a diet supplementation for 14 weeks and collected semen weekly for 23 weeks

	Control	14-week of supplement	S.E.M.	P-values		
				group	week	group×week
Total lipids, μg /10^9 sperm	153.07	153.44	10.33	0.85	0.21	0.21
Cholesterol, %	33.10	33.08	1.84	0.56	0.23	0.25

Cholesterol ester, %	1.24	1.23	0.12	0.23	0.45	0.12
Phospholipids, %	62.57	63.58	2.31	0.21	0.65	0.09
Phospholipid fatty acids (%)						
Saturated	25.97	25.48	2.34	0.29	0.14	0.09
Monounsaturated	11.92	11.06	1.27	0.45	0.09	0.84
18:2(*n-6*)	9.05	9.30	1.58	0.87	0.25	0.25
20:2(*n-6*)	1.05	0.81	0.54	0.65	0.21	0.32
20:3(*n-6*)	0.75	0.84	0.44	0.32	0.12	0.22
20:3(*n-9*)	5.71	3.61	2.33	0.13	0.11	0.32
20:4(*n-6*)	7.03	7.08	2.61	0.12	0.67	0.28
20:5(*n-3*) EPA	3.19	4.79	1.87	0.13	0.23	0.15
22:4(*n-6*)	7.41	7.22	1.45	0.31	0.25	0.28
22:5(*n-6*) DPA	12.97	13.62	0.98	0.65	0.45	0.07
22:6(*n-3*) DHA	14.91	16.16	1.08	0.45	0.25	0.03
Total (*n-6*)	38.26	37.87	2.56	0.12	0.44	0.08
Total (*n-3*)	18.12	20.95	1.24	0.65	0.08	0.04
Total (*n-6*):(*n-3*)	2.71	2.23	0.35	0.87	0.25	0.08
DPA:DHA	0.87	0.84	0.04	0.25	0.15	0.09

Table 13 Lipid and fatty acid profiles of spermatozoa from boars fed a diet supplementation for 7 weeks or a diet supplementation for 14 weeks and collected semen weekly for 23 weeks

	7-week of supplement	14-week of supplement	S.E.M.	P-values		
				group	week	group×week
Total lipids, μg /10^9 sperm	154.73	153.44	9.21	0.23	0.21	0.12
Cholesterol, %	33.08	33.08	1.58	0.12	0.51	0.36
Cholesterol ester, %	1.22	1.23	0.14	0.36	0.23	0.25
Phospholipids, %	65.51	63.58	2.56	0.25	0.65	0.89
Phospholipid fatty acids (%)						
Saturated	25.26	25.48	1.35	0.23	0.25	0.36
Monounsaturated	12.11	11.06	2.36	0.08	0.13	0.56

18:2(*n-6*)	9.19	9.30	1.58	0.21	0.12	0.26
20:2(*n-6*)	1.32	0.81	0.31	0.22	0.34	0.13
20:3(*n-6*)	0.88	0.84	0.23	0.34	0.12	0.31
20:3(*n-9*)	5.05	3.61	2.12	0.15	0.11	0.45
20:4(*n-6*)	7.20	7.08	2.65	0.65	0.36	0.32
20:5(*n-3*) EPA	3.35	4.79	2.67	0.11	0.35	0.23
22:4(*n-6*)	7.20	7.22	1.98	0.21	0.23	0.25
22:5(*n-6*) DPA	13.46	13.62	2.65	0.09	0.27	0.54
22:6(*n-3*) DHA	16.21	16.16	1.24	0.21	0.76	0.15
Total (*n-6*)	37.64	37.87	2.56	0.65	0.18	0.36
Total (*n-3*)	20.82	20.95	2.78	0.45	0.25	0.25
Total (*n-6*):(*n-3*)	2.24	2.23	0.35	0.25	0.36	0.14
DPA:DHA	0.83	0.84	0.12	0.32	0.32	0.56

Subsequent analysis demonstrated the effect of group×period for total sperm; Group I increased the total sperm (P=0.03) from 69.21±3.21×10^9 during Control Period and 70.53±3.21×10^9 during Period I to 72.35±3.21×10^9 during Period II, Group II increased the total sperm (P=0.04) from 69.65±2.87×10^9 during Control Period and 70.98±2.87×10^9 during Period I to 72.49±2.87×10^9 during Period II. In Control Group, the total sperm was 71.23×10^9 during Control Period, 70.24×10^9 during Period I and 70.45×10^9 during Period II (S.E.M= 3.21, 2.87 comparing with Group I and II respectively). For semen volume, group×period affected on Group I increased the volume (P=0.01) from 300.24±27.23 ml during Control Period and 318.37±27.23 ml during Period I to 400.21±27.23 ml during Period II, Group II increased the volume (P=0.04) from 295.69±31.43 ml during Control Period and 309.54±31.43 ml during Period I to 348±31.43 ml during Period II. In Control Group, semen volume was 278.65 ml during Control Period, 288.36 ml during Period I and 295.23 ml during Period II (S.E.M= 27.23, 31.43 comparing with Group I and II respectively).

Table 14 Sperm lipid peroxidation, total antioxidant status and glutathione peroxidase in seminal plasma from boars fed a control diet or fed a diet supplementation for 7 weeks and collected semen in week -7, 0, 7 and 15 for 4 weeks

Parameter	Control	7-week of supplement	S.E.M.	P-values		
				group	Period[a]	Period×week
Lipid peroxide, mmol /10^9 sperm	8.41	8.97	1.27	0.21	0.36	0.54
TAS, mmol/L	1.43	1.58	0.21	0.51	0.23	0.06
GPX, mmol /ml	1.22	1.45	0.19	0.35	0.05	0.02

[a]Control Period= week-7, Period I= week 0,7, Period II= week 15

Table 15 Sperm lipid peroxidation, total antioxidant status and glutathione peroxidase in seminal plasma from boars fed a control diet or fed a diet supplementation for 14 weeks and collected semen in week -7, 0, 7 and 15 for 4 weeks

Parameter	Control	14-week of supplement	S.E.M.	P-values		
				group	Period[a]	Period×week
Lipid peroxide, mmol /10^9 sperm	8.41	8.55	1.35	0.54	0.23	0.14
TAS, mmol/L	1.43	1.63	0.19	0.21	0.36	0.05
GPX, mmol /ml	1.22	1.51	0.21	0.32	0.12	0.009

[a]Control Period= week-7, Period I= week 0,7, Period II= week 15

Table 16 Sperm lipid peroxidation, total antioxidant status and glutathione peroxidase in seminal plasma from boars fed a diet supplementation for 7 weeks or fed a diet supplementation for 14 weeks and collected semen in week -7, 0, 7 and 15 for 4 weeks

Parameter	7-week of supplement	14-week of supplement	S.E.M.	P-values		
				group	Period[a]	Period×week
Lipid peroxide, mmol /10^9 sperm	8.97	8.55	0.45	0.32	0.25	0.23
TAS, mmol/L	1.58	1.63	0.16	0.21	0.18	0.35
GPX, mmol /ml	1.45	1.51	0.21	0.11	0.65	0.36

[a]Control Period= week-7, Period I= week 0,7, Period II= week 15

There were no significant effects of group, period or group×Period on Sperm lipid peroxidation and total antioxidant status comparing among Control Group, Period I and Period II (Table 14,15,16; $P>0.05$). However, we found the effect of group×Period for glutathione peroxidase in seminal plasma; Group I increased glutathione peroxidase in seminal plasma (P=0.02) from 1.28±0.29 mmol/ml during Control Period and 1.31±0.29 mmol/ml during Period I to 1.49±0.29 mmol/ml during Period II (Table 14), Group II increased glutathione peroxidase in seminal plasma (P=0.009) from 1.32±0.31 mmol/ml during Control Period and 1.34±0.31 mmol/ml during Period I to 1.55±0.31 mmol/ml during Period II (Table 15). In Control Group, glutathione peroxidase in seminal plasma was 1.18 mmol/ml during Control Period, 1.21 mmol/ml during Period I and 1.22 mmol/ml during Period II (S.E.M= 0.29, 0.31 comparing with Group I and II respectively).

Table 17 Sexual behavior characteristic of boar fed a control diet or a diet supplementation for 7 weeks and collected weekly for 23 weeks

Parameter	Control	7-week of supplement	S.E.M.	P-values		
				group	Period[a]	Period×week
T1, sec	360.19	364.47	19.52	0.24	0.87	0.35
T2, sec	374.82	439.01	50.23	0.21	0.06	0.001

[a]Control Period= 7 weeks prior to study, Period I= week 0-7, Period II= week 8-15

[b]T1=the time between entrance in the collection area and onset of ejaculation

[c]T2=the duration of ejaculation

Table 18 Sexual behavior characteristic of boar fed a control diet or a diet supplementation for 14 weeks and collected weekly for 23 weeks

Parameter	Control	14-week of supplement	S.E.M.	P-values		
				group	Period[a]	Period×week
T1, sec	360.19	358.29	21.35	0.89	0.25	0.36
T2, sec	374.82	419.30	40.23	0.12	0.21	0.04

[a]Control Period= 7 weeks prior to study, Period I= week 0-7, Period II= week 8-15

[b]T1=the time between entrance in the collection area and onset of ejaculation

[c]T2=the duration of ejaculation

Table 19 Sexual behavior characteristic of boar fed a diet supplementation for 7 weeks or a diet supplementation for 14 weeks and collected weekly for 23 weeks

Parameter	7-week of supplement	14-week of supplement	S.E.M.	P-values		
				group	Period[a]	Period×week
T1, sec	364.47	358.29	22.13	0.54	0.22	0.14
T2, sec	439.01	419.30	45.56	0.23	0.15	0.45

[a]Control Period= 7 weeks prior to study, Period I= week 0-7, Period II= week 8-15

[b]T1=the time between entrance in the collection area and onset of ejaculation

[c]T2=the duration of ejaculation

There was no effect of group, group×Period at any period (Table 17,18,19; $P > 0.05$) on the time between entrance in the collection area and onset of ejaculation. However, the duration of ejaculation was affected by group×Period on Group I (Table 17; $P=0.001$) and II (Table 18; $P=0.04$) comparing with Control Group. There was no

effect from group×Period on the duration of ejaculation comparing between Group I and II.

Table 20 Correlations between *n-3* PUFA in sperm composition, and semen volume, total sperm and the duration of ejaculation

	Semen volume		Total sperm		T2	
	r	*P*	r	*P*	r	*P*
DHA	0.69	0.003	0.61	0.01	0.76	0.09
Total (*n-3*)	0.73	0.002	0.68	0.02	0.72	0.1

Significant positive correlations were detected between *n-3* PUFA in sperm composition (DHA and total *n-3*) and semen volume and total sperm (Table 20). There was no correlation between *n-3* PUFA in sperm composition (DHA and total *n-3*) and the duration of ejaculation

4.5 Discussion

Boar spermatozoa contain high proportions of *n-3* PUFA (Poulos et al., 1973), which play an important role in sperm function (Wathes et al., 2007). Several studies have investigated the effect of dietary *n-3* PUFA on boar reproductive performance. Some have reported beneficial effects of *n-3* PUFA supplementation on total sperm (Estienne et al., 2008; Strzezek et al., 2004) and quality (Mitre et al., 2004; Rooke et al., 2001). In mammal species, fatty acid profiles in blood plasma and semen reflect the fatty acid supplementation. The same relationship applies also with the fatty acid profile of the semen (Wathes et al., 2007). For fish oil supplementation, total *n-3* PUFA and *n-3*/*n-6* ratio are enhanced in sperm composition of human, pigs, poultry (Conquer et al., 2000; Rooke et al., 2001). In this present study, proportions of DHA and *n-3* PUFA were significantly increased in both supplement groups after feeding by fish oil supplementation and agreed with previous reports by Maldjian et al. (2005) and

Strezezek et al. (2004), the fatty acid composition of boar sperm indicates an effective transfer between diet and sperm cells. Additionally, fish oils on-top diet may promote *n-3* biosynthesis, thereby decreasing the *n-6/n-3* ratio as observed in this present study. Furthermore, the proportions of DHA (C22:6*n-3*) and DPA (C22:5 *n-6*) in sperm are inversely related such that an increase in the proportion of DHA in sperm phospholipids is balanced by a decrease of DPA on fatty acids profile in sperm (Rooke et al., 2001).

Semen production from this present study was influenced by intersection between group and Period, agreed with more recent studies in (Estienne et al., 2008; Maldjian et al., 2005; Strzezek et al., 2004), stallions (Harris et al., 2005) and chicken (Surai et al., 2000) reported a positive effect of a *n-3* PUFA diet on sperm production. Any reports have observed positive effects on sperm motility and morphology (Mitre et al., 2004; Rooke et al., 2001). However, some report observed no benefits in reproductive performance (Maldjian et al., 2005; Paulenz et al., 1995).

The effect of *n-3* PUFA supplementation was confounded with antioxidant supplementation and it is unclear which of that dietary component contributes to the improvement of boar performance as compared to the control treatment. Furthermore, evidence of the impact of antioxidants on sperm production in a variety of livestock was indeed reported (Audet et al., 2004; Castellini et al., 2007; Cerolini et al., 2000; Marin-Guzman et al., 1997). An improvement of sperm viability was reported after supplementation with fish oil and antioxidants (Maldjian et al., 2005). It was also demonstrated that a diet supplemented with vitamin E enhanced sperm quality (Brezezinska-Slebodzinska et al., 1995). Therefore, as for semen production, it is unclear which component improves sperm quality.

Some association was made between the high content of brain *n-3* PUFA and the possible role of *n-3* in the regulation of the libido (Bourre, 2005). A study in pigs indicated that the duration of ejaculation was increased by 300 g/d of a dietary supplement (Estienne et al., 2008). The type of *n-3* fatty acids and their impact on male sexual behavior characteristics remains to be investigated as present in this study.

In summary, the number of total sperm, semen volume, proportion of DHA, and total *n-3* in sperm composition was increased, duration of ejaculation was altered and glutathione peroxidase in seminal plasma were improved, in the boars fed a diet supplemented with combination of fish oil, vitamin and selenium.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

Lipids play an important role in cell membrane fluidity and flexibility. The sperm plasma membrane has been demonstrated to contain a high content of 20 (C20) and 22 (C22) carbon atoms of polyunsaturated fatty acids (PUFAs). Phospholipids of mammalian sperm cell membranes characteristically contain very high proportions of long-chain (C22) polyunsaturated fatty acids, particularly *n-3* series. In most mammals, docosahexaenoic acid (DHA, 22:6*n-3*) is the dominant polyunsaturated fatty acid, although, in several species docosapentaenoic acid (DPA, 22:5*n-6*) is also a major component of the sperm cell membranes (Alvarez and Storey, 1992; Cerolini et al., 2000; Mazur et al., 2000; O'Flaherty et al., 1997; Park and Graham, 1992). The importance of C22 polyunsaturates in relation to male fertility has been illustrated by studies in humans demonstrating that the amount of DHA in spermatozoa is positively correlated with sperm motility (Conquer et al., 1999; Nissen and Kreysel, 1983; Zalata et al., 1998). Moreover, PUFAs which are concentrated in the head and tail membrane regions of spermatozoa have been shown to play an important role in both sperm capacitation and the interaction between spermatozoa and uterine surface environment (Conquer et al., 1999; Zalata et al., 1998). However, the sperm plasma membranes are susceptible to lipid peroxidation because of the high content of PUFA (Miller et al., 1993; O'Flaherty et al., 1997; Sprecher, 1989).

In order to investigate the fatty acid composition of boar semen and study effects of feed supplementation on semen quality in our experiment, semen was collected from the boars having normal motility (>60) and low motility (<60%) to compare lipid composition in the semen as first study, which were used as basic information in boars. Second study, the differences of lipids, lipid peroxide and total antioxidants status in semen after cool storage were investigated the alteration of lipids and antioxidants in semen during cooling period. Third study, fish oil, vitamins and

selenium were supplemented on boar feed to affect the boar sperm lipid composition and semen quality.

5.1 Correlation between lipid profiles of semen an the boar semen quality

The present results clearly indicated an association between the conventional measures of sperm quality we employed and lipid composition of the washed sperm and their seminal plasma. Lipid content of sperm plasma membranes are correlated with sperm motility, viability and a functional plasma membrane (Ahluwalia and Holman, 1969; Aksoy et al., 2006; Conquer et al., 2000; Parks and Lynch, 1992; Rooke et al., 2001; Zalata et al., 1998), which is consistent with the positive correlation of sperm total lipid content with sperm motility, viability and normal plasma membrane noted in this study. Cholesterol is an important component of the lipid fraction in the sperm plasma membrane, playing an important role in promoting sperm membrane permeability and fluidity in bulls (Beer-Ljubic et al., 2009; Moraes et al., 2009), stallions (Glazar et al., 2009; Oliveira et al., 2009) and rams (Moce et al., 2009). Fluidity and permeability of sperm plasma membranes contributes to the maintenance of their motility and viability (Beer-Ljubic et al., 2009; Glazar et al., 2009; Moce et al., 2009), which is consistent with our results showing the low motility sperm to have a lower cholesterol content. It has been reported that boar sperm lose up to half of the cholesterol from their plasma membranes after cryopreservation (Cerolini et al., 2001). It is known that a loss of cholesterol from the plasma membrane is one of the first events in capacitation which decreases the stability of the membrane (Tulsiani et al., 1997). Further, when boars were fed a diet supplemented with tuna oil, the cholesterol content of sperm was increased as was sperm motility, viability, and normal acrosomes (Rooke et al., 2001). In contrast, some study found no differences in sperm motility after fed the boars (Estienne et al., 2008). The increment of antioxidants requirement after adding fat to the diet must be considered (Beck et al., 1994). Interestingly, with human sperm there was a negative correlation of cholesterol content with sperm motility, viability, permeability and normal morphology (Zalata et al., 2009). In the present study, phospholipids, PUFA and the *n-3* PUFA including DHA correlate positively with sperm motility, viability, normal

morphology and normal plasma membrane. DHA and other *n-3* PUFA are important for sperm development, motility and viability (Kelso et al., 1997; Safarinejad et al., 2010). DHA was decreased in sperm membrane phospholipids of asthenospermic men (Aksoy et al., 2006; Zalata et al., 1998) and this was related to oxidative damage (Comhaire et al., 2000). However, *n-6* PUFA is the main PUFA of sperm plasma membranes of sperm (Rooke et al., 2001; Moraes et al., 2009; Douard et al., 2000; Aksoy et al., 2006; Zalata et al., 1998). The increasing of PUFA content in sperm plasma membranes make them vulnerable to ROS-induced lipid peroxidation (Aitken et al., 1989; Aitken et al., 1993a; Aitken et al., 1993b; Alvarez and Storey, 1995; Awda et al., 2009). However, extra-cellular antioxidants in seminal plasma can protect sperm from lipid peroxidation by and, consistent with this, a low antioxidant capacity has been associated with human male infertility (de Lamirande and Gagnon, 1992a; Fraga et al., 1991; Khosrowbeygi and Zarghami, 2007; Lewis et al., 1997; Murawski et al., 2007). Previous reports showed that high level of antioxidants presented in boar seminal plasma are derived from low molecular weight fractions (Strzezek, 2002; Strzezek et al., 1999). Thus, a deficiency of these fractions can affect the overall protection of sperm from oxidative damage, which can have a negative effect on sperm motility and fertilization (Strzezek et al., 2004). The present data conform to this in that we note a lower total antioxidant status in seminal plasma derived from the low motility boars.

5.2 The changes of the level of lipids, lipidperoxide and antioxidants in semen after cool storage

This study was reported from cooling semen of boars which was divided into two groups by sperm motility after cool storage at 18ºC for 24h. The increased LPO demonstrated in the present study agrees with previous reports where increased LPO was documented after storage of liquid boar semen (Kumaresan et al., 2009) and frozen-thawed boar sperm (Roca et al., 2004). Reduced sperm motility, membrane permeability and vitality after storage of sperm were performed after 24 h in BTS. The amount of LPO was increased after 24-h storage with the increase being much larger for the low motility group. One factor influencing sperm motility and viability is the

production of ROS (Cummins et al., 1994) which are known to induce lipid peroxidation of sperm membranes, inhibit sperm metabolism, deplete ATP, and cause leakage of intracellular enzymes (Brezezinska-Slebodzinska et al., 1995; Cerolini et al., 2000; Chatterjee and Gagnon, 2001). ROS are a normal byproduct of cellular metabolism but become injurious when produced in excess. In particular, an aromatic amino acid oxidase enzyme system activated following sperm death has been identified as a major source of ROS production in semen (Upreti et al., 1998), which is consistent with the higher sperm mortality and LPO content with storage in the present study, especially in the low motility group. The increasing ROS was produced via the aromatic amino acid oxidase enzyme system and so accentuated the LPO effect, reducing motility and membrane permeability resulting in further sperm mortality. This would be further accentuated by a lower antioxidant capacity of seminal plasma from the low motility group. (Aitken et al., 1989; Alvarez and Storey, 1992; Audet et al., 2009; Chatterjee and Gagnon, 2001; de Lamirande and Gagnon, 1992a; Kumaresan et al., 2009).

DHA in sperm membrane phospholipids of asthenospermic men was decreased (Aksoy et al., 2006; Zalata et al., 1998) and this was related to oxidative damage (Comhaire et al., 2000). In the present study, the *n-3* PUFA including DHA have positive results on sperm motility, viability. However, *n-6* PUFA are the main PUFA of sperm plasma membranes (Rooke et al., 2001; Moraes et al., 2009; Douard et al., 2000; Aksoy et al., 2006; Zalata et al., 1998), but *n-6* PUFA do not have positive effects on sperm quality as shown in the current study and those of previous workers (Aksoy et al., 2006; Cerolini et al., 2000; Conquer et al., 1999; Douard et al., 2000; Rooke et al., 2001; Wathes et al., 2007; Zalata et al., 1998). It is known that a loss of cholesterol from the plasma membrane is one of the first events in capacitation which decreases the stability of the membrane (Tulsiani et al., 1997). Cholesterol is an important component of the lipid fraction in the sperm plasma membrane, playing an important role in promoting sperm membrane permeability and fluidity to contribute the maintenance of their motility and viability in sperm (Beer-Ljubic et al., 2009; Moraes et al., 2009; Glazar et al., 2009;

Oliveira et al., 2009; Moce et al., 2009) which is consistent with our results showing the low motility sperm to have a lower cholesterol content.

5.3 Effect of fish oil, vitamins and selenium on-top feed supplemented on lipid composition of the boar spermatozoa and extend semen quality

PUFA (*n-3*) are predominant PUFA in boar spermatozoa (Poulos et al., 1973), which play an important role in sperm function which was contributed by improving sperm membrane fluidity and flexibility (Wathes et al., 2007). In mammal species, fatty acid profiles in blood plasma and semen reflect the fatty acid supplementation. There are the studies of the effect of dietary *n-3* PUFA on boar sperm production, which has reported beneficial effects of *n-3* PUFA supplementation on total sperm (Estienne et al., 2008; Strzezek et al., 2004) and quality (Mitre et al., 2004; Rooke et al., 2001). The same relationship applies also with the fatty acid profile of the semen (Wathes et al., 2007). In this present study, proportions of DHA and *n-3* PUFA were significantly increased in both supplement groups after feeding by fish oil supplementation agreed with previous reports by Maldjian et al. (2005) and Strezezek et al. (2004), the fatty acid composition of boar sperm indicates an effective transfer between diet and sperm cells. Additionally, fish oils on-top diet promoted *n-3* biosynthesis, thereby decreasing the *n-6/n-3* ratio as observed in this present study. Semen production from this present study was influenced by intersection between group and Period, agreed with more recent studies in (Estienne et al., 2008; Maldjian et al., 2005; Strzezek et al., 2004), stallions (Harris et al., 2005) and chicken (Surai et al., 2000) reported a positive effect of a *n-3* PUFA diet on sperm production. Furthermore, the proportions of DHA (C22:6*n-3*) and DPA (C22:5 *n-6*) in sperm are inversely related such that an increase in the proportion of DHA in sperm phospholipids is balanced by a decrease of DPA on fatty acids profile in sperm (Rooke et al., 2001). Any reports have observed positive effects on sperm motility and morphology (Mitre et al., 2004; Rooke et al., 2001). However, some report observed no benefits in reproductive performance (Maldjian et al., 2005; Paulenz et al., 1995).

The evidence of the impact of antioxidants on sperm production in a variety of livestock was indeed reported (Audet et al., 2004; Castellini et al., 2007; Cerolini et al.,

2000; Marin-Guzman et al., 1997). However, the effect of *n-3* PUFA supplementation confounded with antioxidant supplementation is unclear which of that dietary component contributes to the improvement of boar performance as compared to the control treatment. Not only the study which presented the improvement of sperm viability after supplementation with fish oil and antioxidants (Maldjian et al., 2005) but also the study which demonstrated a diet supplemented with vitamin E enhanced sperm quality (Brezezinska-Slebodzinska et al., 1995). Therefore, as for semen production, it is unclear which component improves sperm quality.

Some association was made between the high content of brain *n-3* PUFA and the possible role of *n-3* in the regulation of the libido (Bourre, 2005). A study in pigs indicated that the duration of ejaculation was increased by the *n-3* PUFA supplement (Estienne et al., 2008), which these association remains to be investigated as present in this study.

Conclusion

After comparison between normal and low motility of fresh boar sperm, we found that the differences of sperm motility were associated with the *n-3* PUFA content in sperm plasma membranes, which play an important role in fluidity and flexibility to improve sperm motility, viability. In semen, the balance between ROS and extra-cellular antioxidants in seminal plasma maintain the stability of cell membrane and also protect sperm plasma membrane from LPO during oxidative stress. The LPO level, total lipid, proportion of cholesterol in sperm plasma membranes and TAS have an involvement in reductions in motility, viability, membrane permeability and hence, storability of sperm. The LPO significantly increased after storage for 24 h can reduce sperm motility and fluidity resulting in further sperm mortality. Cholesterol in sperm plasma membrane promotes plasma membrane fluidity and flexibility to contribute sperm motility and viability, which is consistent with our results showing in the sperm having low motility to have low cholesterol content. It is possible that in the event of lower sperm motility and fertility, supplementing semen with antioxidants or adding a source of *n-3* PUFA to the

boar diet may alleviate these problems. In the boars fed a diet supplemented with combination of fish oil, vitamin and selenium, the number of total sperm, semen volume, and proportion of DHA, total *n-3* in sperm composition and glutathione peroxidase in seminal plasma were increased. Moreover, the duration of ejaculation was longer resulting in improving semen volume.

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