USE OF BIOACTIVE COMPOUNDS TO PRESERVE BOAR SPERM

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Zhiying Zhang

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Abstract

Boar semen must be stored in liquid extenders over 15 °C for up to 7 d in order to obtain a satisfactory sow farrowing rate and litter size. Many factors contribute to the storage limitation; however, sensitivity to oxidative stress plays a vital role. The overall objective of this study was to investigate the use of natural antioxidants found in lowbush blueberries to increase the storage time of boar semen and decrease sensitivity to cold shock.

Four experiments were conducted to study the influence of lowbush blueberries on sperm lipid peroxidation, viability, motility, and capacitation status. In the first experiment, concentrations of total phenolics and total anthocyanins of lowbush blueberry products were compared using 3 different extraction solutions. In the second experiment, lowbush blueberry raw juice, frozen fruit, freeze-dried powder, and leaf extracts were incubated with boar semen in the presence of both ferrous sulphate and ascorbate. The control group without blueberry products or ferrous sulphate and ascorbate was set to exhibit the baseline of lipid peroxidation, whereas the oxidative group containing ferrous sulphate but not blueberry products was set to exhibit the maximum of lipid peroxidation. Sperm lipid peroxidation was quantified by the concentration of malondialdehyde (MDA) using thiobarbituric acid reaction substances (TBARS) fluoremetry and the concentrations of TBARS were measured at 37 °C at 1, 3, and 5 h.

In the third experiment, boar semen was incubated in the commercial X-cell™ extender alone, in the X-cell™ extender in the presence of blueberry leaf extract or powder extract at 5, 16, and 22 °C, respectively. Sperm viability and motility on day 7 were measured using SYBR-14/PI fluorescent microscopy and microscopy, respectively. In the next experiment, sperm capacitation status in the 3 semen extenders at the 3 temperatures on day 2 was identified using chlortetracycline (CTC) fluorescent assay. In addition to the blueberry extracts, heparin and/or calcium ionophore were added to semen extenders in order to induce capacitation and acrosome reaction. Sperm capacitation status was identified using the same CTC fluorescent assay.

In conclusion, acidified ethanol and methanol contained a stronger ability to extract phenolic compounds than acidified water. Concentration of total phenolics was highest in blueberry leaf extract followed by powder and fruit extracts. Concentration of total anthocyanins, however, was highest in powder extract followed by fruit and leaf extracts. Blueberry fruit extract, powder extract, leaf extract, and raw juice had active antioxidant capacity to retard iron-induced sperm lipid peroxidation. Concentrations of TBARS in the semen samples containing blueberry products remained lower than that in the oxidative group from 1 to 5 h. However, the antioxidant capacity did not differ among four blueberry products. None of the extenders protected boar spermatozoa from cold shock at 5 °C. None of blueberry-supplemented extenders preserved sperm viability better than the commercial X-cell™ extender at 16 and 22 °C. All blueberry-supplemented extenders inhibited sperm motility. Spermatozoa in blueberry-supplemented exhibited an equally high percentage of acrosome reaction (AR) pattern as spermatozoa in the control samples. Therefore, blueberry extracts did not prevent heparin-induced and/or calcium ionophore-induced capacitation and acrosome reaction.

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List of Abbreviations

AI	Artificial insemination
BHT	Butylated hydroxytoluene
CASA	Computer automated semen analyzer
CAT	Catalase
CTC	Chlortetracycline
DABCO	1,4-Diazabicyclo[2,2,2]octane
DDW	Deionized distilled water
DFBS	Dye-free buffer system
DMSO	Dimethyl sulfoxide
EtBr	Ethidium bromide
Fe/ASc	Ferrous sulphate/ascorbate
FITC-PNA	Fluorescein isothiocyanate-peanut agglutinin
FITC-PSA	Fluorescein isothiocyanate-pisum agglutinin
GAE	Gallic acid equivalent
HOST	Hypoosmotic swelling test
HPLC	High-performance liquid chromatography
IVF	*In vitro* fertilization
JC-1	5,5',5,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide
LC-MS	Liquid chromatography-mass spectrometry
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate-buffered saline
PI	Propidium iodide
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
R-PE	R-phycoerythrin
SOD	Superoxide dismutase
TALP	Tyrode's complete medium
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reaction substances
TCA	Trichloroacetic acid
TEAC	Trolox-equivalent antioxidant capacity

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1.0 General introduction

1.1 Importance of swine industry

The swine industry plays a vital role in the world economy. The total consumption of pork in the world exceeded 87 million tonnes in 2003 (Quarterly Pork Report, January-March 2004, Agriculture and Agri-Food Canada). World pork production exceeded 88 million tonnes in 2003 and is expected to exceed 90 million tonnes in 2004 (Pork Summary Selected Countries, 2004). The number of pigs in Canada increased after the Second World War, with 2 rapid increases from 1976 to 1981 and from 1996 to 2001 (Statistics Canada, 2001). The Canadian pig herd was 14.8 million animals by July 2004 (Myles, 2004). Canadian pork production was approximately 1.9 million tonnes in 2004 (Myles, 2004). For Prince Edward Island, annual sales of pork are approximately $35 million (CBC, 2004b). Pork, the most widely eaten red meat in the world, contains protein, vitamin B, and several important minerals (e.g. phosphorus, magnesium, potassium, zinc and iron); all these nutrients are beneficial to human health (Meat Nutrition & Cookery, 2003). Additionally, some breeds of pigs, similar to humans in size and weight, are used in various kinds of medical research, e.g. organ transplantation, insulin-deficient diabetes, pediatric surgery, cardiovascular disease, wound healing and essential hypertension (Swindle et al., 1992). Artificial insemination is widely used to meet the demand for pigs and pork products.

1.2 Artificial insemination

Advantages and disadvantages of artificial insemination

Artificial insemination (AI) was first documented in 1780 by the Italian physiologist, Spallanzani; he discovered that a bitch could be fertilized with the cellular portion of semen (reported by Bearden et al., 2004, p156-157). The first attempts at artificial insemination in pigs were done in 1907 by Ivanov (reported by Gadea, 2003). Artificial insemination has many advantages, including: 1) increasing use of germ plasma with superior genetics, even after a boar's death (Bearden et al., 2004, p159); 2) facilitating insemination of a large group of sows and gilts after synchronization of estrus, thereby enhancing farm management (Hafez and Hafez, 2000, p376); 3) avoiding inbreeding by establishing breeding records; 4) facilitating crossbreeding; 5) reducing the risk of transmitting semen-borne diseases (Foote, 1980, p521); 6) reducing the potential risks of injury so that any size boar can mate any size sow; 7) making it easier to identify infertile boars; and 8) eliminating the risk associated with the presence of boars on the farm. Artificial insemination has a few disadvantages, including the need to synchronize and detect estrus, to train young boars, and to have skilled personnel to perform artificial insemination (Bearden et al., 2004, p159).

Semen can be collected from a boar 2-3 times per wk without affecting semen volume or sperm concentration (Foote, 1980, p525). An ejaculate includes 3 fractions: pre-sperm, sperm-rich, and post-sperm fraction. The average ejaculate (gel free portion) is 125 mL, containing 45 billion spermatozoa (Bearden et al., 2004, p174). Since fertility is optimal when an

insemination dose contains 2 to 3 billion spermatozoa (Hafez and Hafez, 2000, p382), a typical ejaculate provides 15 to 22 insemination doses. Ejaculates are diluted with semen extender to achieve an appropriate volume for insemination.

1.3 Male reproduction

Spermatocytogenesis and spermiogenesis

Spermatozoa are produced by the testes by a series of processes, including spermatocytogenesis and spermiogenesis (Figure 1-1); and are released from the testes constantly (Senger, 1997, p169). The seminiferous epithelium is composed of Sertoli cells and germ cells. Germ cells undergo mitosis to form spermatogonia and then primary spermatocytes, which undergo meiosis to become secondary spermatocytes and then haploid spermatids (Hafez and Hafez, 2000, p99). The round haploid spermatids then transform into highly differentiated spermatozoa (Senger, 1999, p172; Hafez and Hafez, 2000, p99). Spermatozoa can be divided into 2 major anatomical sections: sperm head and tail. The sperm head is composed of a haploid nucleus; the anterior end of the sperm head is the acrosome, containing acrosin and other hydrolytic enzymes. The sperm tail is composed of the neck, midpiece, principal piece, and end piece. The midpiece is packed with the mitochondria, which provide energy needed for sperm motility (Senger, 1999, p177; Hafez and Hafez, 2000, p96; Bearden et al., 2004, p80).

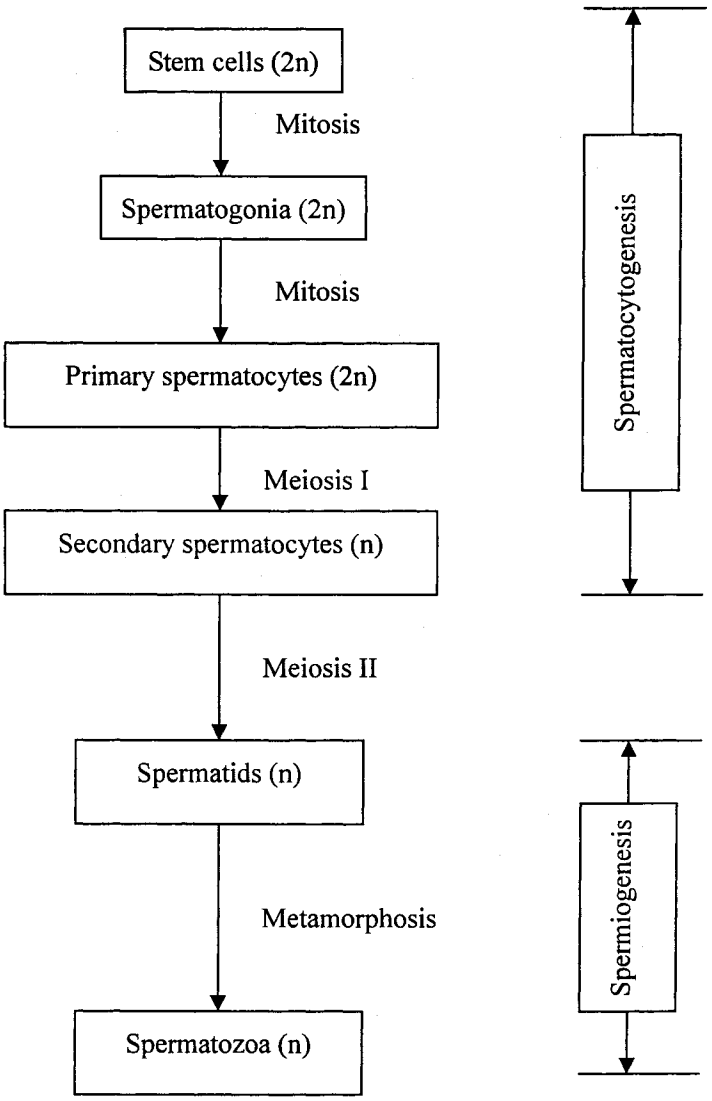

Figure 1-1. Spermatocytogenesis and spermiogenesis.

Sperm maturation and fertilization

Unlike invertebrate spermatozoa, mammalian spermatozoa from the epididymis or freshly ejaculated spermatozoa are unable to fertilize oocytes, even though they are motile. Spermatozoa have to traverse the uterus to the oviduct, overcome retrograde loss, complete capacitation,

achieve hyperactive motility, bind to the oocyte, undergo the acrosome reaction, penetrate the zona pellucida, fuse with the oocyte plasma membrane, and enter the oocytes. Thereafter, the sperm nucleus decondenses and forms a male pronucleus (Senger, 1999, p207-218).

Austin and Chang both reported capacitation in 1951 (Austin 1951; Chang 1951). It is believed that seminal fluid contains decapacitating factors, which are able to inhibit capacitation. *In vivo*, however, female genital tracts have a series of mechanisms to remove these decapacitating factors (Senger, 1999, p213). Briefly, capacitation helps spermatozoa reorganize plasma membrane lipids and proteins, promote sperm hyperactivation and the acrosome reaction. The molecular mechanisms of capacitation are not fully understood. However, it is known that capacitation includes changes in sperm surface, movement of intracellular and extracellular ions, and changes in proteins, including tyrosine phosphorylation (Hardy, 2002, p84).

Cholesterol is an important sterol component of sperm membranes. Cholesterol efflux from the plasma membrane enhances membrane fluidity and induces capacitation (de Lamirande et al., 1997). Mammalian sperm membranes contain many lipids, different lipids located in different membrane leaflets, resulting in membrane asymmetry (Johnson et al., 2000). During capacitation, membrane asymmetry is reorganized, which affects the activity of membrane proteins and ATPase (Hardy, 2002, p84). The alteration of membrane Ca^{2+}-ATPase activity increases intracellular Ca^{2+}; increasing intracellular Ca^{2+} concentrations indirectly or directly activate adenylyl cyclase, increasing the concentration of cAMP, which promotes capacitation-associated protein tyrosine phosphorylation and capacitation. This mechanism is believed to accelerate sperm hyperactivation, characterized by increased velocity but decreased linearity of movement

(Hardy, 2002, p93-98). *In vivo*, hyperactivation helps capacitated spermatozoa penetrate mucoid oviductal secretions, the cumulus oophorus, and the zona pellucida (Longo, 1997, p21-22; Hardy, 2002, p93-98).

The acrosome reaction starts after spermatozoa bind to the zona pellucida of the oocyte (Senger, 1999, p213-217). Spermatozoa bind to ZP3 (ZP3α and ZP3β in pigs, Yurewicz et al., 1998) *in vivo* and initiate the acrosome reaction; hydrolytic and proteolytic enzymes released from the acrosome digest the zona pellucida. Once a spermatozoon has penetrated the zona pellucida, the entire anterior surface of the sperm head is denuded. Spermatozoa that have lost their acrosome cannot bind to the zona pellucida and therefore cannot fertilize an oocyte. Consequently, assessment of acrosomal integrity of ejaculated spermatozoa is an important component of semen analysis.

Storage methods

There are 2 systems for livestock sperm preservation: frozen-storage (cryopreservation) and liquid-storage. The advantage of frozen storage is that semen can be cryopreserved in liquid nitrogen (-196 ^{o}C) for several years, thereby facilitating storage and distribution (Bearden et al., 2004, p211-212). Although cryopreservation of bull semen has been highly successful, AI of frozen-thawed boar semen usually results in low pregnancy rates and small litters (Yoshida, 2000). Boar sperm membranes contain higher concentrations of polyunsaturated fatty acids than other mammalian spermatozoa; these retain membrane fluidity and instability (Flesch and Gadella, 2000). Conversely, boar sperm membranes have a low cholesterol:phospholipid ratio

(Holt, 2000). Collectively, these characteristics make boar spermatozoa highly sensitive to cryopreservation. Additionally, boars produce large volumes of semen with low sperm concentration, further increasing the difficulties associated with frozen-thawed spermatozoa for AI (Bearden et al., 2004, p215).

The advantages of using liquid-stored boar semen are that a lower number of spermatozoa can be used ($3x10^9$/dose compared to $6x10^9$/dose for frozen-thawed semen; Bearden et al., 2004, p215) and the spermatozoa are viable for several days if stored at 15-20 °C. Liquid semen can result in good fertility (Yoshida, 2000) and is in widespread use; more than 99 % of the inseminations conducted worldwide each year are done with liquid extended semen (Johnson et al, 2000). It is noteworthy that storage temperatures below 12 °C resulted in cold shock, with reductions in fertility (Althouse et al., 1998). Although Mulberry extender was effective to store boar semen at lower temperature (5-15 °C; Yoshida, 2000), more efficient methods for prolonged storage, particularly at lower temperatures, are still needed.

1.4 Semen extender

Boar seminal plasma contains fructose, sorbitol, glycerylphosphorylcholine, inositol, citric acid, sodium, potassium, chlorine, calcium, and magnesium (Bearden et al., 2004, p174). These components serve as a buffer and help spermatozoa maintain fertility for a few hours after ejaculation. However, spermatozoa cannot survive for a long time without an appropriate extender. Semen extender media have the following functions: 1) provide nutrients as an energy

source; 2) reduce metabolic rate; 3) minimize the effects of temperature changes (especially cold shock); 4) provide a buffer system to minimize changes in pH; 5) maintain osmotic pressure; 6) inhibit bacteria; and 7) increase semen volume (Foote, 1980, p531; Bearden et al., 2004, p199-200). Extenders can be classified into 2 major groups: short-term (1-3 d) and long-term (> 4 d; Althouse, 1997b). Commonly used extenders include Beltsville Liquid (BL-1), Beltsville Thawing Solution (BTS), and Kiev for short-term storage, and Acromax, Androhep, Mulberry III, X-cell, Zorlesco, and Zorpva for long-term storage (Gadea, 2003).

Extender components

Although the components of most extenders are uncertain due to withholding of proprietary information, basic components are presented in Table 1-1 (Gadea, 2003). Glucose is included in many extenders; it can generate ATP (via many metabolic pathways) to provide energy for spermatozoa. The concentration of fructose in boar seminal plasma is lower than that in bull and ram spermatozoa (Bearden et al., 2004, p178); therefore, a higher concentration of glucose is included in porcine semen extenders. Additionally, higher glucose concentrations can reduce intracellular pH, thereby, helping spermatozoa survive for several days (Johnson et al., 2000).

Sodium citrate and sodium bicarbonate are always used as buffer components. Sodium and potassium are important to maintain equilibrium of ion exchange. Potassium plays a vital role in sperm viability (Bearden et al., 2004, p177-178), and a high potassium:sodium ratio helps maintain membrane integrity. Since calcium is a key factor in fertilization events (including capacitation and the acrosome reaction), it is often absent in semen extender. Furthermore,

ethylenediamine-tetra-acetic acid (EDTA) is included to chelate divalent metal ions, especially calcium ions (Aisen et al., 2000).

Table 1-1. Components of commonly used boar semen extenders (g/L).

	IVT	Kiev	BTS	Zorlesco	Reading	Modena	Androhep
Glucose	3	60	37	11.5	11.5	25	26
Sodium citrate	24.3	3.7	6.0	11.7	11.65	6.90	8.0
EDTA		3.7	1.25	2.3	2.35	2.25	2.4
Sodium bicarbonate	2.4	1.2	1.25	1.25	1.75	1.00	1.2
Potassium chloride	0.4		0.75				
Acetylcysteine	0.05						
HEPES							9.0
BSA				5.0		3.00	2.5
TRIS				6.5	5.5	5.65	
Citrate				4.1	4.1	2.00	
Cysteine				0.1	0.7	0.05	
Trehalose					1		
PVA					1		
mOsm	290	380	330	240	300	282	309
pH		7.2	7.2			6.9	6.8

Source: Reviewed by Gadea, 2003.

Egg yolk and milk are added to semen extenders to protect against cold shock (Foote, 1980, p531; Bearden et al., 2004, p199). Cold shock occurs when semen is cooled rapidly from body temperature down to 15 °C (Johnson et al., 2000); the most critical range of cold shock is from 15 to 0 °C (Bearden et al., 2004, p180). Because of its special membrane characteristics, boar spermatozoa are much more susceptible to cold shock than other spermatozoa from other

domestic animals, e.g. bulls, rams, and stallions. Egg yolk and milk may provide boar spermatozoa with some protection against cold shock. When 2-hydroxypropyl-beta-cyclodextrin (HBCD) was added to BF5 extender, sperm motility, viability, and acrosome membrane integrity were much higher than in unmodified BF5 incubated at 5 °C (Zeng and Terada, 2000). However, none of the semen extenders presently used provides boar spermatozoa with comprehensive protection against cold shock.

In addition to maintaining ion exchange, inorganic ions such as sodium and potassium are included to maintain osmotic pressure. The osmotic pressure for domestic semen is approximately 300 mOsm (Foote, 1980, p531). Both hypotonic and hypertonic conditions result in movement of water through cell membranes and disrupt membrane integrity; therefore, an isotonic extender is required to maximize sperm survival.

Bacterial contamination during semen collection is unavoidable (Althouse et al., 2000). Furthermore, extender components (e.g. glucose) and storage temperature can promote the growth of bacteria. To prevent the growth of microorganisms, antibiotics, e.g. penicillin and streptomycin, are routinely added to extenders (Althouse, 1997b; Bearden, 2004, p202). The pH of freshly ejaculated boar spermatozoa is approximately 6.8 to 7.5 (Bearden et al., 2004, p174), which is close to the pH of body fluids; however, the presence of microorganisms decrease extender pH. Bacterial contamination also decreases motility and alters the acrosome (Althouse et al., 2000).

1.5 Sperm aging and oxidation

The metabolic rate of spermatozoa is proportional to temperature. For example, when bull semen is frozen in liquid nitrogen (-196 °C) the metabolic rate is very slow (Bearden et al., 2004, p180) and semen can be maintained for decades. In boars, however, spermatozoa are usually incubated in extenders above 15 °C, resulting in much higher metabolic rates and accelerated aging of spermatozoa than spermatozoa held at lower temperatures or frozen. In most studies, boar spermatozoa lost motility, viability, mitochondrial activity, and fertility after prolonged storage (Althouse et al., 1998; Cerolini et al., 2000; Huo et al., 2002a; Dubé et al., 2004).

Source of ROS

In addition to aging, oxidative stress has adverse effects on spermatozoa. Reactive oxygen species (ROS) include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), and nitric oxide radical ($NO^{\cdot}$; Alvarez and Storey, 1995; Papas, 1998, p7). Generation of ROS is associated with leukocytes during contamination and inflammation (Baker and Aitken, 2004) and with mitochondrial respiration (Vernet et al., 2004). Low concentrations of ROS are important for cell signal transduction during fertilization (Hardy, 2002, p85). For example, low concentrations of nitric oxide can enhance sperm motility, increase capacitation, and promote sperm-oocyte binding in both human (Zini et al., 1995) and boar spermatozoa (Kodama et al., 1996), whereas superoxide anion initiates capacitation and hyperactivation in human spermatozoa (de Lamirande and Gagnon, 1993). However, leukocytes

kill foreign cells or organisms through the NADPH-oxidase pathway, and may generate superoxide anion and hydrogen peroxide. Hydrogen peroxide and metal ions (e.g. Fe^{2+}), or superoxide anion generate hydroxyl radicals through Fenton reaction or Haber Weiss reaction; these are energetic, highly active radicals (Sikka, 2001) with a short lifespan, but in high concentration they have the potential to impair sperm viability. Superoxide anion also forms the hydroperoxyl radical ($OOH^{\cdot}$), which can cause widespread damage to biomembranes through protonation (Storey, 1997).

$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + HO + HO^{\cdot}$ (Haber Weiss)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO + HO^{\cdot}$ (Fenton)

Excessive generation of ROS results in irreversible oxidative stress in spermatozoa, including loss of motility (Alvarez and Storey, 1982; Alvarez and Storey, 1983; Misro et al., 2004), enhanced premature damage of sperm DNA (Aitken et al., 1998; Potts et al., 2000; Kumar et al., 2002; Sanocka and Kurpisz, 2004), and lipid peroxidation (Twigg et al., 1998; Misro et al., 2004). The sperm midpiece contains mitochondria that provide energy (ATP) for sperm motility; however, the midpiece is also very sensitive to the effects of ROS. Although spermatozoa with damaged nuclear DNA may fertilize oocytes, the damaged DNA may be associated with infertility or with cancer in the offspring (Aitken and Krausz, 2001). As mentioned previously, boar spermatozoa contain high concentrations of polyunsaturated fatty acids (PUFAs), which make them very sensitive to lipid peroxidation. Lipid peroxidation is believed to affect membrane structures and impair membrane integrity (Twigg et al., 1998; Potts et al., 2000). Furthermore, lipid peroxidation is believed to affect protein tyrosine residues and enhance

sperm-tyrosine-kinase-induced signalling, thus inducing premature sperm capacitation (Ford, 2004).

Seminal plasma and antioxidant properties

Seminal plasma contains ascorbate, α-tocopherol, and some antioxidant enzymes such as catalase, superoxide dismutase (SOD), and glutathione reductase to counteract the negative effect of ROS (Alvarez and Storey, 1995). Generally, these substances help protect spermatozoa against oxidative stress (Potts et al., 2000). For example, superoxide anion can be decomposed by SOD, whereas hydrogen peroxide can be decomposed by catalase or glutathione peroxidase (Baumber et al., 2000). However, to maintain fertility, semen must be diluted soon after collection; this dilutes antioxidant concentrations as well as sperm concentration, making spermatozoa more susceptible to damage by ROS.

The longer boar semen is stored *in vitro*, the greater the loss of antioxidants (Cerolini et al., 2000). To extend sperm viability *in vitro*, synthetic antioxidants, e.g. butylated hydroxytoluene (BHT; Roca et al., 2004); have been used as extender additives. Although these synthetic antioxidants may help protect spermatozoa from the deleterious effects of ROS, they are potentially cytotoxic (Ito et al., 1986). Therefore, there is considerable impetus to find products with high antioxidant capacity but low cytotoxicity as additives for semen extenders.

1.6 Lowbush blueberry industry and their bioactivity

Distribution of lowbush blueberries

Blueberries (*Vaccinium spp.*) are perennial crops. Although there are several varieties of blueberries growing in North America and Europe, there are only 6 lowbush blueberry crop areas in the world (including three North American jurisdictions: Quebec, Maritime Canada and Maine; Kalt et al., 2001). It is difficult for wild blueberries to grow in hilly and rocky areas (Prouse, 1997); in Prince Edward Island, most commercial lowbush blueberry fields are concentrated in the Tignish, Wood Islands, Mt Stewart, and Souris areas (Prouse, 1997). Unlike highbush blueberries, lowbush blueberries are usually not well-planted or well-cultivated (Prouse, 1997); therefore, genotype and phenotype of lowbush blueberries differ among areas (Kalt et al., 2001).

Bioactive components and their utilities

Blueberries have a long history of use by aboriginals. Food and medical use of lowbush blueberries were practiced by indigenous people in North America. Currently, lowbush blueberries are becoming a popular fruit in Canada, with export from Atlantic Canada earning over 83.5 million dollars in 2002 (Agriculture and Agri-Food Canada, 2002).

In addition to the well-known antioxidants in fruits and vegetables, such as vitamin C, vitamin E, and carotenoids, many other compounds, e.g. polyphenolics, contribute to the antioxidant capacity of this food, including flavones, isoflavones, flavonones, anthocyanins, and catechins (Wang et al., 1996; Ehlenfeldt and Prior, 2001). The red and blue colors of blueberries

are due to anthocyanin pigment. Anthocyanins are a group of over 500 polyphenolics with diverse structures (McGhie et al., 2003); like other phenolics, they act as antioxidants by scavenging free radicals and chelating metal ions.

Of the 4 cultivar blueberries, *Vaccinium corymbosum* L (highbush), *Vaccinium angustifolium* Aiton (lowbush), *Vaccinium ashei* Reade (Rabbiteye), and *Vaccinium myrtillus* (Bilberry), lowbush blueberries are reported to have the strongest antioxidant capacity, as measured by the oxygen radical absorbance capacity (ORAC) assay (Prior et al., 1998). Based on this fact, a number of studies have examined this fruit for potential health effects against diseases or oxidative stress. Blueberries, spinach, and strawberries were given as dietary supplements to 40 male Fischer 344 rats (19-month-old) for 8 wks (Joseph et al., 1999). The rats had reversals in motor behavioral deficits. Lowbush blueberries protected rats from ischemia-induced brain damage (Sweeney et al., 2002). The intake of anthocyanins through red wine or berry extract has been reported to reduce the risk of coronary heart disease and improve visual function in humans (Renaud and De Logeril, 1992). Six berry extracts (wild blueberry, bilberry, cranberry, elderberry, raspberry seeds, and strawberry) were compared for antioxidant capacity and anti-angiogenic property (Bagchi et al., 2004), the wild blueberry extract and the commercial blueberry product (OptiBerry), which had high antioxidant capacity and low cytotoxic potential, significantly inhibited oxidant-induced expression (such as $H_2O_2^{\cdot-}$ and the tumor necrosis factor-alpha) of human vascular endothelial growth factor. The polyphenols from blueberries were able to protect human endothelial cells against oxidation and inflammation (Youdim et al., 2002). The research discussed above supports the supposition that blueberries, especially lowbush blueberries,

protects against oxidative stress, possibly due to their strong antioxidant capacity. Therefore, it may be possible to use blueberries in other biological systems that require antioxidants such as semen extenders.

Total phenolic contents in highbush blueberries were higher in leaves than in berries (Ehlenfeldt and Prior, 2001); therefore, highbush blueberry leaves may have greater antioxidant capacity. Also, blueberry leaves are left in the field as an underutilized source of phenolics. Therefore, it would be novel to use the lowbush blueberry leaves as the source of polyphenols. Since the relative antioxidant capabilities of lowbush blueberries and leaves are not known, both fruit and leaves were used in this study.

Evaluation of antioxidant capacity

There are many methods to assess antioxidant capacity. High-performance liquid chromatography (HPLC) is used to separate and quantify multiple antioxidant compounds in foods (Kalt et al., 1999; Wada and Ou, 2002). The oxygen radical absorbance capacity (ORAC) is also used to estimate antioxidant capacity in fruit and vegetable samples. R-phycoerythrin (R-PE) is a protein isolated from *Porphyridium cruentum*, which is used as a fluorescent probe. Free radicals damage this probe; the change in the intensity of fluorescence is a measure of the degree of free radical damage. Therefore, this probe can be used to measure antioxidant capacity (Brunswick Laboratories, 2003a). At present, the improved ORAC assay can be used to measure the antioxidant capacity against both peroxyl radical and hydroxyl radical. Researchers use Trolox, an analogue of vitamin E, as the standard to estimate phenolic antioxidant activity

against peroxyl radical (Prior et al., 1998; Kalt et al., 1999; Mazza et al., 2002; Zheng and Wang, 2003); gallic acid, however, is used as the standard to estimate antioxidant activity against hydroxyl radical (Brunswick Laboratories, 2003b).

At present, estimation of total phenolics and total anthocyanins are commonly used to quantify polyphenols of edible plants (Zheng and Wang, 2003; Prior et al., 1998; Kalt et al., 2001). The Folin-Ciocalteu assay (Slinkard and Singletonon, 1977) evaluates total phenolics in fruit and vegetables using gallic acid equivalent (Ehlenfeldt and Prior, 2001) or ferulic acid equivalent (Sellappan et al., 2002), whereas the pH differential assay (Wrolstad, 1993) evaluates total anthocyanins using malvidin-3-glucoside equivalent (Kalt et al., 1999) or cyanidin-3-glucoside equivalent (Wada and Ou, 2002; Howard et al., 2003). Phenolic compounds can be oxidized by the yellow molybdotungstophosphoric heteropolyanion reagent and generate the molybdotungstophosphate blue pigment; the pigment is measured to estimate the concentration of phenolic compounds. At pH 1.0, anthocyanins exist in highly colored oxonium or flavilium forms; however, at pH 4.5, they turn to the colorless carbinol form. Therefore, the difference in absorbance between pH 1.0 and pH 4.5 at different wavelengths is used to estimate the concentrations of anthocyanin compounds in samples (Wrolstad, 1993).

1.7 Evaluation of sperm quality

The following measurements are routinely performed to assess sperm quality: semen morphology, motility and viability, lipid peroxidation, mitochondrial activity, capacitation,

acrosome intactness, and fertility.

Semen morphology, motility, and viability

Sperm morphology is routinely assessed by a light microscope. In boars with good fertility, > 60% of spermatozoa are morphologically normal (Bearden et al., 2004, p173-174). Morphologically abnormal spermatozoa usually have functional deficits and may result in reduced fertility.

Sperm motility can be evaluated by light microscopy; however, accuracy and precision can be increased using a computer automated semen analyzer (CASA; Suzuki and Nagai, 2003). For boar spermatozoa, 50 to 80 % progressive motility is acceptable (Bearden et al., 2004, p185). Although criteria for evaluating the rate of progression varies among laboratories, it can usually be divided into the following categories: 0) no sperm movement; 1) slight tail undulation without forward motion; 2) slow tail undulation with slow or stop and start forward motion; 3) forward progression at a moderate speed; 4) rapid forward progression; 5) very rapid progression (Interpretation of semen evaluation, University of Saskatchewan).

Sperm viability can be determined by many methods. Trypan Blue stains dead spermatozoa, but is unable to stain live spermatozoa (Zou and Yang, 2000). The hypoosmotic swelling test (HOST) is another method to determine viability. Spermatozoa with intact membranes have coiled tails after incubation with a hypoosmotic solution, whereas spermatozoa with damaged membranes do not exhibit coiled tails (Garcia-Lopez et al., 1996; Zou and Yang, 2000). Ethidium bromide (EtBr) and digitonin was widely used in 1980's and 1990's (Bilgili and Renden, 1984;

Bakst et al., 1990). The principle of this method is that the ethidium bromide penetrates damaged sperm membranes, binds to the double-stranded DNA and fluoresces measured with a fluorometer. Digitonin is used to disrupt sperm membranes, helping the ethidium bromide penetrate the membranes, and bind to the double-stranded DNA. The SYBR-14/propidium iodide (PI) staining assay is a new and accurate method that has been widely used to assess sperm viability for many species (Maxwell and Johnson, 1997; Zeng et al., 2001). Spermatozoa with intact membranes are stained by SYBR-14 and exhibit a bright green fluorescence, whereas spermatozoa with damaged membranes allow entry of the PI and have a bright red fluorescence. Rowland et al. (2003) reported the use of Hoechst 33342/PI to evaluate sperm viability and DNA apoptosis. Hoechst 33342 is a bisbenzimide stain; it stains damaged spermatozoa and provides a bright blue fluorescence, whereas live cells are not stained by Hoechst 33342 dye exhibiting unstained or only slightly blue. Different laboratories use either fluorescent microscopy (Huo et al., 2002a, b) or flow cytometry (Garner and Johnson, 1995) to measure stained spermatozoa. To use flow cytometry increases precision because this technique allows counting of thousands of sperm cells, whereas only 200 sperm cells are counted when using fluorescent microscopy.

Lipid peroxidation

Lipid peroxidation during *in vitro* storage is measured by the thiobarbituric acid reaction substances (TBARS) assay (Alvarez and Storey, 1982; Alvarez et al., 1987). Lipids decompose in the presence of free radicals; lipid peroxidation can be accelerated in the presence of ferrous ions and ascorbic acid. A major substance of the TBA (thiobarbituric acid) reaction is

malondialdehyde (MDA; Aitken et al., 1993); 1 mole of MDA reacts with 2 moles of TBA and forms a trimethine colored substance (Aitken et al., 1993). There is a linear increase in pigment concentration due to increasing concentrations of MDA (Kodama et al., 1996); this can be measured with a fluorometer as absorbance (MDA standard). In addition to MDA, other products, e.g. aldehyde, can bind to TBA and fluoresce at the same wavelengths. However, the limitation can be improved by HPLC technique (Sim et al., 2003). By HPLC, the peak of MDA-TBA adducts exhibit in a certain retention time, and can be separated from other complexes.

Mitochondrial activity

The energy driving the synthesis of ATP is derived from the oxidation reactions of the mitochondrial respiratory chain. Rhodamine 123 (R123) is used with propidium iodide (PI) or ethidium bromide (EtBr) to evaluate mitochondrial activity. R123 is a cationic compound; it accumulates in the mitochondria and fluoresces green, indicating the presence of an active membrane potential. However, dead or membrane-damaged spermatozoa can be stained by PI or EtBr (Garner et al., 1997). Unlike R123, the 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) indicates the presence of a mitochondrial membrane potential from low to high, according to its alteration from the monomer (fluorescent green) to the J-aggregate (fluorescent red-orange); a high proportion of red-orange relative to green fluorescence indicates a high mitochondrial membrane potential (Cossarizza et al., 1996; Garner et al., 1997).

Sperm capacitation status

Sperm capacitation is measured by the chlortetracycline (CTC) staining method. The molecular mechanism of the CTC assay is unknown. However, CTC combines with Ca^{2+}, due to the massive calcium alterations during capacitation; the CTC-Ca^{2+} complex reveals the distribution of calcium and capacitation status under a fluorescence microscope (Huo et al., 2002a,b). Capacitation status can be categorized as: 1) F, a uniform fluorescence of the entire head, consistent with uncapacitated, acrosome-intact spermatozoa; 2) B, a fluorescence-free band in the postacrosomal area, indicating capacitated but acrosome-intact spermatozoa; and 3) AR, fluorescence absent from the head, indicating acrosome-reacted spermatozoa. Hoechst 33258 is used with CTC; the dual-stain assay enables evaluation of both viability and capacitation status, thus enhancing sensitivity (Wang et al., 1996; Maxwell and Johnson, 1997; Green and Watson, 2001).

Fluorescein isothiocyanate-peanut agglutinin (FITC-PNA) or fluorescein isothiocyanate-pisum agglutinin (FITC-PSA) are used to measure acrosome integrity. After permeabilizing with Triton X-100, spermatozoa with uniform bright green (using PNA) or blue (using PSA) fluorescence have an intact outer acrosome membrane; spermatozoa with disrupted fluorescence of the acrosome cap have partial disruption of the acrosome membrane, whereas spermatozoa with no fluorescence have lost their acrosome membrane (Zeng and Terada, 2000; de Cuneo et al., 2004). Coomassie blue staining is also used to measure acrosome integrity (Zou and Yang, 2000); spermatozoa with an intact acrosome membrane are blue, whereas spermatozoa with a disrupted acrosome membrane cannot be stained by the Coomassie blue dye. Although

subjective visual assessment of spermatozoa is commonly done, flow cytometry can be used to objectively evaluate large numbers of spermatozoa, thereby improving precision.

Assessment of fertility parameters

The end point of semen preservation is to fertilize oocytes; therefore *in vitro* fertilization (IVF) can be used to evaluate sperm fertility (Kidson et al., 2003; Schoevers et al., 2003). Cumulus-free oocytes are matured *in vitro*, transferred to IVF medium, and incubated with capacitated spermatozoa at 38.5 ^{o}C in the presence of 5% CO_2. After co-incubation for 48 h, oocytes are collected, and sperm fertility can be determined by measuring the cleavage rate (Roca et al., 2004).

1.8 Objectives

As previously stated, boar semen is difficult to cryopreserve, therefore, it is usually stored at 15-18 ^{o}C (Althouse, 1997a). At these temperatures, there are many alterations, including lipid peroxidation of membranes, degeneration of mitochondrial ATP, premature capacitation, and the acrosome reaction. To compensate for loss of fertilizing spermatozoa, a large number of spermatozoa are used in the insemination dose, which decreases the number of doses per ejaculate and increases cost. In addition, to store boar spermatozoa for < 1 wk makes long-distance transportation more difficult. Therefore, it is critical to develop extenders that protect boar spermatozoa from oxidative injury and improve sperm viability during long-term

storage *in vitro*. Lowbush blueberries are edible fruits with high concentrations of antioxidants that have been shown to protect against both *in vitro* and *in vivo* free radicals. Therefore, lowbush blueberries may be a new and effective additive in semen extenders to retard lipid peroxidation, cold shock, and other oxidation-induced injuries.

The overall objective of this study was to determine the efficacy of using lowbush blueberries, blueberry powder, and leaves to protect boar spermatozoa during *in vitro* storage. Our hypothesis was that, by acting as an antioxidant, lowbush blueberry products can protect boar spermatozoa from oxidation, membrane damage and premature capacitation during long-term storage better than commercial extenders.

The specific objectives of this study were to determine:

1) the concentration of the bioactive compounds in blueberry products (fruits, powder and leaves) using 3 extraction solutions (modified ethanol, methanol and water)
2) the effects of blueberry products on sperm lipid peroxidation, viability, motility, and capacitation status during *in vitro* preservation.

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2.0 Analysis of total phenolics and anthocyanins in lowbush blueberry (*Vaccinium angustifolium* Aiton) products

2.1 Introduction

Vegetables and fruit are major food sources for humans. There are approximately 15,000 vegetable growers in Canada; in 2002, Canada produced 7 million tonnes of vegetables, worth CAN $2.4 billion (Sereda, 2004). There are approximately 16,000 fruit growers in Canada; in 2002, Canada produced 677,900 tonnes of fruit, worth $517 million (Leclair, 2004). The per capita consumption of fresh fruits, canned fruits, frozen fruit, and fruit juice among Canadians in 2002 were 67.5, 5.2, 1.9, and 27.7 kg, respectively (Canadian Statistics, 2004a). The per capita consumption of fresh vegetables, canned vegetables, frozen vegetables and juice vegetables in Canada were 139.5, 13.4, 5.7, and 1.5 kg, respectively in 2002 (Canadian Statistics, 2004b).

Increasing consumption of vegetables and fruit is associated with a decreasing evidence of many chronic diseases, including cardiovascular disease (Bazzano et al., 2002; Mennen et al., 2004), lung cancer (Knekt et al., 1997), breast and colon cancer (Grandics, 2003), leukemia (Katsube et al., 2003; Kennedy et al., 2004), diabetes (Sabu and Kuttan, 2002; Ylönen et al., 2003), stroke (Sweeney et al., 2002), and neurodegenerative disease (Joseph et al., 1999). Some of these diseases are associated with the presence of free radicals and oxidative stress; therefore, it is expected that vegetables and fruits containing favourable concentrations of antioxidants can help mitigate free radicals and oxidative stress (Ehlenfeldt and Prior, 2001).

The antioxidant properties of fruit and vegetables were classically attributed to vitamin C, vitamin E, and carotenoids (Prior et al., 1998). However, more recently, it was shown that the content of antioxidant substances is poorly related to total antioxidant capacity. Prior et al., (1998) and Kalt et al., (1999) reported that both highbush blueberries and lowbush blueberries have substantial concentrations of antioxidant activity as measured by the oxygen radical absorbance capacity (ORAC) method. The concentration of ascorbic acid was lower than expected. This has led to the search for other antioxidants in vegetables and fruit. In addition to vitamin C, vitamin E and carotenoids, other potential antioxidant compounds e.g. polyphenols, including flavonoids, flavones, anthocyanins, and catechins (Wang et al., 1996) are present in substantial concentrations in these edible plants. Polyphenol antioxidants have a strong capacity to donate electrons to terminate the redox reaction and they have recently been identified as major antioxidants of vegetables and fruit (Prior et al., 1998; Wada and Ou, 2002).

At present, estimation of total phenolics, total anthocyanins and oxygen radical absorbance capacity are commonly used methods to quantify antioxidant capacity of edible plants (Prior et al., 1998; Kalt et al., 2001; Zheng and Wang, 2003). High-performance liquid chromatography (HPLC) is used to identify and quantify multiple antioxidant compounds in these foods (Kalt et al., 1999; Sellappan et al., 2002; Wada and Ou, 2002). Another method to quantify antioxidant capacity is the Trolox-equivalent antioxidant capacity (TEAC) method (Sellappan et al., 2002); both the ORAC and TEAC were strongly associated with total phenolics and total anthocyanins.

Blueberry fruits compare favourably in antioxidant capacity with numerous other berries, e.g., raspberries (*Fragaria* x *ananassa* Duch.), strawberries (*Rubus idaeus* Michx) (Kalt et al.,

1999), bilberries (*Vaccinium myrtillus*), and rabbiteye blueberries (*Vaccinium ashei*) (Prior et al., 1998). Kalt et al. (2001) compared highbush blueberries (*Vaccinium corymbosum* L.) and lowbush blueberries (*Vaccinium angustifolium* Aiton); they reported that lowbush blueberries were higher in total anthocyanins and total phenolics than highbush blueberries. Because the antioxidant capacity has a strong relationship with the concentration of total phenolics and anthocyanins (Kalt et al., 1999), it is possible that lowbush blueberries contain higher antioxidant capacity. Therefore, lowbush blueberries may be more useful than highbush blueberries to prevent or decrease oxidative damage in *in vitro* systems.

Both the method of extraction and the extraction solution influence the concentration of bioactive compounds in blueberry extracts (Kalt et al., 2001). To maintain the concentration of bioactive compounds during storage, acids were added to some extraction solutions because some bioactive compounds, e.g. anthocyanins, are unstable in an alkaline environment (Kalt et al., 2000). For example, 3 extraction solutions: 1) 96 % acetonitrile and 4 % acetic acid; 2) 88 % methanol, 12 % water and 0.1 % formic acid; and 3) 40 % methanol, 20 % water, 40 % acetone and 0.1 % formic acid, were used to extract bioactive compounds from lowbush and highbush blueberries (Kalt et al., 2001). Ninety-six percent acetonitrile and 4 % acetic acid were used to obtain extracts from highbush blueberries, lowbush blueberries, rabbiteye, and bilberries (Prior et al., 1998). Pure acetone (1:7 w/v) was used to obtain extracts from grapes, strawberries and plums (Wang et al., 1996). Eighty percent aqueous methanol and 1 % hydrochloric acid were used to obtain extracts from blueberries and cherries (Velioglu et al., 1998). In addition, various mechanical methods have been used to extract bioactive compounds from berries. For example,

an orbital shaker (Velioglu et al., 1998), a blender, and a homogenizer (Heinonen et al., 1998; Prior et al., 1998; Zheng and Shetty, 2000) are usually used for compound extraction, and clear supernatant was obtained by filtration (Zheng and Shetty, 2000) or a centrifuge (Prior et al., 1998).

Like methanol, acetonitrile and acetone, ethanol is another organic solvent that is commonly used when measuring the antioxidant capacity of fruit (Zheng and Shetty, 2000). Water might be less efficacious than an organic solvent for extracting organic compounds. However, water is less cytotoxic than most organic solvents. The overall goal of this study was to use semen extender containing blueberry extracts to culture sperm cells *in vitro*; therefore, the blueberry additive in semen extender should be minimally toxic. Since the chemical molecular formulae of methanol, ethanol and water are somewhat similar (same hydroxyl group linked with different chemical chains), these 3 extraction solutions were used in this study.

The antioxidant content of both highbush and lowbush blueberry fruit has been reported by many researchers. Enlenfeldt and Prior (2001) evaluated the antioxidant capacity of highbush blueberries and their leaves. However, the direct comparison of lowbush blueberry fruit and their leaves has not been reported. Therefore, both blueberry fruit and leaves were used in this study. Blueberry powder made from freeze-dried fruit was also used. The objectives of this study were to compare: 1) the ability of acidified methanol, acidified ethanol, and acidified water solutions to extract antioxidant compounds from blueberry samples; and 2) the concentrations of antioxidant compounds in blueberry fruit, leaves, and powder when using the same extraction solution.

2.2 Materials and methods

Sampling Procedures

Lowbush blueberries were harvested fresh from PEI wild grower farms in late August, 2003. Green, overripe and damaged berries were discarded and berries from multiple sources combined to minimize variation from phenotypic diversity (Kalt et al., 2001). Samples were transferred to the laboratory at the Atlantic Veterinary College and frozen (-20 °C) in plastic bags until they were used for extraction. Blueberry leaves that were green were also harvested fresh from the same farms, and damaged leaves were discarded before they were extracted. Samples were combined to minimize variation, transferred to the laboratory, and frozen (-20 °C) in plastic bags until they were used for extraction.

Blueberry powder was made using the following procedure. Two-hundred-and-fifty grams of frozen berries were weighed, brought to the Food Technology Center (Charlottetown, PEI, Canada), deeply frozen (-80 °C) and transferred into a freeze dryer (FD8 LABCONCO Freeze-dryer 8, LABCONCO Co., Kansas City, MO, USA) with the vacuum pump (D8A LEYBOID TRIVAC, LABCONCO Co., Kansas City, MO, USA) for 4 d to remove water. Dried berries were ground using a ZM 100-grinding mill (Retsch Inc., Newtown, PA, USA) with 1 L of liquid nitrogen to maintain a low temperature. Blueberry powder was frozen (-20 °C) in a sealed plastic bag until it was used for extraction.

Reagents

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Acidified methanol was made by mixing anhydrous methanol (Fisher Scientific, Nepean, ON, Canada), deionized distilled water and acetic acid (normality approx. 17.45N; Fisher Scientific) in a ratio of 80:19:1 (v:v:v). Acidified ethanol was made by mixing anhydrous ethanol (Commercial Alcohols Inc, Brampton, ON, Canada), deionized distilled water and acetic acid in a ratio of 80:19:1 (v:v:v). Acidified deionized distilled water was made by mixing deionized distilled water and acetic acid in a ratio of 99:1 (v:v).

The pH 1.0 working solution was made by mixing 0.2 M potassium chloride solution and 0.2 N hydrochloric acid in a ratio of 25:77 (v:v); and the pH was adjusted to 1.0 by adding 0.1 N hydrochloric acid by the pH meter (Accument® Model 815MP, Fisher Scientific, Pittsburgh, PA, USA). A pH 4.5 working solution was made by mixing 1.0 M sodium acetate solution, 1.0 N hydrochloric acid and deionized distilled water in a ratio of 10:6:9 (v:v:v). The pH was adjusted to 4.5 (Wrolstad, 1993) by adding 1.0 N hydrochloric acid. Folin-Ciocalteau (2.0 N), sodium carbonate, and gallic acid were purchased for the Folin-Ciocalteau assay.

Extraction procedure

A 10 g fresh frozen blueberry fruit sample was transferred to a 50 mL centrifuge tube, 20 mL extraction solution (acidified methanol, acidified ethanol or acidified deionized distilled water) was added to the tube, and the sample was homogenized using a homogenizer (Brinkmann, PT10-35 Polytron, Lucerne, Switzerland) for 2 min. The centrifuge tube was

incubated in an ice bath during homogenization to minimize potential loss of bioactive compounds. The mixture was separated by centrifugation (Allegra 6KR centrifuge, Beckman Inc., Palo Alto, CA, USA) at 4 °C for 15 min at 1,667 x g. All of the clear supernatant was transferred to a 50 mL volumetric flask. The pellet was resuspended with another 20 mL of extraction solution. The pellet was again homogenized and centrifuged as above, and the supernatant was transferred to the same volumetric flask. Extraction solution was added to the same volumetric flask to bring the volume of the extract up to 50 mL. Aliquots of fruit extracts were stored at -20 °C in 15 mL centrifuge test tubes or 2 mL microcentrifuge tubes.

A 1.5 g sample of frozen blueberry powder was extracted by the same procedure as the fruit. Powder extract was stored at -20 °C in several 15 mL centrifuge test tubes or 2 mL microcentrifuge tubes for later use.

Blueberry leaves were ground in a coffee grinder and a 1.5 g sample was extracted using the same procedure as for fruit and powder. Leaf extract was stored at -20 °C in several 15 mL centrifuge test tubes or 2 mL microcentrifuge tubes for later use.

Experimental design

The antioxidant activity of 3 different extraction solutions (acidified methanol, acidified ethanol and acidified water) with 3 blueberry products (blueberries, leaves and powder from freeze-dried blueberries) was estimated by 2 methods (total phenolics and total anthocyanins). Each extracted sample of 9 combinations (3 extraction solutions by 3 blueberry products) was measured with 2 replications to improve precision.

Experiment 1: Estimation of total phenolics in 3 blueberry products using 3 extraction solutions. Total phenolics were estimated colorimetrically using the Folin-Ciocalteau method (Singleton and Rossi, 1965; Sellappan et al., 2002). A 200 μL aliquot of sample (fruit extract, powder extract or leaf extract; 2 replicates) was pipetted into a 15 mL test tube and mixed with 800 μL of deionized distilled water, 5 mL of 0.2 N Folin-Ciocalteau solution, and 4 mL of saturated sodium carbonate solution (75g/L). The test tube was covered with parafilm, mixed by inversion and incubated in a cool dry place for 2 h in the dark. Thereafter, 3 mL of the mixture was transferred to a 4 mL cuvette and absorbance was measured at 765 nm with a UV-visible Shimazdu spectrophotometer (UV-1601PC, Mandel Scientific Co. Ltd., Kyoto, Japan). Quantification of gallic acid equivalents (3 replicates) in the extract was based on the gallic acid standard curve generated with 0.02, 0.04, 0.06, 0.08, and 0.10 g per 100 mL of gallic acid (Appendix A). Since leaf extracts had a substantially higher concentration of total phenolics, leaf extract samples were diluted 1:1 before they were mixed with other reagents.

Experiment 2: Total anthocyanins in 3 blueberry products using 3 extraction solutions. Total anthocyanins were estimated using the UV-visible Shimazdu spectrophotometer (UV-1601PC, Mandel Scientific Co. Ltd., Kyoto, Japan) by the pH-differential method (Wrolstad, 1993; Kalt et al., 1999). A 100 μL aliquot of blueberry sample (fruit, leaf or powder extract; 2 replicates) was added to a 4 mL cuvette and mixed with 3900 μL of either the pH 1.0 or pH 4.5 working solution. Cuvettes were covered with parafilm and mixed by inversion. The mixture was incubated for 2 h

in the dark at room temperature. Absorbance of both buffers at 520 nm and 700 nm was read from the spectrophotometer.

Absorbance was calculated as (Wrolstad, 1993):

$A = (A_{520nm}-A_{700nm})_{pH\ 1.0} - (A_{520nm}-A_{700nm})_{pH\ 4.5}$

Concentration of anthocyanins (mg/L) in solution was calculated as malvidin 3-glucoside equivalents using the following formula (Wrolstad, 1993):

A x MW x DF x 1000 / (ε*1)

Where A=absorbance, MW= molecular weight of malvidin 3-glucoside (493.5), DF = dilution factor and ε= molecular coefficient for malvidin 3-glucoside (28000). Then, the concentration of anthocyanins (mg/L) in the extraction solution was back-transformed as mg/g of sample weight.

Statistics

The relationship between the concentration of gallic acid equivalent and absorbance was analyzed using a linear regression model (Minitab Version 13). For total phenolics and total anthocyanins, 2-way ANOVA (general linear mixed model; Minitab Version 13) was used to determine the effects of extraction solutions, blueberry products, and their interaction. Results were presented as means ± standard error of the means (S.E.M.). If the effects of extraction solutions, blueberry products or their interaction were statistically significant, Bonferroni comparisons were utilized to locate differences. Statistical significance was set at $\alpha=0.05$.

2.3 Results

Experiment 1: Estimation of total phenolics in 3 blueberry products using 3 extraction solutions.

The linear regression between the GAE concentration and the absorbance units is presented in Appendix A. The absorbance units had a strongly positive association with the GAE concentration (R^2=99.9 %, P<0.001). There were effects of extraction solutions, blueberry products, and their interaction on the concentration of total phenolics (P<0.001 for each). The concentration of total phenolics in blueberry fruit, blueberry leaves and blueberry powder using the 3 extraction solutions is presented in Table 2-1. Generally, concentrations were higher when using acidified ethanol or acidified methanol as extraction solutions than using acidified water (P<0.05). In blueberry fruit samples, the value of total phenolics was higher in acidified ethanol than in acidified methanol (P<0.05). However, in leaf samples and powder samples, the values of total phenolics were higher in acidified methanol (P<0.05). Considering the effect of blueberry products, the values of total phenolics were higher in blueberry leaf samples (P<0.05) than in blueberry powder samples and blueberry fruit samples (P<0.05), regardless of the extraction solutions.

Experiment 2: Total anthocyanins in 3 blueberry products using 3 extraction solutions.

There were effects of extraction solutions, blueberry products and their interaction (P<0.001 for each). The concentration of total anthocyanins in blueberry fruit, blueberry leaves, and blueberry powder using the 3 extraction solutions is presented in Table 2-2. The concentrations

were higher when using acidified ethanol or acidified methanol than using acidified water ($P<0.05$). Considering the effect of blueberry products, concentrations were higher in blueberry powder samples ($P<0.05$) than in blueberry fruit samples and leaf samples, regardless of extraction solutions.

Table 2-1. Total phenolics of blueberry fruit, powder and leaves in 3 extraction solutions (mg of gallic acid equivalent/g of product weight[1,2])

Product	Acidified water[3]	Acidified ethanol[4]	Acidified methanol[5]
Fruit	$1.98\pm0.10^{a\,A}$	$3.95\pm0.09^{b\,A}$	$3.83\pm0.03^{b\,A}$
Powder	$14.05\pm0.06^{a\,B}$	$24.43\pm0.26^{b\,B}$	$26.48\pm0.32^{c\,B}$
Leaves	$52.50\pm0.41^{a\,C}$	$60.60\pm0.13^{b\,C}$	$66.83\pm0.78^{c\,C}$

Values are presented mean $\pm$ S.E.M.

There were effects of products, extraction solution, and their interaction ($P<0.001$) for each.

[a-c] Within a row, means without a common superscript were different ($P<0.05$).

[A-C] Within a column, means without a common superscript were different ($P<0.05$).

[1]Blueberry fruit and leaves were evaluated by wet weight.

[2]Blueberry powder was evaluated by dry weight.

[3]Acidified water: 99 % deionized distilled water, 1 % acetic acid.

[4]Acidified methanol: 80 % methanol, 19 % deionized distilled water, 1 % acetic acid.

[5]Acidified ethanol: 80 % ethanol, 19 % deionized distilled water, 1 % acetic acid.

2.4 Discussion

Effect of extraction solutions

In the present study, 3 extraction solutions were used to extract bioactive compounds from blueberry products. Concentrations of total phenolics were higher in acidified methanol followed by acidified ethanol, whereas concentrations in acidified water were significantly lower; these

relationships persisted, regardless of blueberry product tested. For total anthocyanins, the relationships also persisted, regardless of blueberry products. Therefore, acidified methanol and acidified ethanol were more efficacious than acidified water for extraction of antioxidant compounds.

Table 2-2. Total anthocyanins of blueberry fruit, powder and leaves in 3 extraction solutions (mg of malvidin 3-glucoside equivalent/g of product weight[1,2]).

Product	Acidified water[3]	Acidified ethanol[4]	Acidified methanol[5]
Leaves	$0.10 \pm 0.01^{a\,A}$	$0.62 \pm 0.03^{b\,A}$	$0.49 \pm 0.01^{b\,A}$
Fruits	$0.54 \pm 0.03^{a\,B}$	$2.35 \pm 0.02^{b\,B}$	$2.45 \pm 0.13^{b\,B}$
Powder	$2.79 \pm 0.04^{a\,C}$	$16.21 \pm 0.08^{b\,C}$	$16.96 \pm 0.10^{c\,C}$

Values are mean $\pm$ S.E.M.

There were effects of products, extraction solution, and their interaction ($P<0.001$) for each.

[a-c] Within a row, means without a common superscript were different ($P<0.05$).

[A-C] Within a column, means without a common superscript were different ($P<0.05$).

[1]Blueberry fruit and leaves were evaluated by wet weight.

[2]Blueberry powder was evaluated by dry weight.

[3]Acidified water: 99 % deionized distilled water, 1 % acetic acid.

[4]Acidified ethanol: 80 % ethanol, 19 % deionized distilled water, 1 % acetic acid.

[5]Acidified methanol: 80 % methanol, 19 % deionized distilled water, 1 % acetic acid.

Currently, many solutions are used to extract antioxidant compounds for blueberries, e.g. 4 % acetic acid in acetonitrile (Sellappan et al., 2002), 80 % aqueous methanol (Velioglu et al., 1998), 70 % aqueous acetone (Smith et al., 2000), and methanol mixed with formic acid and water (70:2:28; Gao and Mazza, 1995). Ethanol is another efficient organic extraction solvent; it dissolved total phenolics which are also organic. The chemical molecular formulae of ethanol, methanol and water are CH_3CH_2OH, CH_3OH and HOH, respectively. Although all 3 have an

identical hydroxyl group, this hydroxyl group is linked with different chains. With longer carbon chain solvents, the extraction capacity for organic molecules will be stronger. This may account for the different extraction properties of these three extraction solutions with regard to total phenolics and total anthocyanins from blueberry products.

Kalt et al. (2001) estimated the total phenolics in lowbush blueberries using 3 extraction solutions: 1) 96 % acetonitrile with 4 % acetic acid; 2) 88 % methanol, 12 % water and 0.1 % formic acid; and 3) 40 % methanol, 20 % water, 40 % acetone with 0.1 % formic acid. They reported that the total phenolics were 2.97, 3.74, and 3.24 mg GAE/g of fresh fruit weight, respectively. Our results agreed with those of Kalt et al., although the value of total phenolics in our acidified methanol (3.83 mg GAE/g of fresh fruit weight) seemed higher than theirs. Many factors, including growing season, variety, climatic conditions, geographic locations, soil type, plant disease and maturity can influence the concentration of phenolic compounds (Sellappan et al., 2002). In addition, modified methods and extraction processing may also influence the values of total phenolics. In the previous study (Kalt et al., 2001), the total phenolic concentration in the 2 acidified methanol extraction solutions were both one-fold greater than in acetonitrile extraction solutions; perhaps the extraction capacity of acidified methanol was greater than acidified acetonitrile.

Visible color differences may be a direct sign of extraction capacity of the 3 extraction solutions. For example, leaf extract in acidified water was light red, whereas leaf extract in acidified ethanol and acidified methanol was bright green (chlorophyll). Furthermore, the color of leaf extract in acidified ethanol was always darker than the leaf extract in acidified methanol.

The color of blueberry fruit extract and powder extract in the 3 extraction solutions was red or purple. However, the color in ethanol was darker than the color in methanol and the color in methanol was darker than the color in water. Therefore, the 3 extraction solutions might have different abilities to extract bioactive compounds from blueberry products. Further studies including the use of HPLC, should be done to clarify whether the different values of total phenolics and total anthocyanins are related to the different visible color.

Effect of blueberry products

Blueberry leaves are of considerable interest due to their high level of bioactive compounds. Ehlenfeldt and Prior (2001) reported concentrations of total phenolics in highbush blueberry leaf tissues; however, no previous study has estimated the concentrations of total phenolics and anthocyanins in lowbush blueberry leaves. In Ehlenfeldt and Prior's study, the average value for total phenolics in leaves was 44.8 mg GAE/g of leaf wet weight, and the average value for total phenolics in fruits was 0.95 mg GAE/g of fruit wet weight; therefore, the concentrations of total phenolics in blueberry leaves were 47-fold greater than that in blueberry fruits. In our study, both total phenolics in lowbush blueberry leaves and fruits seemed higher than Ehlenfeldt's, and the concentration of total phenolics in blueberry leaves was 20-fold (average) greater than that in blueberry fruits. Our leaf/fruit ratio seemed lower than Ehlenfeldt's.

Although they share the name "blueberry", highbush and lowbush blueberries are distinctly different in their concentration of phenolic compounds (Kalt et al., 2001). Therefore, differences between the present study and the previous study might be attributed to species. Also, some

influence might derive from environmental effects, such as soils, growing season and plant maturity (Howard et al., 2003). In both the present study and in the work of Ehlenfeldt and Prior (2001), the concentration of total phenolics in blueberry leaves was several fold higher than that in blueberry fruits, regardless of their species. Ideally, total phenolics in lowbush (and highbush) blueberry leaf extract and in lowbush (and highbush) blueberry fruit extract should be determined simultaneously under identical conditions.

In the present study, the concentration of total phenolics in blueberry powder was approximately 7-fold greater than that in fruits. Since the total phenolics in blueberry powder were back-transformed to dry weight, but the total phenolics in blueberry fruits were back-transformed to wet weight, it is somewhat arbitrary to directly compare their total phenolic levels. However, in the present study, the overall goal was to use the extracts from blueberry products (fruit, powder and leaves) and determine whether they enhanced the preservation of boar spermatozoa; the concentrations of bioactive compounds in the 3 blueberry extracts were of greatest interest. Therefore, we did not back-transform powder to wet weight. On the contrary, we measured the powder by dry weight.

Kay and Holub (2002) reported that 100 g of dried blueberry powder was the equivalent of approximately 500 – 650 g of whole blueberries; therefore, blueberry fruits lost approximately 80 to 85 % (weight) of aqueous components during freeze-drying. The bioactive content in 1 g of blueberry powder should equal that from 5 to 6.5 g of blueberry fruits. This background could provide some information on powder/fruit ratio. In future studies, powder weight should be recorded and compared to fresh fruit weight. Also, it should be more accurate to freeze-dry

blueberry leaves as well and all 4 blueberry products (fresh fruits, freeze-dried fruits, fresh leaves and freeze-dried leaves) can be estimated under the same conditions.

The anthocyanins are a large class of antioxidant compounds. To estimate anthocyanin concentrations in lowbush and highbush blueberries, cyanidin-3-glucoside (Prior et al., 1998; Kalt et al., 2001; Sellappan et al., 2002) and malvidin 3-glucoside (Kalt et al., 1999) are the 2 equivalents that are commonly used. In the present study, malvidin 3-glucoside was used as the equivalent to estimate total anthocyanins in lowbush blueberries, powder, and leaves.

In the present study, powder had the highest concentration of anthocyanins, followed by fruit and then leaves. Anthocyanins, a color pigment, are reported only in lowbush blueberry peel (Kalt and Dufour, 1997). Both blueberry fruits and blueberry powder contain peel content, and a dark blue color, which was less pronounced in leaves. Therefore, the concentrations of total anthocyanins in fruits and powder should be higher than that in leaves. The average ratio of total anthocyanins in powder and fruits was approximately 6.3, the average ratio of anthocyanins in fruits and leaves was approximately 4.7, and the ratio of anthocyanins in powder and leaves was approximately 29.5. These results seem to reveal the distributions of anthocyanins in fruits powder and leaves in nature. For blueberry powder, since the aqueous content was removed from berry fruits, the same weight of powder should contain higher anthocyanins than fruit. Furthermore, blueberry peel had a higher concentration of anthocyanins than leaves.

Disadvantages of the Folin-Ciocalteu assay and the pH differential method

At present, most laboratories use the Folin-Ciocalteu reagent to estimate total phenolics in

fruit and vegetables (Slinkard and Singletonon, 1977). Although the Folin-Ciocalteu assay is sensitive and easy to control, this method has some drawbacks. This method uses certain phenolic acids like gallic acid or ferulic acid as standards to quantify phenolic substances in extracts. The total phenolic content is expressed in gallic acid equivalent or in ferulic acid equivalent. However, a single phenolic acid cannot represent the entire concentration of phenolic compounds. To hasten the oxidation reaction, phenolics must be in an alkaline state. However, the reagent is not stable in alkali and the blue color is not very stable either, especially if the reaction temperature is increased (Slingkard and Singletonon, 1977). In addition, crude extracts contain some substances such as sulphur dioxide, ascorbic acid and ferrous ion, which can react with the molybdotungstophosphoric heteropolyanion reagent and contribute to the apparent total phenolic compounds (Slingkard and Singleton, 1977).

Anthocyanin pigments are responsible for the red, purple or blue colors of many fruits and vegetables. At present, malvidin-3-glucoside and cyaniding-3-glucoside are 2 standards that are used as equivalents to estimate total anthocyanins. The pH differential assay is used to estimate the monomeric anthocyanin pigments. Since no technology can completely separate all the monomeric anthocyanins from fruit extract or vegetable extract, this assay cannot detect the entire content of total anthocyanins in extracts. Because of these drawbacks of the pH differential method, blueberry extracts (fruit, powder and leave) need to be purified for further testing.

Use of other assays to estimate bioactivity

It is not enough to estimate antioxidant capacity of lowbush blueberries only considering

the values of total phenolics and total anthocyanins. There are many assays that are frequently used to assess antioxidant capacity.

The ORAC is another method to estimate antioxidant capacity in fruit and vegetable samples. R-phycoerythrin (R-PE) is a protein isolated from *Porphyridium cruentum*, which is chosen as a fluorescent probe; free radicals will damage the fluorescent probe and thus influence the intensity of fluorescence (Brunswick Laboratories, 2003). The change of the fluorescence intensity is associated with the degree of free radical damage. Antioxidants, scavenge free radicals, influencing the fluorescent intensity. The fluorescence intensity of the mixture (free radical and fluorescent probe) is measured at specific time intervals and has a decay curve. Furthermore, the fluorescence of the mixture (free radical, fluorescence probe and hydrophilic antioxidant or lipophilic antioxidant) can be concurrently measured, and another decay curve can be determined. The antioxidant capacity is calculated as the difference of the area under the 2 decay curves (Brunswick Laboratories, 2003). At present, the improved ORAC assay can be used to measure the antioxidant capacity against both peroxyl radical and hydroxyl radical. Researchers use Trolox, an analogue of vitamin E, as the standard to estimate phenolic antioxidant activity against peroxyl radical (Prior et al., 1998; Kalt et al., 1999; Mazza et al., 2002; Zheng and Wang, 2003). Furthermore, they use gallic acid as the standard to estimate antioxidant activity against hydroxyl radical (Brunswick Laboratories, 2003).

In the present study, phenolic compounds were not separated. Therefore, it is not known which specific compounds are potentially useful for storage of semen. In the future, separation and purification of compounds should be done. Zheng and Wang (2003) used high-performance

liquid chromatography (HPLC) to determine the chemical structures of some phenolic compounds. Phenolic acids in berries are hydroxybenzoic acids and hydroxycinnamic acids (Hakkinen et al., 1998). Hydroxycinnamic acids include caffeic acid, chlorogenic acid, ferulic acid, sinapic acid, p-coumaric acid. Hydroxybenzoic acids include gallic acid, p-hydroxybenzoic acid, vanillic acid, and syringic acid (Zheng and Wang, 2003). Chlorogenic acid, myricetin-3-arabinoside, quercetin-3-galactoside, quercetin-3-glucoside and other quercetin derivative are major phenolics in blueberries. Furthermore, kaempferol-3-glucoside and other kaempferol derivatives, delphinidin-3-galactoside, delphinidin-3-arabinoside, petunidin-3-arabinoside, malvidin-3-galactoside, malvidin-3-glucoside and malvidin-3-arabinoside are major anthocyanins in blueberries (Zheng and Wang, 2003). Knowledge of the specific compounds present in blueberries should be useful information when individual bioactive compounds are identified and measured.

2.5 Conclusions

In the present study, previously reported procedures and assays were used to determine the extraction efficacy of 3 frequently used extraction solutions and to determine the concentrations of antioxidants in 3 blueberry products by estimating total phenolics and total anthocyanins. Acidified methanol and acidified ethanol were much more efficient than acidified water; among the 3 blueberry products, the total phenolic concentration in blueberry leaves was significantly higher than blueberry powder and blueberry fruit. However, total anthocyanin concentrations in

blueberry powder were significantly higher than blueberry fruits and leaves.

In the present study, acidified methanol and acidified ethanol had strong extraction capacity. However, since water may be less toxic than ethanol or methanol for cell culture, we chose acidified water to extract bioactive compounds from blueberry products for use in subsequent research. Although the antioxidant concentrations varied among the 3 blueberry products tested, we chose all 3 products for subsequent research.

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3.0 Analysis of lipid peroxidation of boar semen during incubation

3.1 Introduction

Artificial insemination (AI) has been used in swineherds for many years (Johnson et al., 2000). Compared to natural-service, the advantages of AI and the choice to use AI, as stated in Chapter 1, can be made for many reasons, including: 1) increased use of germ plasm with superior genetics, even after a boar's death (Bearden et al., 2004, p159); 2) make it possible to inseminate a large group of sows and gilts after synchronization of estrus, therefore facilitating farm management (Hafez and Hafez, 2000, p376); 3) avoid inbreeding by establishing breeding records; 4) make crossbreeding easier and change a production trait; 5) reduce the risk of transmitting semen-borne diseases; and 6) reduce potential risk of injury so that any size boar can mate any size sow. There are few disadvantages of artificial insemination, such as the need to synchronize and detect estrus, to train young boars to allow sperm collection, and to have skilled personnel to conduct artificial insemination (Bearden et al., 2004, p159). There are 2 ways to store semen: liquid and frozen. Unlike cattle where frozen-thawed semen is routinely used, the outcome of using frozen-thawed boar semen to inseminate sows is generally unsatisfactory, with a farrowing rate of less than 50 % and a litter size of approximately 7 piglets per litter (Johnson et al., 2000). The average farrowing rate of sows using liquid extended semen is 82 % and average litter size is 10 piglets (Benchmarking for Canadian Herds, 2004). Therefore, boar semen is usually processed as liquid extended semen.

To be useful for AI, spermatozoa in diluted extenders must maintain satisfactory motility. For instance, semen in Androhep® extender can be stored for 3 d (83 % grossly motile) or in X-cell™ extender can be stored for 6 d (77 % grossly motile) and utilized for AI without a significant decrease in litter size and farrowing rate (Kuster and Althouse, 1999). Sperm viability over time and therefore the success of AI is very dependent on the semen extender used. Many of semen extenders have been developed to extend the interval that spermatozoa maintain satisfactory viability (Althouse, 1997). Semen extenders contain substances to supply energy to the spermatozoa, to maintain a physiological environment, to protect spermatozoa from cold shock, to control microbial contaminants (Bearden et al., 2004, p199-202), and to supply antioxidants to protect spermatozoa from oxidative stress (Szczęśniak-Fabiańczyk et al., 2003). For boars, commercially processed semen should be able to withstand storage at 15-20 °C for 1 to 5 d (Johnson et al., 2000); some semen extenders such as X-cell ™ (Kuster and Althouse, 1999) can be used to store boar semen up to 6 d without reducing reproductive outcomes. However, the storage period for liquid semen is still short compared with that of frozen semen. Therefore, improvements in the quality of semen extender for liquid storage would be beneficial; extending the period of sperm viability could increase the utilization of swine AI in the future.

Oxidative stress occurs in biological systems when there is an excess production of free radicals and/or a decrease in the concentration or activity of antioxidants. The quantity of oxidative stress in tissues is determined by the balance of reactive oxygen species (ROS) generation and degradation (Baumber et al., 2000). The ROS generation originates from normal metabolic oxidative activity of cells (de Lamirande et al., 1997). However, excessive ROS may

be produced by oxidase systems (Baumber et al., 2000) or by iron/ascorbate combination (Aitken et al., 1989). Like other mammalian spermatozoa, boar sperm membranes contain a high concentration of polyunsaturated fatty acids (Flesch and Gadella, 2000). These compounds maintain membrane fluidity, which is required for membrane fusion events such as the acrosome reaction (Flesch and Gadella, 2000). However, the polyunsaturated fatty acids in sperm membranes are very sensitive to damage by ROS, and excessive lipid peroxidation may induce irreversible damage to sperm membranes (Flesch and Gadella, 2000). ROS can damage the sperm midpiece, which contains mitochondria; a reduction in the number of functional mitochondria results in loss of sperm motility (Kodama et al., 1996; Baumber et al., 2000). Motility plays a vital role during fertilization, because there is high correlation between number of motile sperm and fertility (Bearden et al., 2004, p184). ROS can also damage double-stranded DNA and induce DNA breaks. Spermatozoa with damaged DNA have reduced fertility (Szczęśniak-Fabiańczyk et al., 2003) and can be manifested as idiopathic male infertility (Lopes et al., 1998; Twigg et al., 1998).

Seminal plasma contains antioxidant enzymes, which function to protect spermatozoa against ROS, including superoxide dismutase (SOD) (Alvarez and Storey, 1983a; Alvarez et al., 1987), the glutathione (GSH) peroxidase/reductase system (Alvarez and Storey, 1989; Kaneko et al., 2002), and catalase (CAT) (Jeulin et al., 1989). In addition, a number of components with low molecular weight, such as vitamins C and E, act as antioxidants by scavenging ROS (Christova et al., 2004; Sanocka and Kurpisz, 2004). In pigs, antioxidant enzymes, e.g. superoxide dismutase, glutathione peroxidase, and glutathione reductase were found in seminal

plasma. However, catalase was not found (reviewed by Strzeżek 2002). In addition, low molecular weight antioxidants, e.g. L-glutathione and L-ascorbic acid were also found in boar seminal plasma (reviewed by Strzeżek 2002). The antioxidant systems available to protect spermatozoa from oxidative damage are both numerous and complex.

Phenolic compounds are commonly found in plants; they have been reported to have antioxidant activity by scavenging free radicals and chelating metal ions (Prior et al., 1998; Kahkonen et al., 1999). Crude extracts of fruit and plants rich in phenolics have the capacity to retard oxidative degradation of lipids (Kahkonen et al., 1999). Lowbush blueberry (*Vaccinium angustifolium* Aiton) fruit and leaves contain a wide range of phenolic acids that may have higher antioxidant capacity than vitamins C and E (Prior et al., 1998; Kalt et al., 1999). Their antioxidant activity results from scavenging metal ions and free radicals (Yoshino and Murakami, 1998). Therefore, extracts of blueberry fruit and leaves may retard the oxidative degradation of lipids. However, the usefulness of antioxidants from blueberry products to retard the lipid peroxidation of boar spermatozoa has not been reported. Therefore, we hypothesized that blueberry extracts rich in phenolic compounds could retard lipid peroxidation of boar spermatozoa.

The most widely used method for monitoring lipid peroxidation in mammalian spermatozoa is the assay of thiobarbituric acid reaction substances (TBARS; Alvarez et al., 1987; Alvarez and Storey, 1982). Free radicals oxidize lipids into breakdown products, the most important of which is malondialdehyde (MDA; Aitken and Buckingham, 1993). One mole of MDA reacts with 2 moles of TBA in the presence of acid and forms a trimethine colored substance (Aitken and

Buckingham, 1993). Color intensity of the reactants rise as the concentration of MDA increases; the relationship between the concentration of MDA and the fluorometric absorbance is linear at 520-550 nm (Kodama et al., 1996).

Lipid peroxidation in any biological system can be induced or accelerated in the presence of transition metal ions such as ferrous ions and cupric ions (Aitken et al., 1989). The ferrous ions catalyse the breakdown of precursor lipid peroxides and accelerate the production of alkoxyl and peroxyl radicals, which propagate the lipid peroxidation process (Aitken and Buckingham, 1993). Ascorbate reduces ferric ions to ferrous ions, thus maintaining the iron-induced lipid peroxidation (Ratty and Das, 1986; Niki, 1991). Kodama et al. (1996) used 4 concentrations of a ferrous/ascorbate combination to accelerate lipid peroxidation of mouse spermatozoa; with increasing concentrations of ferrous/ascorbate, TBARS concentration was also increased. Although a ferrous/ascorbate model could be useful for the study of boar sperm lipid peroxidation, it is important to determine the optimal concentration of these reagents to induce sperm lipid peroxidation. The relationship between boar sperm lipid peroxidation and the concentration of ferrous/ascorbate combination has not been reported.

Because of the relatively constant concentration of PUFAs in boar semen (Flesch and Gadella, 2000), concentrated semen contains a higher level of the polyunsaturated fatty acids than diluted semen. Therefore, if the concentrations of ferrous ion/ascorbate are fixed, concentrated semen may produce more TBARS. However, the relationship between boar semen concentration and the lipid peroxidation has not been reported.

The objectives of this study were to investigate: 1) the ability of 3 concentrations of

ferrous/ascorbate to accelerate lipid peroxidation of boar spermatozoa; 2) the degree of lipid peroxidation in boar semen at 2 sperm concentrations, in the presence of ferrous ion and ascorbate; and 3) the ability of blueberry extracts to retard lipid peroxidation of boar semen in the presence of ferrous ions and ascorbate.

3.2 Materials and methods

Semen collection

The sperm-rich fraction of ejaculates was collected from Yorkshire boars at a commercial Prince Edward Island swine herd using a gloved-hand technique. Each different single ejaculate was used in each trial (see each experiment for details). Boar identification was recorded to ensure that a specific boar was not used more than once. The gel fraction was removed and the sperm concentration of the sperm-rich fraction was estimated using a SpermaCue^{TM} spectrophotometer (Minitube Inc., Ingersoll, ON, Canada) at the farm. Semen was transported to the laboratory immediately in a Styrofoam box with 38 ^{o}C water packs. Raw semen was diluted with saline to achieve a final concentration of $50x10^6$/mL or $100x10^6$/mL, allowing for the later addition of pro-oxidant solution, saline and blueberry extracts.

Blueberry products

Extracts of blueberry fruit, powder and leaf were made as described in Chapter 2. Briefly, 10 g of frozen blueberry fruit were mixed with 20 mL of acidified water extraction solution (1 %

acetic acid, v:v), homogenized for 2 min in an ice bath and centrifuged for 15 min at 1,667 x g at 4 °C, using a homogenizer (Brinkmann, PT10-35 Polytron, Lucerne, Switzerland). The supernatant was transferred to a 50 mL volumetric flask. The pellet was resuspended with another 20 mL of 1 % acidified water extraction solution, homogenized and centrifuged as above, and the supernatant transferred to the volumetric flask. The same extraction solution was added to bring the volume to 50 mL. Powder extract was obtained according to the same process as above, using 1.5 g blueberry powder (processing is presented in Chapter 2). Leaf extract was obtained according to the same process as above using 1.5 g of frozen blueberry leaves. Raw blueberry juice, made by compressing blueberry fruit, was obtained from the Food Technology Center (Charlottetown, PEI, Canada).

Reagents

Ferrous sulphate (heptahydrate, 99.0 %), L-ascorbic acid, phosphotungstic acid, 2-thiobarbituric acid (TBA) (4, 6-dihydroxypyrimidine-2-thiol; 98 %), butylated hydroxytoluene (BHT) (2, [6]-di-tert-Butyl-p-cresol, 99.0 %), sodium dodecyl sulphate (SDS), malonaldehyde bis-(dimethyl acetal, 99 %) (MDA) and sodium chloride were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Hydrochloride acid, 1-butanol and pyridine were obtained from Fisher Scientific (Nepean, ON, Canada).

Experimental design

Experiment 1: The effects of 3 concentrations of ferrous/ascorbate on sperm lipid peroxidation.

One ejaculate was collected from 1 boar, and Experiment 1 was conducted once. The stock ferrous/ascorbate solution (6/30 nM) and/or saline were mixed with diluted semen, according to the protocol presented in Table 3-1. Two replications were conducted for each of 4 treatments described in Table 3-1. The product of TBARS of each sample was measured using the TBARS assay. The concentration of ferrous/ascorbate that induced the highest value of the TBARS was used in Experiment 2.

Table 3-1. Sample preparation for TBARS assay of boar semen exposed to various concentrations of ferrous/ascorbate.

	Control[1]	Fe/ASc (0.02/0.1 mM)[2]	Fe/ASc (0.1/0.5 mM)[3]	Fe/ASc (0.4/2.0 mM)[4]
Semen (μL)[5]	2600	2600	2600	2600
Saline (μL)	400	200	200	200
Fe/ASc (6/30 mM) (μL)[6]	0	10	50	200
Water (μL)	0	190	150	0
Total (μL)	3000	3000	3000	3000

[1]Lipid peroxidation baseline sample

[2]Final concentration of ferrous/ascorbate 0.02/0.1 mM

[3]Final concentration of ferrous/ascorbate 0.1/0.5 mM

[4]Final concentration of ferrous/ascorbate 0.4/2.0 mM

[5]57.7×10^6/mL of boar semen diluted to give a final concentration of 50×10^6/mL

[6]6/30 mM of ferrous/ascorbate stock solution to give final concentrations of 0.02/0.1 mM, 0.1/0.5 mM, and 0.4/2.0 mM, respectively.

Experiment 2: The effects of ferrous/ascorbate concentration and semen concentration on lipid peroxidation. One ejaculate was collected from 1 boar, and Experiment 2 was conducted once. Concentrated semen contains more lipids per unit volume. Therefore, concentrated semen mixed with a pro-oxidant such as ferrous/ascorbate would be expected to increase the production of TBARS per unit time. Therefore, TBARS production from 2 semen concentrations, $50x10^6$/mL and $100x10^6$/mL were compared using the model developed in Experiment 1. The ferrous/ascorbate combinations with the highest ability to accelerate sperm lipid peroxidation from Experiment 1 were used to accelerate the lipid peroxidation in Experiment 2 (Table 3-2; 2 replications were conducted for each of 6 treatments). The semen concentration that produced highest value of TBARS when incubating with ferrous/ascorbate combination was used in Experiment 3.

Table 3-2. Sample preparation for TBARS assay of 2 concentrations of boar semen exposed to various concentrations of ferrous/ascorbate.

	Control[1]	Fe/ASc (0.1/0.5 mM)[2]	Fe/ASc (0.4/2.0 mM)[3]
Semen (μL)[4,5]	2600	2600	2600
Saline (μL)	400	200	200
Fe/ASc (6/30mM) (μL)[6]	0	50	200
Water (μL)	0	150	0
Total (μL)	3000	3000	3000

[1]Lipid peroxidation baseline sample

[2]Final concentration of ferrous/ascorbate 0.1/0.5 mM

[3]Final concentration of ferrous/ascorbate 0.4/2.0 mM

[4]$57.7x10^6$/mL of boar semen to give a final concentration of $50x10^6$/mL

[5]$115.4x10^6$/mL of boar semen to give a final concentration of $100x10^6$/mL

[6]6/30 mM of ferrous/ascorbate stock solution to give final concentration of 0.1/0.5 mM, 0.4/2.0 mM, respectively.

Experiment 3: The effect of blueberry products on sperm lipid peroxidation. One ejaculate was collected from 1 boar, and Experiment 3 was conducted twice. Extracts of 3 blueberry products (blueberry fruit, blueberry powder and blueberry leaves) and blueberry juice were incubated with semen and ferrous/ascorbate to determine their ability of decreasing sperm lipid peroxidation, using the model developed in Experiment 2. The semen concentration that produced the highest value of TBARS in Experiment 2 ($50x10^6$/mL) was incubated without the addition of ferrous/ascorbate and blueberry products to provide the baseline (control) of sperm lipid peroxidation. The same concentration of semen incubated in the presence of ferrous/ascorbate (0.1/0.5 mM) was used as the pro-oxidant treatment to accelerate sperm lipid peroxidation (Aitken et al., 1989). Another 4 treatments were made by mixing the same amount of 4 blueberry products with the same concentration of semen in the same concentration of ferrous/ascorbate, respectively (Table 3-3; 2 replicates were conducted for each of 6 treatments).

To measure the potential TBARS-like contribution of blueberry products in the absence of semen, 4 additional samples were made by mixing blueberry products (fruit extract, powder extract, leaf extract, and juice) and ferrous/ascorbate with saline, as outlined in Table 3-4. Readings of these samples in the absence of semen were subtracted from those of the blueberry samples with semen. Concentrations of total phenolics and total anthocyanins in the blueberry samples used in this experiment were estimated according to the methods presented in Chapter 2.

Table 3-3. Sample preparation for TBARS assay of boar semen exposed to ferrous/ascorbate and various blueberry products

	Control[1]	Fe/ASc[2]	Fruit[3]	Leaf[4]	Powder[5]	Juice[6]
Diluted semen (μL)[7]	2600	2600	2600	2600	2600	2600
Saline (μL)	400	350	0	0	0	0
Fe/ASc (6/30 mM) (μL)[8]	0	50	50	50	50	50
Fruit extract (μL)[9]	0	0	350	0	0	0
Leaf extract (μL)[10]	0	0	0	350	0	0
Powder extract (μL)[11]	0	0	0	0	350	0
Juice (μL)[12]	0	0	0	0	0	350
Total (μL)	3000	3000	3000	3000	3000	3000

[1]Lipid peroxidation baseline sample

[2]Lipid peroxidation accelerate group without blueberry products

[3]Ferrous/ascorbate combination group mixed with blueberry fruit extract

[4]Ferrous/ascorbate combination group mixed with blueberry leaf extract

[5]Ferrous/ascorbate combination group mixed with blueberry powder extract

[6]Ferrous/ascorbate combination group mixed with blueberry juice

[7]$57.7X10^6$/mL of semen to give the final concentration of $50x10^6$/mL

[8]6/30 mM of ferrous/ascorbate combination to give the final concentration of 0.1/0.5 mM

[9]Fruit extract made from 10 g frozen blueberry fruit

[10]Leaf extract made from 1.5 g frozen blueberry leaves

[11]Powder extract made from 1.5 g freeze-dried blueberry powder

[12]Raw juice obtained from the Food Technology Center (Charlottetown, PEI).

Table 3-4. Sample preparation for TBARS assay of blueberry products exposed to ferrous/ascorbate.

	Fruit[1]	Leaf[2]	Powder[3]	Juice[4]
Diluted semen (μL)[5]	0	0	0	0
Saline (μL)	2600	2600	2600	2600
Fe/ASc (6/30mM) (μL)[6]	50	50	50	50
Fruit extract (μL)[7]	350	0	0	0
Leaf extract (μL)[8]	0	350	0	0
Powder extract (μL)[9]	0	0	350	0
Juice (μL)[10]	0	0	0	350
Total (μL)	3000	3000	3000	3000

[1]Blank for fruit extract

[2]Blank for leaf extract

[3]Blank for powder extract

[4]Blank for juice

[5]57.7X10^6/mL of semen to give a final concentration of 50x10^6/mL

[6]6/30 mM of ferrous/ascorbate combination to give the final concentration of 0.1/0.5 mM

[7]Fruit extract that made from 10 g frozen blueberry fruits

[8]Leaf extract that made from 1.5 g frozen blueberry leaves

[9]Powder extract that made from 1.5 g freeze-dried blueberry powder

[10]Raw blueberry juice that obtained from the Food Technology Center (Charlottetown, PEI).

Determination of lipid peroxidation by TBARS production

Each semen sample preparation was placed in a 15 mL test tube sealed with parafilm. Tubes were incubated in a water-bath incubator at 37 °C. After 1, 3 and 5 h of incubation, a 0.5 mL aliquot of each semen suspension from the same test tube was pipetted into a Teflon screw-cap test tube, and mixed with 1 mL of TBA working solution: 1 mL of 7 % SDS + 10 mL of 0.1M HCl + 1.5 mL of 10 % phosphotungstic acid + 5 mL of 0.67 % TBA + 0.5 mL of 0.2 mM BHT. Samples were incubated in a water bath for 30 min at 100 °C. After test tubes cooled, 3 mL of a butanol extraction solution (15:1 (v:v) of 1- butanol: pyridine) were added to each of the test

tubes and mixed thoroughly. Test tubes were then centrifuged at 1,667 x g for 15 min to separate the butanol and aqueous phases, and the liquid in the butanol phase was aspirated into cuvettes. Pigments were measured using a Shimadzu spectrofluorophotometer (RF-5301 PC, Mandel Scientific Co. Ltd., Kyoto, Japan) with excitation and emission wavelengths of 520 nm and 550 nm. A standard curve was generated using MDA after incubation with 0.1 M HCl for 30 min under the same conditions as the experimental samples (Kodama et al., 1996).

Statistics

A split-plot design was used to estimate the differences in TBARS production (Christensen, 1996). The main-plot factor was the TBARS value of treatments, whereas the sub-plot factor was incubation time. Statistical significance of difference in TBARS production among treatments was assessed using analysis of variance (general linear mixed model; Minitab Version 13). If the treatment, the incubation time, or their interaction were significant, Bonferroni comparisons were utilized to locate differences. To approximate a normal distribution, a natural logarithmic transformation of the TBARS estimate was used. Statistical significance was set at α=0.05.

3.3 Results

Experiment 1: The effects of 3 concentrations of ferrous/ascorbate on sperm lipid peroxidation.

The production of TBARS by boar semen when incubated with the 3 concentrations of ferrous/ascorbate, 0.02/0.1 mM, 0.1/0.5 mM, and 0.4/2.0 mM is presented in Figure 3-1. All 3

concentrations of ferrous/ascorbate accelerated (P<0.05) the lipid peroxidation of boar semen compared with the control sample. The values of TBARS in all samples were associated with the incubation time (P<0.05). Ferrous/ascorbate 0.1/0.5 mM had the greatest capacity to accelerate lipid peroxidation (P<0.05), whereas the ability of ferrous/ascorbate 0.02/0.1mM had the lowest response (P<0.05). There was also an interaction of treatment and incubation time (P<0.05).

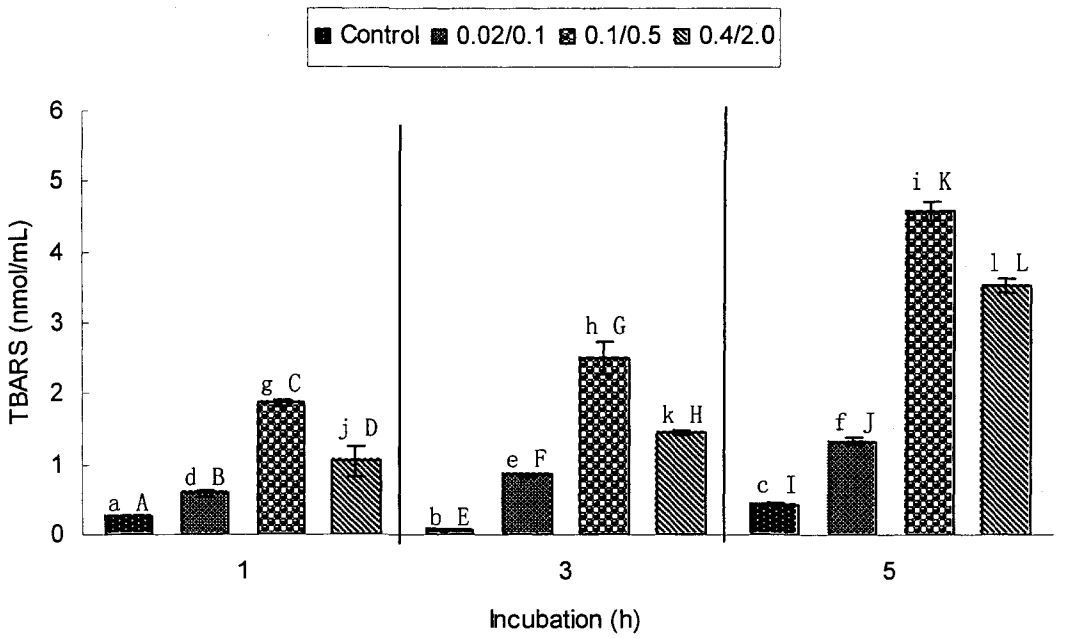

Figure 3-1. Effects of 3 concentrations of ferrous/ascorbate on sperm lipid peroxidation. Mean (± S.E.M. for 2 replications of 1 ejaculate) TBARS concentration were measured at 1, 3, and 5 h in the presence of 0.02/0.1 mM, 0.1/0.5 mM, and 0.4/2.0 mM ferrous/ascorbate concentrations. A semen sample (control) without ferrous ion and ascorbate was monitored simultaneously to provide baseline lipid peroxidation. There were effects of treatment, incubation, and their interaction on TBARS concentration (P<0.001 for each). In the presence of ferrous/ascorbate combination, concentrations of TBARS within each treatment increased during incubation (P<0.05), and concentrations of TBARS depended on concentrations of ferrous/ascorbate combination.

[A-L] Within a specific time, groups without a common letter were different (P<0.05).

[a-l] Within a specific treatment, groups without a common letter were different (P<0.05).

Experiment 2: The effects of ferrous/ascorbate concentration and semen concentration on lipid peroxidation.

The 2 ferrous/ascorbate concentrations that provided the higher TBARS values, in Experiment 1 (0.1/0.5 mM and 0.4/2.0 mM), were mixed with 2 concentrations of boar semen, $50x10^6$/mL and $100x10^6$/mL, respectively. The production of TBARS is shown in Figure 3-2. TBARS values in all samples were associated with incubation time ($P<0.05$). When incubated with the ferrous/ascorbate (0.1/0.5 mM), the more diluted semen produced a higher value of the TBARS than the more concentrated semen ($P<0.05$). However, the effect of semen concentration on TBARS production in the samples mixed with ferrous/ascorbate (0.4/2.0 mM) was only significant at 3 h of incubation ($P<0.05$). There was an interaction of treatment and incubation time ($P<0.05$).

Experiment 3: The effects of blueberry products on sperm lipid peroxidation.

The effects of blueberry products on sperm lipid peroxidation are presented in Figure 3-3. After blank correction, the TBARS values of 4 blueberry treatments did not increase significantly within 5 h of incubation, and the differences between blueberry treatments were not significant. The interaction of treatment and incubation time was not significant.

Except for the slight increase in leaf extract, the TBARS-like values of blueberry products in the absence of boar semen did not increase significantly within 5 h incubation. The TBARS-like values of raw juice had the highest response ($P<0.05$); the difference between fruit extract and

powder extract was not significant, and values were lower in leaf extract ($P<0.05$; Figure 3-3).

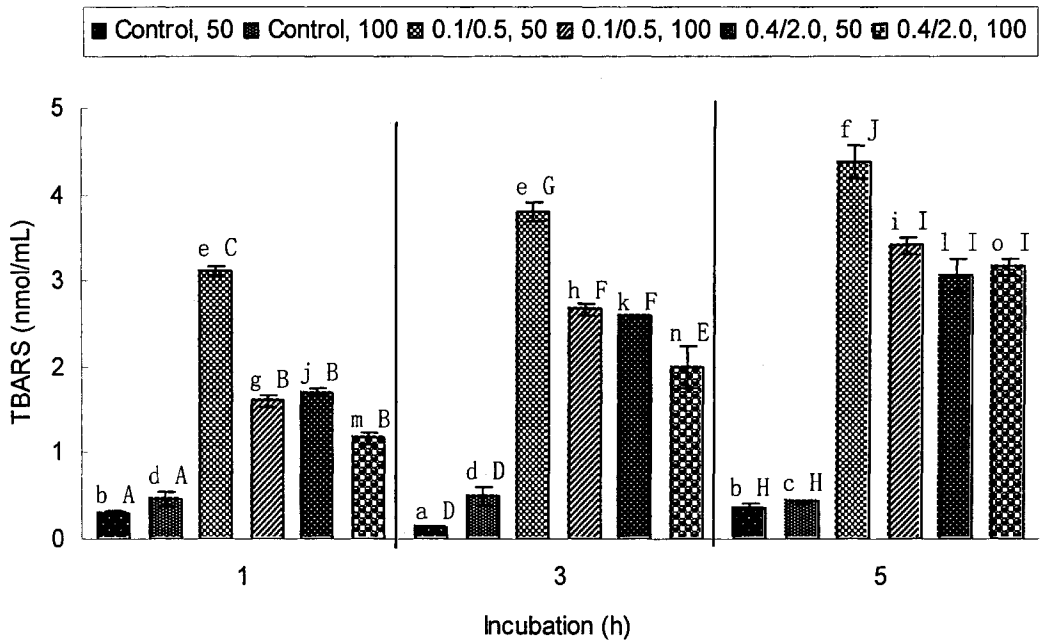

Figure 3-2. Effects of 2 concentrations of ferrous/ascorbate on lipid peroxidation at 2 concentrations of boar semen. Mean (± S.E.M. for 2 replications of 1 ejaculate) TBARS concentrations are presented at 1, 3, and 5 h in the presence of 0.1/0.5 mM and 0.4/2.0 mM ferrous/ascorbate. Semen samples without ferrous/ascorbate combination (control), were monitored simultaneously to provide the baselines of lipid peroxidation. The effects of treatment, incubation, and their interaction on TBARS concentration were significant ($P<0.001$).
$^{A-J}$ Within a specific time, groups without a common letter were different ($P<0.05$).
$^{a-o}$ Within a specific treatment, groups without a common letter were different ($P<0.05$).

The values (including TBARS and TBARS-like) of all 6 treatments are shown in Figure 3-4. The values in the control and leaf treatment were similar, which, however, were lower than other treatments ($P<0.05$); the values between the fruit treatment and the powder treatment were similar; the juice treatment had significantly higher values than other blueberry treatments, which were not significant with the Fe/Asc pro-oxidant treatment.

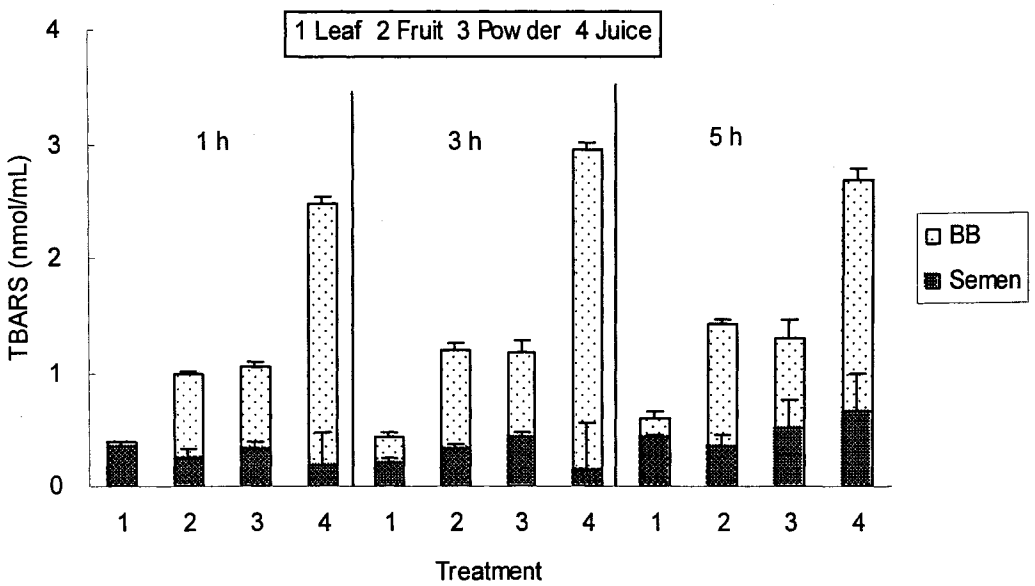

Figure 3-3. Net TBARS values of semen samples, corrected for increase in TBARS-values due to blueberry products. Mean (± S.E.M. for 2 replications of 1 ejaculate) TBARS values of $50x10^6$/mL boar spermatozoa were monitored after 1, 3 and 5 h in the presence of 0.1/0.5mM ferrous/ascorbate combination. The TBARS values were composed of the TBARS from the semen sample (the icon labelled as "semen") and the TBARS-like from the blueberry product (the icon labelled as "BB"). After correction, the effects of treatment, incubation, and their interaction on TBARS concentration were not significant. There were no significant differences among different treatments at the same time period and no significant differences of each treatment at different time periods.

3.4 Discussion

In the present study, boar sperm lipid peroxidation was induced in the presence of ferrous ions and ascorbate. Four blueberry products, raw juice, fruit extract, powder extract and leaf extract, were used to determine their capacity to decrease the lipid peroxidation as measured by

the TBARS assay.

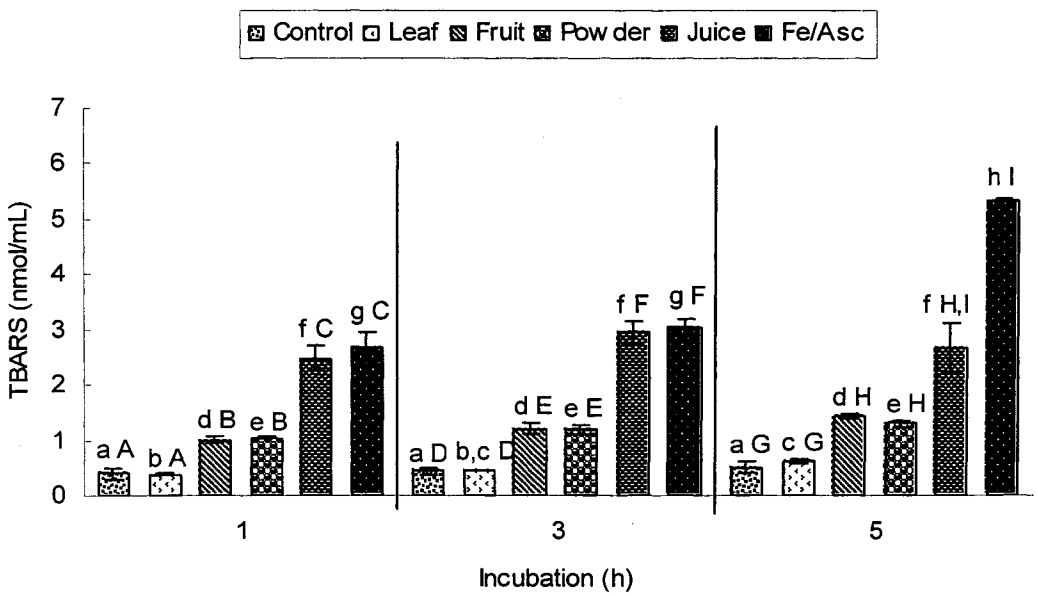

Figure 3-4. Effects of blueberry products on boar sperm lipid peroxidation.Semen sample without ferrous/ascorbate combination (control) was monitored simultaneously to provide baseline lipid peroxidation. Semen sample without blueberry additive but in the presence of ferrous/ascorbate was monitored to accelerate lipid peroxidation. Mean (± S.E.M. for 2 replications of 1 ejaculate) TBARS values of $50x10^6$/mL boar spermatozoa were monitored after 1, 3 and 5 h of incubation.

[A-I] Within a specific incubation time, groups without a common letter were different ($P<0.05$).

[a-h] Within a specific treatment, groups without a common letter were different ($P<0.05$).

Ferrous ions and ascorbate concentration

Spermatozoa generate small amounts of hydrogen peroxide through the NADPH oxidase system, and the hydrogen peroxide reacts with superoxide anion (HaberWeiss equation), or it reacts with the ferrous ion (Fenton) to produce hydroxyl radicals (Sikka, 2001). These substances

initiate a lipid peroxide cascade and cause peroxidative damage to the sperm membrane. The accumulated lipid peroxides break down to small compounds, such as malondialdehyde, in the presence of iron or copper ion (Khanduja et al., 2001). In the present study, 3 concentrations of ferrous/ascorbate were used to induce sperm lipid peroxidation, with the ratio of ferrous/ascorbate held at 1:5, based on the work by Kodama et al. (1996) who used this concentration of ferrous ion and ascorbate to accelerate lipid peroxidation of mouse spermatozoa. In their study, 4 ferrous/ascorbate concentrations were used to promote lipid peroxidation. Ferrous/ascorbate with 0.4/2.0 mM resulted in the highest TBARS value, followed by 0.1/0.5 mM, 0.02/0.1 mM and 0.004/0.02 mM, indicating that higher concentrations of ferrous/ascorbate resulted in higher TBARS values. However, in the present study, the highest TBARS values were with ferrous/ascorbate concentration of 0.1/0.5 mM; 0.4/2.0 mM resulted in lower TBARS values. This occurred at sperm concentrations of $50x10^6$/mL as well as $100x10^6$/mL. Therefore, the concentration of ferrous/ascorbate combination was not linearly related to the production of TBARS values. The reason for this is uncertain. In the future, additional concentrations should be tested to determine that which gives an optimal response.

Semen concentration

In the present study, 2 concentrations of boar semen were used and mixed with 2 combinations of ferrous/ascorbate. Since sperm plasma membrane was the source of lipids, we expected that the more concentrated semen sample would produce a higher value of TBARS during induced lipid peroxidation. However, with 0.1/0.5 mM of ferrous/ascorbate, semen lipid

peroxidation was greater at the lower semen concentration. In contrast, when 0.4/2.0 mM ferrous/ascorbate were used to promote lipid peroxidation, TBARS production in the 2 semen concentrations was similar. Therefore, the concentration of ferrous ion and ascorbate had a greater influence than semen concentration on the degree of semen lipid peroxidation in this experiment.

Effect of blueberry products

In the present study, the ability of 4 blueberry products to decrease induced semen lipid peroxidation was determined. All 4 products decreased lipid peroxidation induced by ferrous ions and ascorbate. Lowbush blueberries possess a high antioxidant capacity, thereby scavenging free radicals and chelating metal ions (Prior et al., 1998; Kahkonen et al., 1999). Lipid peroxidation can be induced in the presence of transition metal ions (Aitken et al., 1989); the ferrous ions accelerate the production of alkoxyl and peroxyl radicals, thus propagate lipid peroxidation (Aitken et al., 1993).

Perhaps the blueberry products in the semen samples chelated the ferrous ions and thereby preventing iron-induced lipid peroxidation (Heijne et al., 2002; Heim et al., 2002). Another possible explanation would be scavenging free radicals. The lipid peroxidation reaction process operates by 3 pathways: 1) non-enzymatic, free radical-mediated chain oxidation; 2) non-enzymatic, non-radical oxidation; and 3) enzymatic reaction (Papas, 1998, p9). The first pathway consists of chain initiation, propagation and termination. Free radicals may be formed in the presence of light, heat, metal ions, and then the radicals attack lipids and generate lipid

radicals (Reaction 1), which react with oxygen, produce lipid peroxyl radicals, and oxidize other lipid molecules (Reaction 2). The chain propagation would not stop until the lipid radicals or lipid peroxyl radicals are scavenged by antioxidants (Reaction 3) or 2 lipid peroxyl radicals react to produce non-radical products (Reaction 4; Papas, 1998, p9-13).

Chain initiation:

Formation of radical $X^{\bullet}$ (in the presence of light, heat or metal ions)

$X^{\bullet} + LH\ (lipid) \longrightarrow XH + L^{\bullet}$ (lipid radicals; Reaction 1)

Chain propagation:

$L^{\bullet} + O_2 \longrightarrow LO_2^{\bullet}$ (lipid peroxyl radical)

$LO_2^{\bullet} + LH \longrightarrow LOOH + L^{\bullet}$ (Reaction 2)

Chain termination:

$LO_2^{\bullet}$ → Scavenged by antioxidants (Reaction 3)

$LO_2^{\bullet}$ → Ketone + alcohol + O_2 (Reaction 4)

Lipophilic vitamin E and hydrophilic vitamin C are 2 well-known antioxidants that are able to scavenge lipid radicals. On the other hand, many plant phenols exert antioxidant effects to inhibit or retard lipid peroxidation by scavenging lipid peroxyl radicals and thus breaking chain reactions (Halliwell and Gutteridge, 1999, p225-231). As discussed, lowbush blueberries contain high concentrations of phenolic compounds other than vitamin E and vitamin C. Perhaps these phenolic compounds scavenged lipid radicals $X^{\bullet}$ at the beginning and inhibited chain initiation; alternatively, they may have scavenged lipid peroxyl radicals $LO_2^{\bullet}$ and terminated chain

propagation. As a consequence, the values of by-products, for example MDA, may decrease.

In the present study, all 4 blueberry products were known to contain considerable amounts of phenolic compounds (details were presented in Chapter 2). These compounds seemed to scavenge radicals, which were propagated by the ferrous/ascorbate combination, in semen cell suspension (the lipid source). Therefore, after samples reacted with TBA, the trimethine colored substance maintained a lower value. Consequently, the values of TBARS were maintained within 5 h of incubation. In short, antioxidants, e.g. phenolic compounds in the blueberry products, may retard iron-induced lipid peroxidation of boar spermatozoa by acting as metal-chelator and/or radical scavenger.

Before correction, leaf extract had the highest antioxidant capacity of all 4 blueberry products, measured by total phenolics and total anthocyanins, resulting in slightly higher TBARS values than the control treatment, with little change over the 5-h incubation. Fruit extract and powder extract also showed antioxidant capacity, although the TBARS values were higher than the control treatment and leaf treatment. Juice treatment had higher TBARS values than the other 3 blueberry product samples. However, the difference with the pro-oxidant (Fe/ASc) treatment was not significant. According to the outcomes, it seems that blueberry juice may not retard lipid peroxidation as well as other blueberry products. However, the TBARS-like values in juice were higher than that in the other 3 blueberry extracts. Perhaps another mechanism is present during lipid peroxidation reaction that interferes with the measurement of TBARS. Therefore, it is necessary and important to understand the roles of blueberry products, semen, ferrous ions and ascorbate during the reaction.

Potential interference of anthocyanins

Sperm lipid peroxidation, which can be induced by ferrous ion and ascorbate, has been estimated in many studies (Kodama et al., 1996; Twigg et al., 1998; Strzezek et al., 2000). In these studies, washed mammalian spermatozoa or spermatozoa in some extenders were used to measure the production of TBARS. However, in the present study, spermatozoa needed to be incubated in the presence of blueberry extracts.

Using the same methods as in Chapter 2, the total anthocyanin contents of powder extract, fruit extracts, leaf extract and juice were 0.084, 0.109, 0.003, and 0.381 mg malvidin 3-glucoside equivalent/mL. Since the concentration of total anthocyanins is associated with antioxidant capacity (Prior et al., 1998), it was possible to predict the antioxidant capacity of blueberry products by total anthocyanins. Blueberry juice had the highest value, followed by the fruit extract and powder extract, but leaf extract had the lowest value. This order was the same as the TBARS-like substances (Figure 3-3). This suggests that anthocyanins themselves may contribute to the TBARS concentration measured by the fluorescent assay.

The interference of plant tissues containing anthocyanins and other compounds on the TBARS assay can be determined by comparing the TBARS values with and without TBA reagent (Hodges et al., 1999). Plants, such as highbush blueberries, eggplants and red cabbage, containing high concentration of anthocyanidin derivatives, absorb at the same wavelength (532 nm) and lead to overestimation of TBARS values (Hodges et al., 1999). Hodge's method was used in our pilot study to measure the potential overestimation of TBARS by measuring TBARS

of blueberry juice without TBA reagent. However, the contribution of blueberry juice without adding TBA reagent was negligible (data not shown). This demonstrates that some compounds in blueberry juice react with the TBA reagent, producing the TBARS-like substances, and contribute to the observed TBARS value. Therefore, Hodges' method cannot be used in our study to modify the overestimation from juice. Since the fruit samples and powder samples are also derived from blueberries, Hodges' method may not adequately correct the overestimation from these blueberry products. Instead, we measured the TBARS-like values of blueberry products in the absence of semen to correct the TBARS values (Figure 3-3).

Interference of sugar

Sugar-rich plant tissue extracts, which contain sucrose, fructose, glucose, lactose, citrus pectin have a positive relationship with the TBA reaction (Du and Bramlage, 1992). The TBA-sucrose reaction can produce a precipitate, a chromogen that generates a higher absorbance at a similar wavelength to the wavelength that is used to estimate TBARS (Du and Bramlage, 1992). Substitution of the trichloroacetic acid (TCA) by hydrochloric acid provides further promotion of the TBA-sucrose reaction (Du and Bramlage, 1992). In the present study, no effort was used to discard sugars; therefore, all sugars in all 4 blueberry products may have interfered with the TBARS assay. Both blueberry powder and juice were made of blueberry fruit, that contain high concentrations of sugar; blueberry leaves, however, contain low concentrations of sugar. Therefore, perhaps the high concentration of sugar in fruit, powder and juice interfered with the measurement of TBARS and/or produced the TBARS-like substances, whereas, with

blueberry leaves, sugars contributed very little to the TBARS values observed. In our study, TBARS assay of blueberry products in the absence of spermatozoa allowed us to correct the TBARS values for TBARS-like substance(s) derived from the blueberry products.

Interaction of iron with phenolic compounds

Blueberries and other edible plants have strong antioxidant capacity due to their abundant phenolic components such as flavonoids (Kalt and Dufour, 1997); these phenolic components contain multiple hydroxyl substitutions that scavenge free radicals (Prior et al., 1998) and chelate metal ions (Laughton et al., 1989). However, in the presence of certain concentrations of copper ion or ferrous ion, flavonoids also demonstrate a pro-oxidant capacity (Cao et al., 1997; Chan et al., 1999). Copper ions directly react with flavonoids in the presence of oxygen; the reaction generates the flavonoids-Cu^{2+}-O_2 compounds and produces reactive species such as hydroxyl radicals, which catalyze lipid peroxidation. Similarly, ferrous ions can generate flavonoids-Fe^{2+}-O_2 compounds, produce hydroxyl radicals and propagate lipid peroxidation (Cao et al., 1997).

In Chapter 2, the flavonoids in blueberries and their chemical structures were discussed. The number of hydroxyl substitutions and their substitution positions affect the antioxidant capacity of flavonoids (Guo et al., 1997; Kalt and Dufour, 1997; Wang et al., 1997; Frankel, 1999). For instance, di-OH substitution at the 3' and 4' positions produces a molecule with stronger antioxidant capacity than molecules that do not have hydroxyl substitution or have single substitution. However, when these flavonoids react with metal ions, the multiple hydroxyl

substitutions enhance their pro-oxidant capacity (Cao et al., 1997). Perhaps the flavonoids in the blueberry products promoted lipid peroxidation, therefore increasing the TBARS values at the concentration used.

HPLC conformation

As stated in the introduction, one mole of MDA reacts with 2 moles of TBA; the MDA-TBA adduct, red or pink, can absorb at specific wavelengths (520-550 nm). However, other adducts rather than the MDA-TBA adduct are also present in the mixture, since the TBARS assay is not specific for MDA. Jardine et al. (2002) reported the identification of thiobarbituric acid/ malondialdehyde/ barbituricasice adducts and the barbituric acid/ malondialdehyde adduct with liquid chromatography-mass spectrometry (LC-MS) method. Sim et al. (2003) reported the use of high-performance liquid chromatography (HPLC) when measuring human plasma malondialdehyde and the measurement was improved by using methyl malondialdehyde as an internal standard. Therefore, a HPLC method could be utilized to specify the MDA-TBA adduct. Therefore, in future studies, the HPLC method could be used with the TBARS assay to decrease the interference from impurities.

Effect of different boars

To minimize variation, research on spermatozoa usually uses different ejaculates from at least 3 different boars each time. However, in our study, semen was used from 1 boar each time. Many effects impact boar sperm quality, including boar age, semen concentration, seminal

plasma components, feed components, collecting procedure, collecting time, or delivery time. All these reasons may result in some differences between trials. In the future, semen should be collected from more than 1 boar at a time, and comparisons should be made on different ejaculates.

Comparison with traditional antioxidants

Superoxide dismutase (Alvarez and Storey, 1983a), catalase (Baumber et al., 2000), albumin, glutathione peroxidase (Alvarez and Storey, 1989), vitamin C and vitamin E (Christova et al., 2004; Sanocka and Kurpisz, 2004), found in the seminal plasma, are biosynthesized antioxidants that decrease lipid peroxidation. No studies indicated that these antioxidants interfere with the TBARS assay. Therefore, some of them could be used as standard antioxidants and can be compared with antioxidant capacity of blueberry products. For instance, antioxidant capacity of blueberry products could be estimated by the ORAC using vitamin E as standard; their capacity to retard sperm lipid peroxidation could be estimated simultaneously with vitamin E as a positive control.

3.5 Conclusions

In the present study, ferrous/ascorbate 0.1/0.5 mM was used to promote the lipid peroxidation of boar spermatozoa (final concentration, $50x10^6$/mL). All blueberry products decreased the lipid peroxidation induced by ferrous ion and ascorbate. However, compounds of

blueberry products interfered with the TBARS assays. The components most likely to result in interference were pigments, sugars and flavonoids. However, the overestimation of TBARS-like substances resulting from these interferences was corrected by performing the TBARS assay on blueberry products in the absence of boar semen. It was noteworthy that all blueberry products had substantial antioxidant capacity to retard sperm lipid peroxidation induced by ferrous ions and ascorbate.

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4.0 Effect of lowbush blueberry products on viability and motility of boar semen

4.1 Introduction

Artificial insemination (AI) plays a vital role in modern livestock breeding. Bull semen can be readily frozen, facilitating both shipping and long-term storage, while still producing acceptable post-thaw fertility (Bearden et al., 2004). However, neither cryopreservation nor chilled storage of boar semen have proved satisfactory, as they result in loss of motility (Althouse et al., 2001), loss of viability (Eriksson et al., 2001), and cell death (Pursel et al., 1973; Johnson et al., 2000), culminating in reduced pregnancy rates and smaller litters (Gillmore et al., 1998). Consequently, AI in the swine industry is limited to fresh semen or semen stored at $\geq$ 15 ^{o}C for 5-7 d (Johnson et al., 2000). However, even with storage above 15 ^{o}C, current semen extenders cannot prevent gradual loss of fertility (Althouse, 1997) due to biochemical and physiological degradation of spermatozoa (Huo et al., 2002).

As stated in Chapter 1, sperm cold shock occurs when ambient temperature changes rapidly from boar body temperature down to 0 ^{o}C (Johnson et al., 2000; Bearden et al., 2004). The sensitivity of boar sperm to cold shock is due, in part, to the high concentration of polyunsaturated fatty acids in sperm membranes; although they enhance membrane fluidity and fertility (Flesch and Gadella, 2000), they are readily attacked by reactive oxygen species (ROS) when the semen is cooled (Chatterjee and Gagnon, 2001; Roca et al., 2004). Spermatozoa attacked by ROS undergo lipid peroxidation (Cerolini et al., 2000), which reduces motility and

viability (Alvarez and Storey, 1982; Alvarez and Storey, 1983; Baumber et al., 2000; Misro et al., 2004). The addition of antioxidants, e.g. the enzymes superoxide dismutase and glutathione peroxidase, improved the viability of frozen-thawed boar spermatozoa (Cerolini et al., 2001; Gadea et al., 2004). The addition of Trolox® (a commercial cell-permeable, water-soluble derivative of vitamin E with antioxidant properties) also improved the motility and mitochondrial activity of boar spermatozoa during cryopreservation (Peña et al., 2003). Therefore, supplementation with antioxidants has proven to be protective to boar spermatozoa against cold shock and reduced the rate of loss of sperm fertility.

Lowbush blueberry (*Vaccinium angustifolium* Aiton) fruit and leaf extracts have a relatively high antioxidant capacity, and thus may protect boar spermatozoa from damage during *in vitro* storage. Lowbush blueberry fruit and leaves contain high concentrations of polyphenols, which are reported to have higher antioxidant capacity than vitamins C and E (Prior et al., 1998; Kalt et al., 1999). Blueberry extracts decreased the thiobarbituric acid reaction substances (TBARS) in boar semen exposed to ferrous/ascorbate, supporting the idea that blueberry extracts can retard sperm lipid peroxidation (Chapter 3). It was hypothesized that antioxidants from blueberry extract may reduce cold shock and may retain sperm viability and motility of boar spermatozoa during *in vitro* storage. However, there are no reports regarding the use of blueberry-supplemented semen extender.

X-cell™ extender is a frequently used semen extender that can preserve boar semen at 15-17 °C up to 6 d without decreases in sperm fecundity (Kuster and Althouse, 1999). In the present study, X-cell™ was the base semen extender, with the addition of blueberry leaf and powder

extracts to enhance antioxidant properties. We hypothesized that blueberry-supplemented X-cell™ extenders would protect boar semen from cold shock and oxidative stress, with sperm viability and motility maintained for up to 7 or 8 d.

Sperm viability can be determined by many methods. The SYBR-14/PI staining assay is one of them used to determine sperm viability. This assay has been used in bull, stallion, boar, monkey, and human spermatozoa (Maxwell et al., 1998; Zeng et al., 2001). Spermatozoa with intact membranes are stained by SYBR-14 dye and display a bright green fluorescence (under the fluorescent microscope), whereas cells with damaged membranes are stained by PI (propidium iodide) and display a bright red fluorescence. Therefore, sperm viability can be expressed as the ratio of SYBR-14 positive cells to total cells.

The objectives of this study were to incubate boar spermatozoa in X-cell™ extender alone or blueberry-supplemented X-cell™ extenders and determine: 1) sperm viability (using the SYBR-14/PI assay); and 2) sperm motility (using light microscopy).

4.2 Materials and methods

Semen collection

The sperm-rich fraction of ejaculates was collected from Duroc boars at a commercial Prince Edward Island herd, using the gloved-hand technique. Each single ejaculate was used in each trial (see each experiment for details). Boar identification was recorded to ensure that no boar was used more than once. The gel fraction was removed and the sperm concentration of the

sperm-rich fraction was estimated using a SpermaCue^{TM} spectrophotometer (Minitube Inc., Ingersoll, ON, Canada) at the farm. The semen sample was diluted 1:1 by X-cell^{TM} extender, and the sample was immediately transported to the laboratory in a Styrofoam box with water packs at 38 °C. Only semen with > 70 % motile spermatozoa was used. Several drops of well-mixed diluted semen (1:1 with extender) were transferred onto a glass slide. Four fields on the same slide were viewed and motility was estimated according to the average of the 4 fields.

Blueberry products

Extracts of blueberry leaves and powder were made as described in Chapters 2 and 3. In short, 1.5 g of ground blueberry leaves were mixed with 20 mL of acidified water extraction solution (1 % acetic acid, v:v), homogenized (Brinkmann, PT10-35 Polytron, Lucerne, Switzerland) for 2 min in an ice bath, and centrifuged for 15 min at 1,667 x g at 4 °C. The supernatant was transferred to a 50 mL volumetric flask. The pellet was resuspended with another 20 mL of 1 % acidified water extraction solution, homogenized and centrifuged as described above, and the supernatant was transferred to a volumetric flask. The same extraction solution was added to the volumetric flask to bring the volume up to 50 mL. Blueberry powder extract was obtained according to the same process as above, using 1.5 g of blueberry powder (processing as described in Chapter 2). Blueberry extracts were adjusted to a pH of 7.3 (by the addition of sodium hydroxide) and the concentration of total phenolics in blueberry extracts was measured (as described in Chapter 2) before use.

Reagents

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). X-cell™ extender was ordered from Gencor IMV (Woodstock, ON, Canada). Syringes (HSW Norm-Ject 20mL) were purchased from Air-Tite Products Company, Inc. (Virginia Beach, VA, USA). The SYBR-14 working solution was prepared by diluting the SYBR-14 stock solution (Live/Dead® Sperm Viability Kit L-7011, component A, Molecular Probes Inc, Eugene, OR, USA) 1:50 with dimethyl sulfoxide (DMSO). Both the SYBR-14 working solution and the propidium iodide (PI) stock solution (Live/Dead® Sperm Viability Kit L-7011, component B, Molecular Probes Inc) were stored at -20 °C in Eppendorf tubes wrapped with foil.

Experimental design

Experimental preparations

The phenolic acid concentrations of the powder extract and the leaf extract were 0.4 and 1.5 mg/mL, respectively, measured in gallic acid equivalent (GAE). The intention was to examine motility and viability of boar spermatozoa in extended semen preparations containing blueberry-derived phenolic acids in concentrations similar to those examined using the ferrous/ascorbate model in Chapter 3, i.e. 0.047 and 0.175 mg GAE/mL, respectively, for powder and leaf preparations. Extenders containing the intended concentrations were used in Experiment 1, because no interference was noted when the SYBR-14/PI assay was used at these

concentrations, and these extenders are referred to as leaf extender and powder extender (Table 4-1).

Table 4-1. Sample preparation for measuring sperm viability and motility using control, leaf extender, and powder extender.

	Semen preparation		
Treatment	Control	Leaf extract concentration	Powder extract concentration
Extended semen (mL)[1]	200	200	200
Blueberry extract (mL)[2]	0	38.25	30
X-cell™ extender (mL)	70	31.75	40
Total (mL)	270	270	270
Phenolic acids ($x10^{-3}$mg GAE/mL)[3]	0	175	47

[1]Boar semen diluted by the addition of X-cell™ extender to achieve a sperm concentration of $67.5x10^6$/mL

[2]Stock blueberry leaf extract containing 1.3 mg GAE/mL, or stock blueberry powder extract containing 0.4 mg GAE/mL (see text for extraction method)

[3]Final concentration of blueberry-derived phenolic acids in the blueberry-supplemented semen preparations, expressed as mg GAE/mL

Table 4-2. Recipe of different ratios of theoretical live:dead sperm cells.

Live:dead[1]	10:0	8:2	6:4	4:6	2:8	0:10
Fresh semen (μL)[2]	1000	800	600	400	200	0
Damaged semen (μL)[3]	0	200	400	600	800	1000

[1] Theoretical ratio that use fresh semen and frozen-thaw semen

[2] 1:1 diluted semen with X-cell™ extender that was transferred back from the pig farm

[3] 1:1 diluted semen with X-cell™ extender after 2 frozen-thaw cycles treatment

Experiment 1: Determination of sperm viability and motility using X-cell™ extender and blueberry-supplemented X-cell™ extender on Day 7. Semen samples were prepared as described

as the semen preparation to yield final concentrations of blueberry-derived phenolic acids identical to those used in Chapter 3 (see Table 4-1). Semen samples, with a final concentration of $50x10^6$/mL, were maintained in 3 pre-warmed beakers (the same temperature as the semen) to avoid cold shock. A 20 mL aliquot of semen sample from the 3 beakers was transferred to a 20 mL syringe. These syringes were stored in 3 Styrofoam boxes at 5, 16, and 22 °C, respectively. All syringes were labelled by series numbers to blind treatments. Each of the 9 combinations (3 extenders by 3 temperatures) was replicated 4 times. Cooling rate of semen samples (0.2 °C/ min) was controlled by altering the amount of Styrofoam material placed in each Styrofoam box. Each syringe was agitated daily to minimize the effects of sperm clumping.

Experiment 1.1: Relationship between theoretical and actual live cell percentage of semen assessed by SYBR-14/PI fluorescent microscopy. To validate the SYBR-14/PI fluorescent microscopy for assessing sperm viability under these conditions, semen samples containing different ratios of live:dead spermatozoa were made. Fresh boar semen was mixed gently and allocated into 2 equal parts. One part was transferred to cryovial tubes; sperm membranes were damaged by putting the cryovial tubes into liquid nitrogen at -196 °C for 5 min and thawing for 2 freeze-thaw cycles. After thawing the second time, the frozen-thawed semen was pooled and mixed well. The remainder of the ejaculate (not subjected to freezing and thawing) was mixed, in triplicate, with the frozen-thawed semen at the following ratios: 10:0, 8:2, 6:4, 4:6, 2:8, and 0:10, respectively (Table 4-2). The staining assay was conducted according to the manual (Molecular Probes Inc, Eugene, OR, USA). In brief, 1 mL aliquots of extended semen were transferred to a

new test tube and were mixed with 1 μL SYBR-14 working solution. The sample was incubated at 36 °C for 5-10 min, then was mixed with 5 μL PI stock solution and incubated at 36 °C for an additional 5-10 min. A 10 μL aliquot of stained semen was pipetted to a slide, covered with a cover glass, and gently pressed. Two-hundred spermatozoa on each slide were assessed under a fluorescent microscope (400x) (ZEISS, West Germany) and were recorded as either green (live) or red (dead). Fresh boar semen was collected as described in the semen collection, and Experiment 1.1 was conducted once using only 1 boar.

Experiment 1.2: Sperm viability in the commercial X-cell™ extender, the leaf-supplemented X-cell™ extender and the powder-supplemented X-cell™ extender on Day 7 at 3 temperatures. On Day 7, sperm samples were transferred to pre-cooled test tubes. Approximately 2 mL of semen sample was transferred to a test tube that was pre-cooled in a Styrofoam box with a water bath and maintained at 5, 16, and 22 °C, respectively. Samples were maintained at these temperatures during incubation. Sperm viability of each sample was measured using the SYBR-14/PI fluorescent microscopy, as presented in Experiment 1.1. Fresh boar semen was collected as described in the semen collection, and Experiment 1.2 was conducted 4 times using a different boar each time.

Experiment 1.3: Sperm motility in the commercial X-cell™ extender and the blueberry-supplemented extenders on Day 7 at 3 temperatures. Sperm motility of the same semen samples in Experiment 1.2 were monitored immediately after testing sperm viability.

Sperm motility was monitored on Day 7 post collection. The stage warmer was adjusted to 35 °C to pre-warm slides, cover glass and capillary tubes. A capillary tube was used to transfer approximately 20 μL of well-mixed semen (from each syringe) to a glass microscope slide. The sample was covered by a cover glass for 30 sec (for samples from 16 °C and 22 °C) or 60 sec (for samples from 5 °C). Four different fields on the same slide were observed with a light microscope (400x) (ZEISS, West Germany). The percentage of motile cells was considered as motility (with 5 % increments). Fresh boar semen was collected as described in the semen collection, and Experiment 1.3 was conducted 4 times using a different boar each time.

Statistics

In Experiment 1.1, the relationship between theoretical and actual live cell percentages was analyzed using a linear regression model (Minitab Version 13). Differences of sperm viability (Experiment 1.2), and sperm motility (Experiment 1.3) were analyzed by 2-way ANOVA (Minitab Version 13) including extender and temperature as main effects. The results were presented as means $\pm$ standard error of the means (S.E.M.). If the effects of extender, temperature, or their interactions were significant, Bonferroni comparisons were utilized to locate differences. To approximate a normal distribution, the proportions of viable spermatozoa and motile spermatozoa were arcsine-transformed prior to analysis. Statistical significance was set at α=0.05.

4.3 Results

Experiment 1.1: Relationship between theoretical and actual live cell percentage of semen assessed by SYBR-14/PI fluorescent microscopy.

The relationship between theoretical proportions of live cells and the actual proportions of live cells in the X-cell^{TM} extender is presented in Figure 4-1. The actual proportions of live sperm cells that were monitored by the SYBR-14/PI fluorescent assay had a strong positive association with the theoretical proportions of live cells in the X-cell^{TM} extender (R^2=96.5 %, P<0.001). The slope was 0.779, and the intercept was 1.905. Sperm average viability in the theoretical 100 % live semen sample was 80 %, thus there was approximately 20 % damaged sperm cells in fresh semen samples. For a 20 % change in theoretical damaged cell percentage, a 15.6 % change was observed in actual damaged cell percentage.

Experiment 1.2: Sperm viability in the commercial X-cell^{TM} extender, the leaf-supplemented X-cell^{TM} extender and the powder-supplemented X-cell^{TM} extender on Day 7 at 3 temperatures.

Sperm viability using SYBR-14/PI fluorescent microscopy is presented in Table 4-3. There were effects of extender and temperature (P<0.001) and a tendency (P=0.067) for an interaction of extender by temperature. The difference among the 3 extenders was not significant at 5 °C; the differences between the control and the powder extender were not significant at 16 and 22 °C, but sperm viability of the leaf extender was lower than the other 2 extenders (P<0.05). The viability at 5 °C was always lower than that at 16 and 22 °C (P<0.05) regardless of extender,

whereas there was no significant difference on Day 7 viability at 16 and 22 °C.

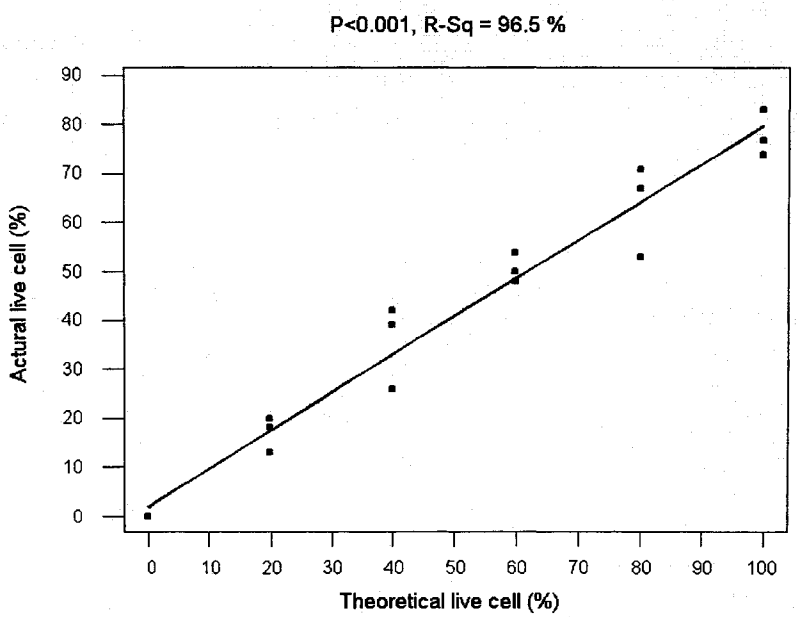

Figure 4-1. The relationship between theoretical and actual live cell percentages of spermatozoa in the X-cell™ commercial extender using SYBR-14/PI fluorescent microscopy (3 replications of 1 ejaculate).

Experiment 1.3: Sperm motility in the commercial X-cell™ extender and the blueberry-supplemented extenders on Day 7 at 3 temperatures.

Sperm motility using light microscopy is presented in Table 4-4. The motility in all 3 extenders was very low at 5 °C; differences in motility among extenders were not significant. At 16 °C, the motility in the leaf extender was lower than the other 2 extenders ($P<0.05$). At 22 °C, motility in the control was higher than the other 2 extenders ($P<0.05$). The difference between 2 blueberry-supplemented extenders was not significant, since motility in both decreased to 0 %.

Table 4-3. Sperm viability (live cell %) of the control, leaf extender, and powder extender stored at 5, 16 and 22 °C on Day 7 using SYBR-14/PI fluorescent microscopy.

	5 °C	16 °C	22 °C
Control	50.6 ± 2.9^{aA}	84.1 ± 2.7^{bB}	85.9 ± 1.4^{bB}
Leaf extender	47.9 ± 0.7^{aA}	69.8 ± 0.7^{bA}	75.0 ± 2.6^{bA}
Powder extender	55.4 ± 1.9^{aA}	82.9 ± 1.2^{bB}	84.3 ± 2.0^{bB}

Values are presented by mean ± S.E.M for 4 replications of 1 ejaculate.

[a-c] Within a row, means without a common superscript were different ($P<0.05$).

[A-C] Within a column, means without a common superscript were different ($P<0.05$).

Table 4-4. Sperm motility of the control, leaf extender, and powder extender stored at 5, 16 and 22 °C assessed with microscopy on Day 7.

	5 °C	16 °C	22 °C
Control	16.3 ± 2.4^{aA}	41.3 ± 4.3^{bB}	42.5 ± 6.0^{bB}
Leaf extender	8.8 ± 1.3^{bA}	15.0 ± 2.0^{bA}	0.0 ± 0.0^{aA}
Powder extender	10.0 ± 2.0^{bA}	30.0 ± 2.0^{cB}	0.5 ± 0.3^{aA}

Values are presented by mean ± S.E.M for 4 replications of 1 ejaculate.

[a-c] Within a row, means without a common superscript were different ($P<0.05$).

[A-C] Within a column, means without a common superscript were different ($P<0.05$).

4.4 Discussion

Effect of semen extenders on sperm viability

Sperm viability was related to the concentration of phenolic compounds. The concentrations of total phenolics in control, powder extract, and leaf extract were 0, 47.0 $x10^{-3}$, and 175.0 $x10^{-3}$ mg GAE/mL, respectively. Sperm viability (SYBR-14/PI) was lower in the leaf-supplemented

extender than in the control or the powder-supplemented extender suggesting that at the concentration used, leaf extender was deleterious to the boar spermatozoa. Khanduja et al. (2001) investigated the relationship between concentrations of quercetin (a flavonoid substance) and human sperm viability. They reported that sperm viability decreased when the concentration of quercetin was > 50 μM (8.45×10^{-3} mg/mL). In the present study, the gallic acid equivalent was used to calibrate antioxidant values in blueberry extract. Gallic acid equivalent (GAE) in the powder-supplemented extender was 47.0×10^{-3} mg/mL; however, the GAE in the leaf-supplemented extender was 175.0×10^{-3} mg/mL. It is clear that the powder extender contained a lower concentration of GAE, whereas the leaf extender contained a higher concentration of GAE. The concentration of GAE in the leaf extender was 3.7-fold greater than that in the powder extender. Therefore, with an increasing concentration of blueberry-supplementation in the extender, sperm viability decreased. This, therefore, may explain why sperm viability in the leaf extender had much lower viability compared to the control, while viability in the powder extender was similar to the control; therefore, a mild concentration of GAE (e.g. in the powder extender) did not affect sperm viability.

Boron plays a vital role in commercial blueberry production. It always binds to oxygen (Çöl and Çöl, 2003), and is sometimes applied to blueberries to avoid boron deficiency. However, a higher concentration of boron may have adverse effects on spermatozoa. The distribution of boron is low in nature, only reaching <0.1-0.5 ppm in fresh surface waters (Çöl and Çöl, 2003). However, the normal concentration of boron in blueberry leaves is approximately 31-80 ppm (Blueberry Nutrition and Fertilization). The adverse effects of excessive boron on animals or

humans are not fully understood, however, excessive boron results in testicular damage and infertility, and it also contributes to low semen production, sperm count and motility (IPCS, 1998). In animals, boron is excreted mainly through urine. In the present study, boar spermatozoa were incubated in the blueberry leaf-supplemented extender. Potentially, the concentration of boron in the extenders was too high for boar spermatozoa, and therefore damaged sperm motility and viability, since spermatozoa may not have the ability to decompose excessive boron. The knowledge of blueberry leaf composition is very limited. Therefore, in future studies boron concentration of blueberry leaf extracts should be determined prior to using it as semen extender supplementation.

Sperm viability at 5 °C was consistently lower than that at 16 or 22 °C, regardless of extender. Therefore, neither the control nor any of the blueberry-supplemented extenders effectively protected spermatozoa against cold shock. The lack of a protective effect may have been due to the pro-oxidant action of blueberry extract (which induced lipid peroxidation), thus resulting in increased sensitivity to cold shock.

Several substances have been used in cryopreservation or cold storage of boar semen. Egg yolk and glycerol are used to protect spermatozoa of bulls (Amirat et al., 2004), boars (Eriksson et al., 2001; Zeng and Terada, 2001), and rams (Gil et al., 2003) from cold shock during cryopreservation. The BTS, KIEV, Zorlesco and Androhep are liquid extenders for boar semen (Johnson et al., 2000). Semen stored in these extenders is usually stored above 15 °C. These extenders do not contain egg yolk or glycerol. The recommended storage temperature of the X-cell™ extender is between 15 and 17 °C. Perhaps X-cell™ extender does not contain egg yolk

or glycerol; this would contribute to unsatisfactory viability and motility at low temperatures. The Beltsville F5 extender allows storing boar semen in liquid nitrogen: 100 mL of the Beltsville F5 extender contains 20 mL of egg yolk and 2 mL glycerol (Bearden et al., 2004, p214-215). However, frozen-thawed semen requires a larger number of spermatozoa per insemination unit compared to liquid semen, and fertility may vary substantially among semen samples. Therefore, frozen-thawed semen is not widely used on commercial farms (Bearden et al., 2004, p216-217). In the future, the Beltsville F5 extender containing egg yolk and glycerol may be used with blueberry extract to determine this combination can decrease the stress of cold shock of boar spermatozoa.

Effect of semen extenders on sperm motility

Sperm motility in the leaf-supplemented extender and powder-supplemented extender was much lower than in the control extender, suggesting an inhibitory effect of leaf and powder extracts on sperm motility. As mentioned in the materials and methods, NaOH was used to increase the pH of blueberry extracts from 3 to 7; this may have changed phenolic compounds. Therefore, the inhibition of motility may have been due to these compounds. In order to elucidate the relationship between concentration of phenolic compounds and boar sperm motility, other experiments were conducted using the same method as stated in Experiment 1.3. Three blueberry leaf extenders containing lower concentrations of leaf extract, and three blueberry powder extenders containing lower concentrations of powder extract were made and compared with the control (Appendix B, Table 1). Sperm motility of the seven extenders was measured at

Day 0, 2, 4, 6, and 8 at the three storage temperatures (Appendix B, Figures 1-3). It was clear that the diluted blueberry leaf extenders and powder extenders did not effectively protect sperm motility at 5 °C, because sperm motility in all blueberry-supplemented extenders decreased after the treatment and the low motility persisted up to Day 8. Sperm motility of the blueberry leaf extenders and powder extenders was lower than that of the control at 16 °C and 22 °C on Day 0. The low motility also persisted up to Day 8. Therefore, the blueberry extracts, even at much lower concentrations in the X-cell™ extender, inhibited boar sperm motility. Pardeep et al. (1989) reported the effects of polyphenolic compounds on sperm motility. Although the motility-inhibiting effect was structure-dependent, the 7 benzoic acid derivatives (salicylic acid, 3-hydroxy benzoic acid, protocatechuic acid, gentisic acid, gallic acid, vanillic acid, and syringic acid), the 7 cinnamic acid derivatives (o-coumaric acid, m-coumaric acid, p-coumaric acid, caffeic acid, ferulic acid, sinapic acid and chlorogenic acid), 4 coumarins (coumarin, umbelliferone, esculetin and scopoletin), and 3 flavonoids (quercetin, morin, and rutin) inhibited rat sperm motility after 5 min of incubation when the final concentration was $1x10^{-4}$ mol/L. Khanduja et al. (2001) investigated the relationship between quercetin concentration and human sperm motility. They reported that sperm motility decreased in the presence of quercetin at concentrations as low as 5 μM ($1.69 x10^{-3}$ mg/mL). It is believed that polyphenols are inhibitors of certain phosphorylations (Way et al., 2004; Wheeler et al., 2004), and they act as inhibitors of glycolytic and Kreb's cycle enzyme activity (Tso and Lee, 1982). Therefore, it can be speculated that the polyphenols from blueberry extracts may inhibit sperm motility at certain concentrations.

Progressive motility is required for the spermatozoa to traverse the reproductive tract and

penetrate the cumulus-oocyte complex, zona pellucida and oocyte membrane (Bearden et al., 2004, p100-105). Sperm motility < 50 % is not satisfactory for artificial insemination (Bearden et al., 2004, p185). Sperm motility in all blueberry-supplemented extenders used was consistently lower than in the control sample. Further research should be done to study the effect of lower concentrations of blueberry-supplemented extender on sperm motility. It was noteworthy that sperm motility was measured by the light microscopy using increments of 5 % in the present study. To increase accuracy, sperm motility should be evaluated with a computer automated semen analyzer (CASA; Suzuki and Nagai, 2003). However, since evaluations were done without knowledge of treatment groups and many of the differences between the control and blueberry-supplemented groups were large, the present findings are considered valid.

Use of flow cytometry

Comparing to the outcomes from the flow cytometry, the SYBR-14/PI fluorescent microscopy assay was more variable. With flow cytometry, the outcomes are from all the spermatozoa on each slide (Garner and Johnson, 1995); while the outcomes from SYBR-14/PI fluorescent microscopy only describe sperm viability in a small sample (200 sperm cells on each slide). Ideally, sperm viability should be measured by the flow cytometry when using SYBR-14/PI fluorescent assay. Unfortunately, flow cytometry was not available for this study. However, it would be a valuable addition to future studies.

4.5 Conclusions

None of the extenders protected boar spermatozoa from cold shock when spermatozoa were stored at 5 °C. None of the blueberry-supplemented extenders improved sperm viability as hypothesized. Both blueberry-supplemented extenders inhibited sperm motility.

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5.0 Effect of lowbush blueberry products on boar sperm capacitation

5.1 Introduction

In vivo, ejaculated mammalian spermatozoa cannot fertilize oocytes until they incubate in the female genital tract for a period of time (Chang, 1951; Austin, 1951). During this incubation, spermatozoa undergo capacitation, the process that helps spermatozoa remove adherent seminal plasma proteins and reorganize the plasma membrane lipids and proteins. Capacitation must occur at the appropriate time to promote sperm hyperactivation, acrosome reaction, and must occur at the appropriate location for spermatozoa to fertilize oocytes. In that regard, premature sperm capacitation induces cellular death, resulting in loss of fertility (Aitken et al., 1995).

Many factors can influence premature capacitation. Boar spermatozoa are very sensitive to cold shock and cryopreservation, probably due to the low cholesterol:phospholipid ratio (Holt, 2000), and the high concentration of polyunsaturated fatty acids in their membranes (Flesch and Gadella, 2000). Cold shock and cryopreservation are associated with the phosphorylation of sperm tyrosine kinases, which trigger capacitation-like alterations (Kaneto et al., 2002). Additionally, capacitation can be initiated in the presence of excessively high concentrations of reactive oxygen species (ROS; de Lamirande and Gagnon, 1993).

Artificial insemination is frequently used in the swine industry. Because of its extreme sensitivity to low temperature, frozen-thawed boar semen usually does not result in satisfactory fertility. Consequently, spermatozoa are stored in liquid extenders at 15-20 ^{o}C (Johnson et al.,

2000). However, spermatozoa stored in this way are exposed to oxidative damage, which may induce premature capacitation. Antioxidants, which are able to scavenge ROS and retard oxidative damage, should protect boar spermatozoa against premature or non-regulated capacitation during *in vitro* storage (Roca et al., 2004). Vitamins C and E have been used to protect bull spermatozoa from oxidative-induced capacitation (O'Flaherty et al., 1997); antioxidant enzymes such as catalase and superoxide dismutase are also reported to protect human spermatozoa from these events (Potts et al., 1999; Baumber et al., 2003). Phenolic compounds from lowbush blueberries have a strong antioxidant capacity to scavenge free radicals and chelate metals (Chapters 2, 3 and 4). However, no investigations have reported the effect of phenolic compounds on boar sperm capacitation. On the other hand, the structures of boar sperm membranes change after cooling, with enhanced fluidity and capacitation-like alterations. Although the sensitivity of boar spermatozoa to cold shock can be decreased by adding antioxidants (Roca et al., 2004), whether phenolic compounds from lowbush blueberries (known to have antioxidant properties) can protect spermatozoa from cold-induced capacitation is not known.

Heparin is a glycosaminoglycan, secreted in the female genital tract of several mammalian species. Heparin is frequently used to enhance capacitation in bull and stallion spermatozoa (Farlin et al., 1993). The calcium ionophore A23187 regulates intracellular distribution of calcium ions and is also used to induce acrosome reaction of capacitated spermatozoa in many species (Giojalas et al., 2004). It is expected that blueberry-supplemented semen extenders are able to protect boar spermatozoa against capacitation during *in vitro* storage. Although the

mechanisms of *in vivo* versus *in vitro* capacitation are different and not completely understood, spermatozoa stored in the blueberry-supplemented extenders will not be able to fertilize ova if they do not undergo capacitation in the presence of heparin and/or calcium ionophore *in vitro*. Therefore, it is important to determine whether blueberry-supplemented extenders inhibit heparin and/or calcium ionophore-induced capacitation.

Although the molecular mechanism is unknown, chlortetracycline (CTC) is widely used to measure sperm capacitation (Huo et al., 2002a,b; Maxwell and Johnson, 1997). Spermatozoa can be stained by the CTC exhibiting non-capacitated (F pattern), capacitated (B pattern), and acrosome reacted (AR pattern), respectively. The CTC staining assay was used to identify sperm capacitation in the present study.

The overall objective of this study was to estimate the potential effects of blueberry extract on boar sperm capacitation. Specific objectives were to determine: 1) sperm capacitation status after 10 min incubation; and 2) sperm capacitation with or without heparin and/or calcium ionophore (A23187), when semen was incubated in X-cell^{TM} extender alone or blueberry-supplemented (powder extract and leaf extract) X-cell^{TM} extenders.

5.2 Materials and methods

Semen collection

The sperm-rich fraction of ejaculates was collected from Duroc boars at a commercial Prince Edward Island swine herd, using the gloved-hand technique. Boar identification was recorded to

ensure that no boar was used more than once. The gel fraction was removed and the sperm concentration of the sperm-rich fraction was estimated using a SpermaCue spectrophotometer (Minitube Inc., Ingersoll, ON, Canada) on the farm. The semen sample was diluted 1:1 with commercial X-cell^{TM} extender, and the sample was transported to the laboratory immediately in a Styrofoam box with 38 °C water packs. Raw semen was diluted with X-cell^{TM} to achieve a final concentration of $50x10^6$/mL, allowing for the later addition of X-cell^{TM} extender and blueberry extracts. Semen only with > 70 % motile spermatozoa was used (Eriksson et al., 2001).

Blueberry products

Extracts of blueberry leaves and powder were made as described in Chapter 4. Blueberry extracts were adjusted to a pH of 7.6 by the addition of sodium hydroxide and the concentration of total phenolics in blueberry extracts was measured before use (as described in Chapter 2).

Reagents

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Heparin working solution was prepared by dissolving 0.01 g heparin (100,000 units) in 10 mL saline. Calcium ionophore (A23187) working solution was prepared by diluting 1 mg of calcium ionophore in 1.9098 mL dimethyl sulphoxide (DMSO). The CTC working solution was prepared by dissolving 0.6304 g (20 mmol/L) Tris-HCl, 1.519 g NaCl (130 mmol/L), 0.1237 g L-cysteine (5 mmol/L), and 0.0966 g chlortetracycline (purity 80 %, HPLC; 750 μmol/L) in 200 mL deionized distilled water (DDW). The chlortetracycline (CTC) working

solution was adjusted to a pH of 7.8 by the addition of sodium hydroxide solution. Paraformaldehyde (12.5 % w/v) was prepared by dissolving 12.5 g paraformaldehyde in 100 mL DDW containing 0.5 M Tris-HCl solution; sodium hydroxide solution was used adjust to a pH of 7.4. The 1, 4-diazabicyclo[2.2.2]octane (DABCO; 0.2468 g, 0.22M) was dissolved in 1 mL PBS; the mixture was then mixed well with 9 mL glycerol.

Experimental preparation

The extended semen samples were prepared to provide a blueberry powder-derived phenolic acid concentration of 0.047 mg/mL gallic acid equivalent (GAE), and a blueberry leaf-derived phenolic acid concentration of 0.175 mg/mL GAE, respectively, as described in Chapters 3 and 4.

Semen processing and storage

Semen processing and storage were done as in Chapter 4. In short, X-cell^{TM} extender was used to further dilute the 1:1 diluted boar semen to provide a cell suspension with $67.5x10^6$/mL. Blueberry leaf and powder extract were added to each semen preparation to provide 2 experimental blueberry-supplemented preparations and a control preparation. Semen samples, with a final concentration of $50x10^6$/mL, were held in 3 pre-warmed beakers (the same temperature as the semen) to avoid cold shock. A 20 mL aliquot of semen sample from the 3 beakers was transferred to a 20 mL syringe. These syringes, labelled by series numbers to blind the operator to treatments, were stored in 3 Styrofoam boxes at 5, 16, and 22 °C, respectively.

Each of 9 combinations (3 extenders by 3 temperatures) had 3 replications to increase precision. Cooling rate of semen samples (0.2 °C/min, see Chapter 4) was controlled by altering the amount of Styrofoam material placed in each Styrofoam box. The day that boar spermatozoa was collected and processed was recorded as Day 0. Each syringe was agitated daily to avoid sperm clumping. Capacitation status of semen samples were assessed on Day 2.

Experimental design

Experiment 1: Sperm capacitation status after incubation in a water bath for 10 min. Semen samples were transferred to new pre-cooled test tubes (5, 16, and 22 °C, respectively) in a water bath to maintain storage temperatures (5, 16, and 22 °C, respectively). Samples were then stained according to the CTC staining assay (Fraser et al., 1995) after incubation in the water bath at 39 °C for 10 min. In brief, a 45 μL aliquot of sperm cell suspension was mixed with a 45 μL aliquot of CTC working solution for 1 min, followed by the addition of 8 μL aliquot paraformaldehyde (12.5 %) in 0.5M Tris-HCl for 60 sec. A 10 μL aliquot of fixed semen cell suspension was placed on a slide and mixed with 1 drop of 0.22 M DABCO to impede fluorescence fading. A coverslip was covered on the glass slide, and was compressed; excess fluid was removed by KimWipes® tissue. Slides were sealed with clear nail polish and were investigated immediately in the dark. One hundred sperm cells were examined on each slide using a fluorescent microscope (450-490 nm, Ft 510, LP 520; Zeiss, West Germany). Three patterns were identified: F, uniform fluorescence of the entire head, characteristic of uncapacitated, acrosome-intact cells; B,

fluorescence-free band in the postacrosomal area, characteristic of capacitated, acrosome-intact cells; and AR, fluorescence absent from the head, characteristic of acrosome-reacted cells. Three replicates were performed for each temperature and extender combination to increase precision, and 3 replicates were performed for each experiment to estimate variation among boars. Semen (1 ejaculate) was collected from 1 boar, and Experiment 1 was conducted 3 times.

Experiment 2: Sperm capacitation status in the absence and/or presence of heparin and/or calcium ionophore. Semen samples stored at 3 temperatures in 3 extenders were transferred to new pre-cooled test tubes (5, 16, and 22 °C, respectively) in a water bath. Samples of each combination (3 temperatures x 3 extenders = 9 combinations) were selected and used in Experiment 2. First, 970 μL of each semen sample was pipetted twice into 2 test tubes. Group 1 comprised 9 test tubes without heparin or calcium ionophore A23187. Group 2 comprised 9 test tubes with 30 μL of heparin solution (0.001g/mL dissolved in saline) without calcium ionophore, to obtain a final heparin concentration of 30 μg/mL. Semen samples of Group 1 and Group 2 were incubated for 4 h at 39 °C in the water bath. After 4 h incubation, a half volume of semen sample (495 μL) from each group was transferred to a new test tube. The samples in new test tubes from Group 1 were mixed with the calcium ionophore working solution (dissolved in dimethyl sulphoxide, DMSO) to obtain a final calcium ionophore concentration of 10 μM, and this group was referred to as Group 3. A 495 μL of semen sample from Group 2 were mixed with the calcium ionophore working solution (the final concentration was 0.01 mM) in new test tubes, and this group was referred to as Group 4. Semen samples in Groups 3 and 4 were incubated for

30 min at 39 °C. During the incubation, the semen samples in Group 1 and Group 2 were stained by CTC solution to determine capacitation status, whereas the semen samples in Group 3 and Group 4 were stained by CTC solution after 30 min incubation to determine their capacitation status. The CTC staining method was done as described in Experiment 1. Four trials were done for each experiment to increase precision. Semen (1 ejaculate) was collected from 1 boar at a time, and Experiment 2 was conducted 4 times.

Statistics

The prevalence of F pattern in Experiment 1 was compared among extender, storage temperature, and their interaction by 2-way ANOVA (general linear mixed model; Minitab Version 13). The prevalence of AR pattern in Experiment 2 was compared among different extenders, storage temperatures, heparin effect, calcium ionophore effect, and their interaction by 4-way ANOVA (general linear mixed model; Minitab Version 13). In Experiment 1, if the effects of extender, storage temperature, or their interaction were significant, Bonferroni comparisons were utilized to locate differences. In Experiment 2, if the effects of extender, storage temperature, heparin, or calcium ionophore were significant, Bonferroni comparisons were utilized to locate differences. Data were subjected to arcsine transformation to obtain an approximately normal distribution. Statistical significance was set at α=0.05.

5.3 Results

Experiment 1. Sperm capacitation status after incubation in a water bath for 10 min

Sperm capacitation status (F pattern percentage) is presented in Table 5-1. Data for F, B, and AR patterns are presented in Appendix C (Figures 1-3). The effect of extender on F pattern was statistically different, with a higher percentage in leaf-supplemented extender than in the other extenders ($P<0.001$). The effect of temperature on F pattern was also statistically different, with a higher percentage at 22 °C than at other storage temperatures ($P=0.02$). The effect of interaction of extender by temperature was profound ($P<0.001$). The percentages of F pattern in powder-supplemented extender were higher than the control at 5 °C ($P<0.05$), but similar to the control at 16 and 22 °C. The percentages of F pattern in the leaf-supplemented extender were similar, regardless of temperatures; in the powder-supplemented extender, F pattern was lower at 16 °C ($P<0.05$); however, in the control, F pattern was lower at 5 °C ($P<0.05$).

Table 5-1. Sperm capacitation status (F pattern %) after incubation in a water bath for 10 min.

	5 °C	16 °C	22 °C
Control	$41.0 \pm 3.1^{a A}$	$51.7 \pm 3.6^{b A}$	$59.7 \pm 1.2^{b A}$
Leaf extender	$75.0 \pm 1.6^{a C}$	$80.0 \pm 0.9^{a B}$	$76.3 \pm 2.1^{a B}$
Powder extender	$63.7 \pm 2.8^{b B}$	$50.0 \pm 2.3^{a A}$	$59.4 \pm 1.4^{a,b A}$

Values are presented by mean ± S.E.M. for 3 different ejaculates (3 replications for each ejaculate).

[a-c] Within a row, means without a common superscript were different ($P<0.05$).

[A,B] Within a column, means without a common superscript were different ($P<0.05$).

Experiment 2. Determination of sperm capacitation with/without heparin and/or calcium ionophore A23187

Storage temperature did not have an effect on the acrosome reaction (P=0.73). However, the proportion of spermatozoa that exhibited acrosome reaction differed among the three extenders (P=0.002). Additionally, this proportion was associated with use of heparin and calcium ionophore (P=0.04, P=0.001, respectively). However, no interactions were significant. Therefore, only the effects of extender, heparin, and calcium ionophore alone were considered, and sperm capacitation status (percentage of AR pattern) is presented in Table 5-2. Complete data for F, B, and AR patterns are presented in Appendix C (Figures 4-15). For the 3 semen extenders, the percentage of AR pattern of spermatozoa was lower in the leaf-supplemented extender than the other 2 extenders (P=0.002). However, control and powder-supplemented extenders were not different (P=0.42). For the effect of heparin, the percentage of AR pattern of spermatozoa was higher in the presence of heparin than in the absence of heparin (P=0.04). The percentage of AR pattern of spermatozoa was higher in the presence of calcium ionophore than in the absence of calcium ionophore (P=0.001).

5.4 Discussion

Effect of semen extenders on sperm capacitation

Sperm capacitation in the X-cell™ extender alone was compared to sperm capacitation in

blueberry-supplemented extenders. In both the blueberry powder-supplemented extender and the leaf-supplemented extender, percentages of spermatozoa with an F pattern were higher than that in the control samples. Therefore, blueberry-supplemented extenders retarded capacitation-like alterations of spermatozoa *in vitro* when boar spermatozoa were incubated at 5 °C.

Table 5-2. Sperm capacitation status (AR pattern %) with/without heparin and/or calcium ionophore A23187.

Variable	Level	AR pattern	P-value
Extender	Control	75.4 ± 1.8^{b}	
	Leaf extender	67.9 ± 1.7^{a}	
	Powder extender	75.1 ± 1.5^{b}	0.002
Heparin	No	71.0 ± 1.2	
	Yes	74.6 ± 1.6	0.04
Calcium Ionophore	No	69.5 ± 1.4	
	Yes	76.1 ± 1.3	0.001

Values are presented by mean ± S.E.M. for 4 different ejaculates.

[a,b] Within the column, means without a common superscript were different ($P<0.05$).

A high concentration of hydrogen peroxide (> 1 mmol/L) can inhibit tyrosine phosphatases; thus increasing tyrosine phosphorylation (Ford, 2004) and enhancing capacitation. On the other hand, oxidants can also increase intracellular calcium concentrations by acting on protein phosphorylation (Ford, 2004). It is unlikely that storage temperature influenced the bioactivity of phenolic compounds, since these antioxidants are non-enzymatic in nature. Roca et al. (2004) reported the use of butylated hydroxytoluene (BHT) on boar spermatozoa survival and fertility after the frozen-thaw process. They confirmed the antioxidant capacity of BHT, and noted that

BHT could incorporate into sperm membranes and prevent the damaging influence of lipid peroxyl radicals. In the present study, the contents of blueberry extract were not analyzed. Therefore, whether all polyphenols are able to penetrate mammalian cell membrane, especially boar sperm membranes, remains uncertain. However, some phenolics such as flavonoids can affect a series of intracellular enzymes, which indicates that these flavonoids can incorporate into mammalian cells (Middleton et al., 2000). Therefore, perhaps the phenolic compounds in the blueberry powder and leaf extracts incorporated into sperm membranes and scavenged excessive induced ROS, thereby inhibiting capacitation-like alteration. On the other hand, sperm membrane destabilization associated with cold shock may change membrane lipid composition, increase membrane fluidity and permeability, thereby elevating the concentration of intracellular calcium ions, which may enhance capacitation (Maxwell and Johnson, 1997; Breitbart and Naor, 1999; Watson, 2000; Green and Watson; 2001). Sperm membrane permeability increases after cooling. It is clear that the higher intracellular calcium concentration induced capacitation.

Although molecular mechanisms of capacitation are not certain, it appears that excessive ROS production accelerates an influx of calcium ions, triggering adenylyl cyclase and increasing the concentration of cAMP, resulting in capacitation and capacitation-associated protein tyrosine phosphorylation (Hardy, 2002, p85; Rivlin, 2004). Consequently, blueberry extracts containing antioxidants may prevent excessive ROS production and therefore inhibit capacitation. According to Chapters 3 and 4, the antioxidant concentration (GAE/mL) in the leaf-supplemented extender was higher than that in the powder-supplemented extender and the control, consistent with the greater percentage of spermatozoa with the F pattern in the

leaf-supplemented extender.

Effect of semen extenders on sperm capacitation in the presence of heparin and calcium ionophore

All semen samples had high percentages of the AR pattern, indicating that blueberry-supplemented extenders did not inhibit heparin-induced or the calcium ionophore-induced capacitation. Heparin is a glycosaminoglycans secreted by specific cells in the female genital tract (Parrish et al., 1988). The molecular mechanisms of heparin-induced capacitation in mammalian spermatozoa are uncertain, and its influence varies among species. It is thought that glycosaminoglycans are able to bind to and remove seminal plasma proteins; they can elevate intracellular pH and increase cAMP synthesis (Parrish et al., 1994), thus inducing capacitation. In the present study, boar spermatozoa stored in the 3 extenders at 3 different temperatures were incubated in the presence or absence of heparin. Based on the CTC staining assay, > 65 % of spermatozoa in each sample underwent an acrosome reaction. Since capacitation is a prerequisite for the acrosome reaction, it is possible that more spermatozoa underwent capacitation then acrosome reaction. This strengthens the assertion that boar sperm capacitation and acrosome reaction can be regulated by heparin in the presence of blueberry extract.

The major function of a calcium ionophore is to assist calcium ions to cross sperm membranes, thereby increasing intracellular calcium concentrations. The molecular mechanisms of the acrosome reaction have not been fully clarified. However, increasing intracellular calcium

concentration accelerates the acrosome reaction (Hardy, 2002, p82-83). Superoxide dismutase (SOD) blocks calcium ionophore (A23187)-induced acrosome reaction (Griveau et al., 1995) or the heparin-induced capacitation in bulls (O'Flaherty et al., 1997). However, in the present study, the CTC assay demonstrated that a higher proportion of acrosome reaction in boar spermatozoa occurred in presence of calcium ionophore, and the percentage that underwent acrosome reaction was significantly higher than that in the absence of calcium ionophore. Therefore, antioxidants from blueberry extracts did not prevent spermatozoa from undergoing capacitation and acrosome reaction.

Effect of incubation time on sperm capacitation and acrosome reaction

The average time required to complete capacitation and an acrosome reaction varies among species (Hardy, 2002, p60). In Experiment 2, semen samples in Group 1 contained neither heparin nor calcium ionophore; however, the percentages of AR pattern in each sample were significantly higher after semen samples were incubated for 4 h. Therefore, incubation time may be another major factor in sperm capacitation and the acrosome reaction. In the future, the relationship among incubation time, heparin and/or calcium ionophore concentrations, and blueberry extract concentrations should be measured.

Limitation of the CTC staining assay

Chlortetracycline (CTC) is a fluorescent antibiotic that chelates membrane-associated divalent cations, especially Ca^{2+} (Hallett et al., 1972). Although the molecular mechanism of

CTC assay is not fully understood (Hardy, 2002, p62-64; Wang et al., 1995), CTC fluorescence changes when sperm capacitation status changes, and this fluorescence is able to distinguish noncapacitated sperm cells, capacitated sperm cells, and acrosome reacted sperm cells. However, potential interference resulting from other divalent cations may weaken fluorescence intensity due to Ca^{2+}. In the present study, components of the X-cell™ extender and blueberry extract from powder and leaves were unknown. Therefore, we cannot evaluate potential interference of the X-cell™ extender and the blueberry-supplemented extenders when using the CTC assay. As mentioned in Chapter 4, semen samples can be washed by PBS buffer using centrifugation (Maxwell et al., 1998; Green and Watson, 2001; Wang et al., 1995; Maxwell and Johnson, 1997) to minimize interference, and can be then stained by CTC assay. Recently, Hoechst 33342/CTC (Maxwell and Johnson, 1997) or Hoechst 33258/CTC (Huo et al., 2002a; Huo et al., 2002b) dual-stain assay was used to improve the CTC assay. Hoechst 33342 or Hoechst 33258, a membrane-penetrating fluorescent stain, can penetrate sperm membranes when membranes are not intact and fluoresce (blue), whereas live spermatozoa with intact membranes do not allow the Hoechst stain to enter. Using the dual-stain assay, it is possible to differentiate live spermatozoa from dead spermatozoa thus increasing sensitivity. Therefore, washed semen samples should be stained by both Hoechst stain and CTC to differentiate live or dead cells and to also determine capacitation status.

5.5 Conclusions

In the present study, using the CTC assay, a high proportion of spermatozoa in blueberry leaf-supplemented X-cell^{TM} had an F pattern in the absence of heparin and calcium ionophore, indicating that this extender protected spermatozoa against capacitation-like alterations induced by cold shock. However, spermatozoa in blueberry powder-supplemented X-cell^{TM} had a high percentage of F pattern at 5 °C, indicating that this extender may also protect spermatozoa against capacitation-like alteration. Although blueberry-supplemented extenders did not fully inhibit heparin-induced and/or calcium ionophore-induced capacitation and the acrosome reaction, blueberry leaf-supplemented extender retarded the sperm acrosome reaction.

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6.0 General discussion and conclusions

The swine industry plays a vital role in modern agriculture and artificial insemination (AI) has been widely used to improve the genetic base of the hog population. Boar semen is extremely sensitive to cold shock; the use of frozen-thawed semen results in suboptimal fertility, relative to other species. Therefore, liquid-storaged semen is the industry standard for artificial insemination of sows and gilts. Fresh semen combined with a good extender has a storage life of only 5 to 7 d. To counteract excessive reactive oxygen species (ROS), antioxidants were added to semen extenders, e.g. catalase, superoxide dismutase (SOD) and glutathione reductase (Alvarez and Storey, 1995; Foote et al., 2002); in addition, a synthetic antioxidant, butylated hydroxytoluene (BHT; Roca et al., 2004) and pyruvate (Bilodeau et al., 2002) were also added to boar and bull extenders, respectively. Many factors are involved in the degeneration of boar spermatozoa. Excessive oxidative stress results in lipid peroxidation of sperm membranes, reduction of motility, loss of viability, and premature capacitation.

It has been hypothesized that many of the degenerative changes in stored boar semen could be prevented or delayed by the addition of antioxidant compounds. Lowbush blueberries contain many bioactive compounds; although not well characterized, some of these have antioxidant properties. The overall goal of this project was to use antioxidant compounds from lowbush blueberries to improve storage life of liquid-stored boar spermatozoa.

In the present study, crude extracts of lowbush blueberries made from frozen fruit, dehydrated powdered fruit and leaves of lowbush blueberry plants were analyzed for total

phenolics and total anthocyanins. Three solutions were made to extract the bioactive compounds from blueberry products, and the ability of 3 extraction solutions (acidified water, acidified methanol, and acidified ethanol) was compared. The 3 blueberry products (from acidified water extraction solutions) plus raw blueberry juice were incubated with boar semen and a pro-oxidant complex (ferrous ions and ascorbate) to determine the antioxidant capacity to retard iron-induced lipid peroxidation. Blueberry powder extract and leaf extract were then mixed with a commercial extender (X-cell^{TM}) to make a series of modified semen extenders, and sperm viability, motility, capacitation were determined during *in vitro* storage of spermatozoa.

The goal was to investigate how bioactive compounds in blueberry extracts protect boar spermatozoa against lipid peroxidation, cold shock, membrane breaking, loss of motility and premature capacitation; spermatozoa were expected to survive in the blueberry-supplemented extenders longer than in the X-cell^{TM} extender alone.

6.1 Extract processing and analysis

Semen extenders play a vital role in storage by reducing cytotoxicity. Because of the fragility of boar spermatozoa, blueberry extracts must be modified before adding the extract to the extenders. In Chapter 2, higher values of total phenolics and total anthocyanins were obtained with acidified ethanol or methanol as an extraction solution, compared with an acidified water extraction solution. Both ethanol and methanol are organic solvents; they had a stronger capacity to extract organic compounds, e.g. polyphenols, than inorganic solvents. However, neither

methanol nor ethanol are compatible with boar spermatozoa and therefore were not used in subsequent experiments. The pH of blueberry powder extract and leaf extract varied from 2.9 to 3.2; it increased from 4 to 6 (depending on the extract volume) after mixing with the X-cell™ semen extender. Consequently, sodium hydroxide solution was used to adjust the pH value of blueberry extracts approximately 7.4. Although the antioxidant activity of polyphenols is greatest at low pH, sperm viability is profoundly compromised. Perhaps the change in pH had adverse effects either on semen or on the antioxidant activity of the blueberry products themselves. Polyphenolic and other bioactive compounds present may have become chelated when exposed to sodium hydroxide or the change in pH may have had inhibitory effects on the antioxidant properties of blueberry products. Furthermore, the chemicals added may have been cytotoxic to the spermatozoa. Therefore, the extraction process, and its effect on spermatozoa require further study.

Several investigators reported the utility of processed extracts of berry fruit for use in cell cultures. Youdim et al. (2002) used an acidified alcohol-based extraction solution (acetone: methanol:water:formic acid; 40:40:20:0.1 v/v/v/v) to obtain crude extracts from blueberries and cranberries. In that study, the extracts were eluted by acidified methanol (formic acid), acidified acetone (formic acid), or ethyl acetate. The fractions including phenolic acids, flavonols, anthocyanins, and tannins were then used to culture human microvascular endothelial cells. Seeram et al. (2004) obtained 3 products from cranberry extracts: sugar-enriched, organic acid-enriched and polyphenol-enriched fractions, when using water, water/methanol (85:15 v/v) and methanol/acetic acid (99:1 v/v) extraction solutions, respectively. The fractions were used to

culture the KB and CAL cancer cells, SW 480, SW620, HT-29, and HCT1116 colon cancer cell lines, and RWPE-1 and RWPE-2 prostate cancer cell lines *in vitro*. In their studies (Youdim et al., 2002 and Seeram et al., 2004), alcohol-based extraction solutions were used to obtain extracts containing a high concentration of polyphenols. However, alcohol has adverse effects on spermatozoa. Human sperm progressive motility and morphology decreased after exposure to ≥ 30 mg/L *in vitro* (Donnelly et al., 1999). Therefore, in the present study, the methods of alcohol-based extraction solutions were discarded; acidified water was used to extract polyphenols from blueberry products. In addition, in the present study, crude extracts from blueberries containing many mixtures were used. Therefore, not only the polyphenols, but other materials may have affected boar sperm preservation, this making it difficult to define the chemical and functional characteristics of the polyphenols on sperm preservation. Therefore, it is necessary to improve the present extraction method. In future study, compositions of blueberry products from water-based extraction solution should be eluted and analyzed.

As stated in Chapter 4, boron concentration is profoundly high in blueberry leaves. A high concentration of boron inhibits sperm motility and causes infertility (IPCS, 1998). Perhaps boron contributed to low boar sperm viability and motility in the present study. On the other hand, composition of blueberry leaves is not fully understood, which means that other compositions in blueberry leaves may have adverse effects on sperm survival. In the future, the concentration of boron should be determined before adding blueberry leaf extract into semen extenders.

Measurements of phenolic compounds

In Chapter 2, the Folin-Ciocalteau method (Sellappan et al., 2002) was used to assess total phenolics, and the pH-differential method (Wrolstad, 1993) was used to determine the value of total anthocyanins in the blueberry products. These methods are frequently used to determine phenolic compounds in various edible plant extracts. However, these 2 measurements are only a surrogate measure of phenolic compounds, since only gallic acid equivalent and malvidin 3-glucoside were used to represent total phenolics and total anthocyanins, respectively. Other methods, such as high-performance liquid chromatography (HPLC) and oxygen radical absorbance capacity (ORAC), are frequently used to determine the antioxidant content of plant extracts. The advantage of HPLC is that more phenolic compounds in blueberry products could be identified. The ORAC technique is used to quantify antioxidant capacity in fruit and vegetable extracts. The antioxidant capacity of blueberries could be measured by the ORAC technique and can be compared to the antioxidant standard e.g. vitamin E. Although more expensive to conduct than the techniques used in this study, these methods would yield more specific knowledge regarding the antioxidant capacity of blueberry products.

6.2 Measurement of lipid peroxidation

In Chapter 3, the thiobarbituric acid reaction substances (TBARS) assay was used to assess the ability of raw blueberry juice, powder extract, fruit extract, and leaf extract to retard lipid peroxidation of spermatozoa. The raw juice, the fruit extract from 10 g of frozen fruit, the

powder extract from 1.5 g blueberry freeze-dried powder, and the leaf extract from 1.5 g ground blueberry leaves were used in the presence of boar semen, ferrous sulphate, and ascorbate; their ability to retard lipid peroxidation was compared. Before correcting for interference, blueberry leaf extract had a higher capacity to retard the iron-induced lipid peroxidation than other blueberry products; the powder extract and fruit extract had similar capacities. The TBARS value of raw juice was extremely high compared to other blueberry products, which had similar values to the TBARS of the pro-oxidant (Fe/ASc) group. However, all blueberry products had similar TBARS values after correction for interference, which confirmed that all 4 blueberry products had similar capacity to retard lipid peroxidation.

Using the Folin-Ciocalteu assay, the concentrations of total phenolics in powder extract, fruit extract, leaf extract and juice were 0.4, 0.4, 0.8, and 0.6 mg GAE/mL; however, the concentrations of total anthocyanins were 0.084, 0.109, 0.003, and 0.381 mg malvidin 3-glucoside equivalent/mL. Since the the concentration of total phenolics (and total anthocyanins) is associated with antioxidant capacity (Prior et al., 1998), blueberry raw juice should have the capacity to retard lipid peroxidation as effectively as other blueberry products. It is obvious that interference due to the presence of blueberry products cannot be discounted when the TBARS assay was used. Although TBARS assay has been frequently utilized to assess increased oxidative stress of spermatozoa, it is also influenced by the presence of other aldehydes which are able to combine with TBA and generate pigment complexes. Whether pigments of blueberry extracts contributed to the TBARS assay was uncertain; furthermore, whether the phenolic compounds of blueberry extracts reacted with other chemicals was also uncertain. Therefore, in

future studies, a more specific assay should be utilized to assess blueberry extracts. High-performance liquid chromatography (HPLC) is an alternative to TBARS fluorometry. The pigment complexes can be separated and analyzed; thus, questions whether the pigments, the sugars or other potential components present in blueberry products contribute to the TBARS assay may be clarified.

6.3 Measurement of motility and viability

In Chapter 4, the X-cell^{TM} commercial extender, the blueberry leaf-supplemented and blueberry powder-supplemented extenders were used to evaluate both motility and viability during *in vitro* storage at 5, 16, and 22 °C. Based on the SYBR-14/PI assay, none of the extenders protected spermatozoa against cold shock at 5 °C. Only in the X-cell^{TM} extender alone and the powder-supplemented extender was sperm viability maintained, however, sperm viability was low in the leaf-supplemented extender.

Sperm motility was very sensitive to temperature and treatment. Sperm motility was no higher than 20 % when the spermatozoa was stored at 5 °C, and motility decreased rapidly to 30 % or less in the extenders that contained either a higher concentration of leaf extract or powder extract. Therefore, none of the blueberry-supplemented extenders were able to retain sperm motility.

As stated in Chapter 3, different concentrations of Fe/ASc combination were used to induce sperm lipid peroxidation, and the combination (Fe/ASc 0.1/0.5 mM) that helped to produce the

highest TBARS value was selected to trigger lipid peroxidation in the presence of blueberry products. Sperm parameters such as viability and motility were not measured at the same time. Perhaps the concentration of Fe/ASc combination was efficacious to produce radicals during incubation and the concentration of blueberry products was also satisfactory to either scavenge or chelate these radicals. However, whether the condition was appropriate to protect boar spermatozoa was uncertain. Perhaps the concentrations of blueberry products were too high for sperm survival, contributing to the unsatisfactory sperm viability and low sperm motility in Chapter 4. Therefore, sperm parameters should be measured concurrently with lipid peroxidation.

The SYBR-14/PI dual fluorescent microscopy can be used to measure sperm viability (Maxwell and Johnson, 1998) as stated in Chapter 4. Waterhouse et al. (2004) reported the use of fluorochrome-conjugated annexin V to evaluate plasma membrane- and acrosome-integrity. The fluorochrome-conjugated annexin V is a membrane-impermeable protein with high binding affinity to phosphatidylserine (PS), which is located within the inner leaflet of mammalian membrane cells; PS will be exposed at the membrane surface when the structure and function of membranes change during exocytosis or apoptosis. As stated in Chapter 4, sperm viability after being washed by PBS can be stained by fluorochrome-conjugated annexin V to evaluate sperm viability.

The DNA in spermatozoa plays a vital role during fertilization. Therefore, sperm DNA should be monitored during incubation or preservation. The combined Hoechst 33342/PI dual fluorescent assay (Rowland et al., 2003) was able to identify viable human sperm (clear or slight

blue at the post-acrosome region), early apoptosis (faintly blue acrosome), apoptosis (completely blue), and necrosis (red-pink, or half blue and half red). Boar sperm DNA parameters should be measured using this assay in the presence of Fe/ASc combination and blueberry products.

6.4 Measurement of capacitation status

In Chapter 5, capacitation status of spermatozoa stored in 3 extenders at 3 temperatures in the absence and/or presence of heparin and/or calcium ionophore was measured using the CTC assay. In the absence of heparin and calcium ionophore, spermatozoa in the leaf-supplemented extender exhibited a higher value of F pattern than that in the X-cell^{TM} extender alone or the powder-supplemented extender. Therefore, antioxidants from the blueberry extracts reduced oxidation-induced premature capacitation. Conversely, higher values of AR patterns of spermatozoa in the presence of heparin or in the presence of calcium ionophore indicated that antioxidants from the lowbush blueberry extracts did not inhibit the effects of heparin and calcium ionophore.

The molecular mechanism of the CTC assay is not fully understood. Furthermore, whether the blueberry extract and the X-cell^{TM} extender might interfere with the CTC assay is uncertain. Therefore, the measurement of sperm capacitation status should be improved by using the PI/CTC or Hoechst 33258/CTC dual-staining assay. In addition, as mentioned in Chapter 1, other methods such as the fluorescein isothiocyanate-peanut agglutinin (FITC-PNA), fluorescein isothiocyanate-pisum agglutinin (FITC-PSA), and Coomassie blue assays can be used to measure

acrosome integrity. Since capacitation is the upstream event of acrosome reaction, these assays could be used to confirm sperm capacitation status.

In conclusion, to our knowledge, this is the first time that lowbush blueberries have been used to preserve boar semen. Lowbush blueberry products (juice, fruit extract, powder extract, and leaf extract) contain substantial antioxidant capacity, which protected boar spermatozoa against iron-induced lipid peroxidation. This indicated that blueberry products may be used as a semen extender additive. No extender protected spermatozoa from cold shock when spermatozoa were stored at 5 °C, regardless of the assay used. No extender (except X-cell™) maintained sperm motility; all extenders containing blueberry supplementation inhibited sperm motility on Day 0, whereas sperm motility in the X-cell™ extender decreased steadily. When using the SYBR-14/PI assay, sperm viability (live cell %) in the control and the powder-supplemented extender remained high, whereas it was significantly lower in the leaf-supplemented extender. The low viability and low motility indicated that the concentrations of leaf extender and powder extender may have been too high to allow sperm survival; adverse effects may exceed the beneficial bioactivity of blueberry products. Spermatozoa in the leaf-supplemented extender exhibited a higher value of F pattern when using the CTC assay, even when they were incubated at 5 °C. However, spermatozoa in the control or the powder-supplemented extender exhibited lower values of the F pattern. Spermatozoa exhibited higher values of the AR pattern in the presence of heparin or calcium ionophore, regardless of temperatures or extenders, indicating that blueberry supplements did not inhibit heparin-induced or calcium ionophore-induced capacitation.

In the future, freeze-dried blueberry powder should be considered as a potential antioxidant. The pH value of blueberry powder extract in nature is profoundly lower than a semen extender required. Therefore, water may replace acidified water as a solution to extract antioxidants from blueberry products. In addition, to measure the concentrations of total phenolics and total anthocyanins, the ORAC assay should be used to measure the antioxidant capacity. The optimal concentration of the blueberry extract in the semen extender should be decided allowing for sperm parameters such as viability, motility, and fertility, when measuring lipid peroxidation. If the crude extract had an adverse effect on sperm parameters, components in the blueberry extract should be analyzed by HPLC, and should be separated by advanced extract methods. Then, different fractions from the blueberry extract may be added to semen extenders to measure sperm parameters.

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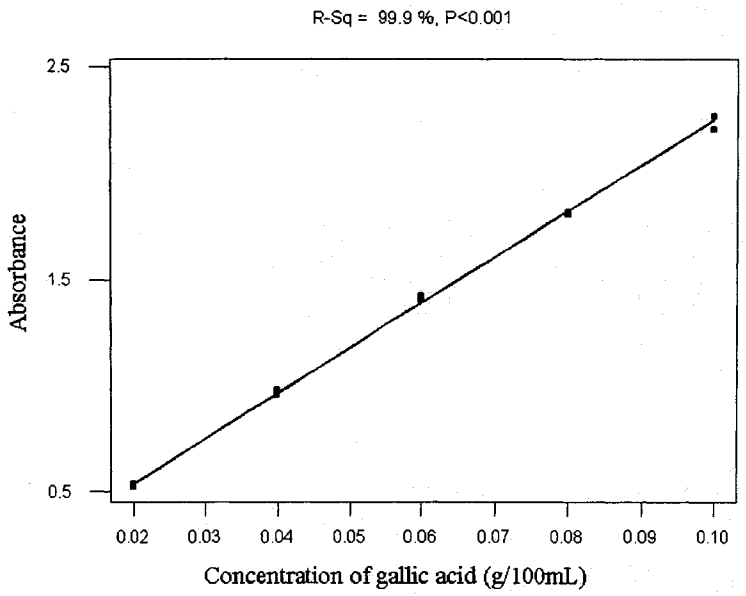
Appendix A-Chapter 2. Relationship between concentrations of gallic acid and the absorbance.



Appendix B-Chapter 4.

Table 1. Sample preparation for measuring sperm viability and motility using control, low, medium and high leaf extenders, and low, medium and high powder extenders.

		Semen preparation					
Treatment	Control[1]	Leaf extract concentration			Powder extract concentration		
		Low	Medium	High	Low	Medium	High
Extended semen (mL)[1]	120	120	120	120	120	120	120
Blueberry extract (mL)[2]	0	1.015	2.03	3.045	0.686	1.373	2.059
X-cell™ extender (mL)	30	28.985	27.97	26.955	29.314	28.627	27.941
Total (mL)	150	150	150	150	150	150	150
Phenolic acids ($x10^{-3}$mg GAE/mL)[3]	0	8.75	17.5	26.3	2.35	4.7	7.05

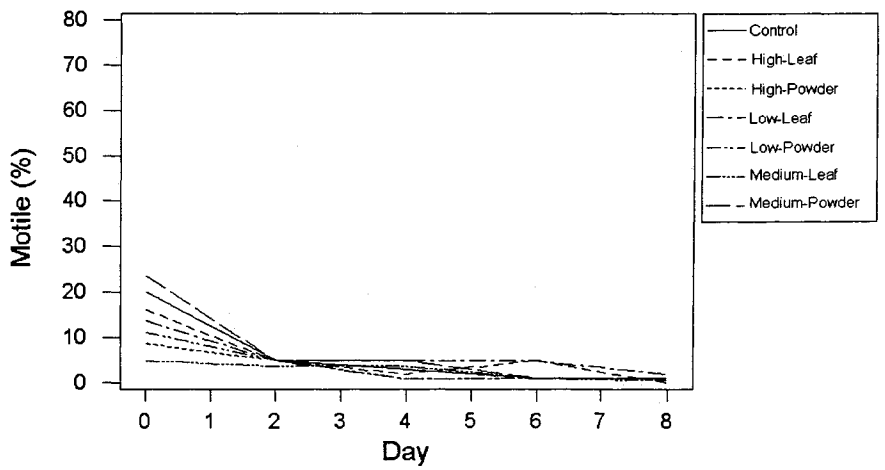
[1]Boar semen diluted by the addition of X-cell™ extender to achieve a sperm concentration of $62.5x10^6$/mL

[2]Stock blueberry leaf extract containing 1.5 mg GAE/mL, or stock blueberry powder extract containing 0.4 mg GAE/mL (see text for extraction method)

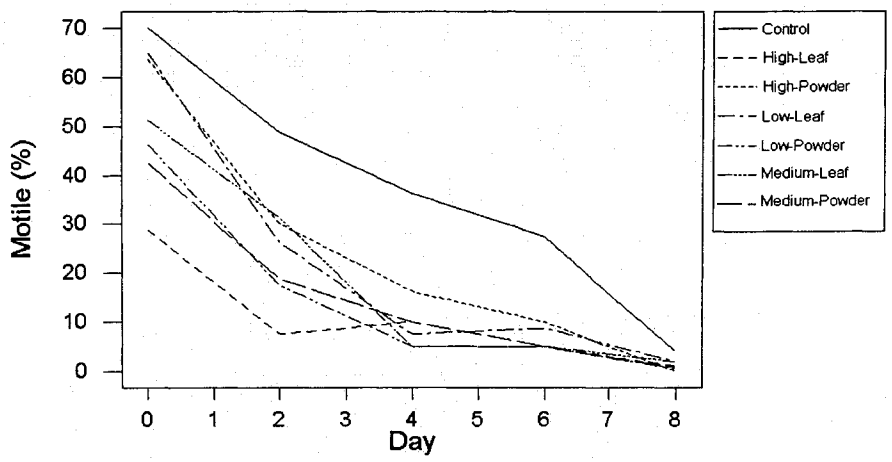
[3]Final concentration of blueberry-derived phenolic acids in the blueberry-supplemented semen preparations, expressed as mg GAE/mL

Appendix B-Chapter 4.

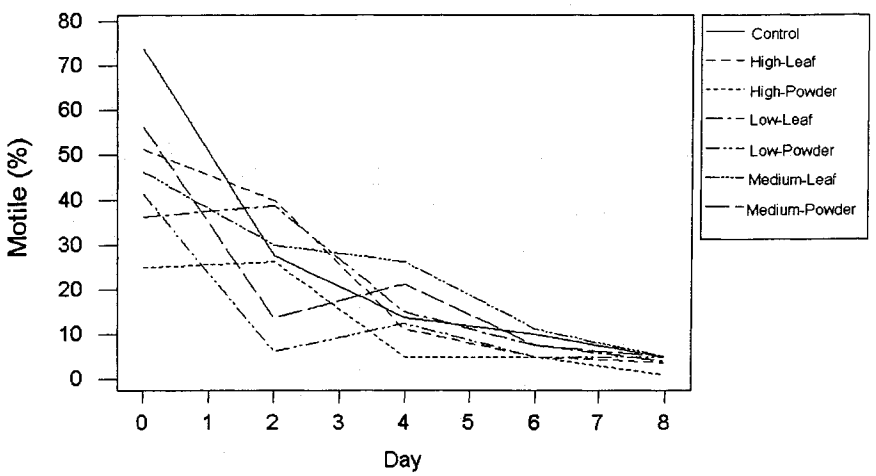
Figure 1. Sperm motility from Day 0 to Day 8 in 7 semen extenders at 5 °C.



Appendix B-Chapter 4.
Figure 2. Sperm motility from Day 0 to Day 8 in 7 semen extenders at 16 °C.

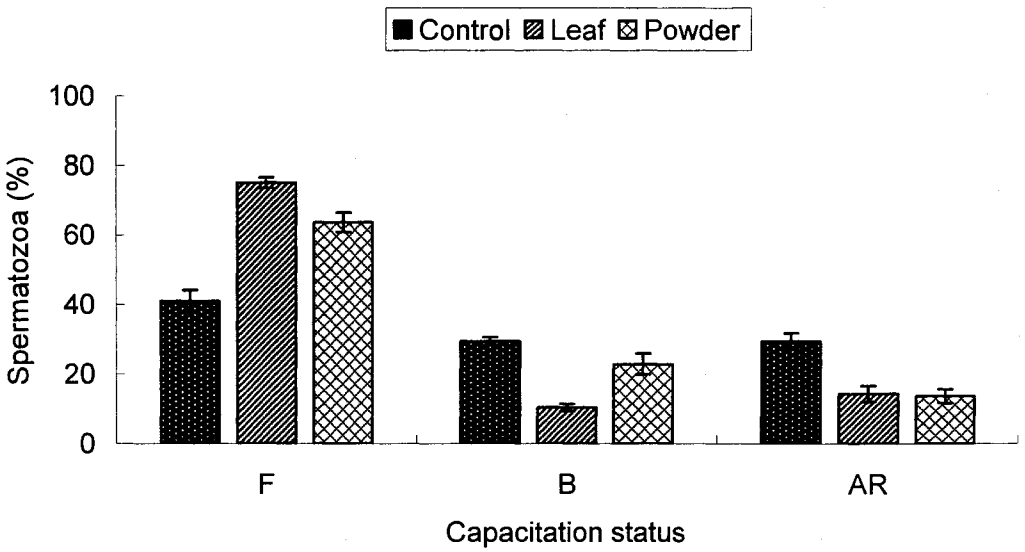


Appendix B-Chapter 4.
Figure 3. Sperm motility from Day 0 to Day 8 in 7 semen extenders at 22 °C.



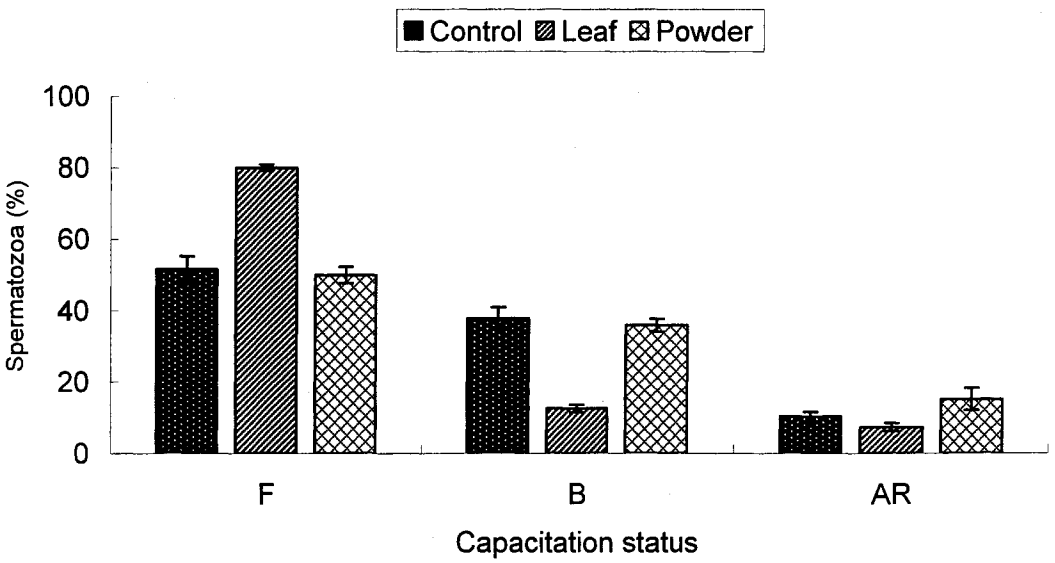
Appendix C-Chapter 5.

Figure 1. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 5 °C after 10 min incubation (39 °C).



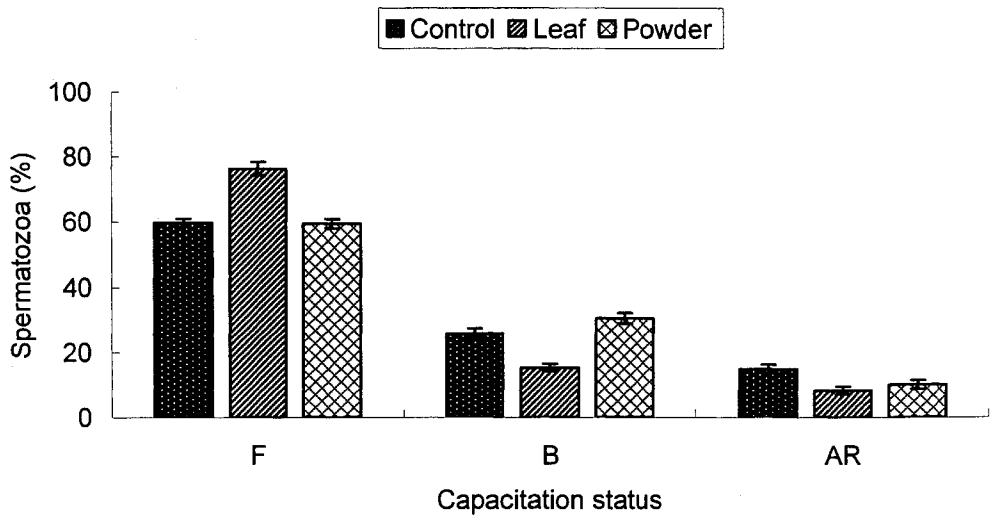
Appendix C-Chapter 5

Figure 2. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 16 °C after 10 min incubation (39 °C).



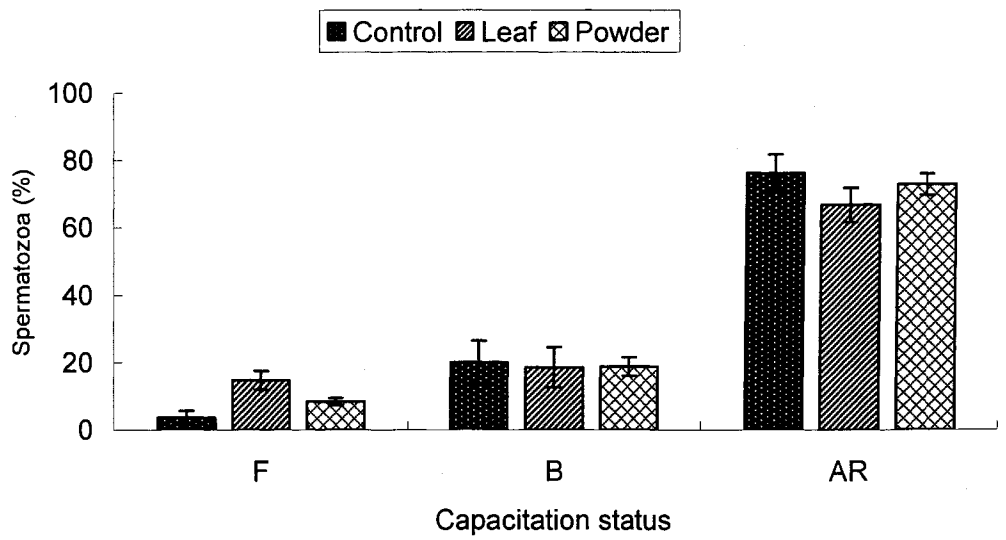
Appendix C-Chapter 5

Figure 3. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 22 °C after 10 min incubation (39 °C).



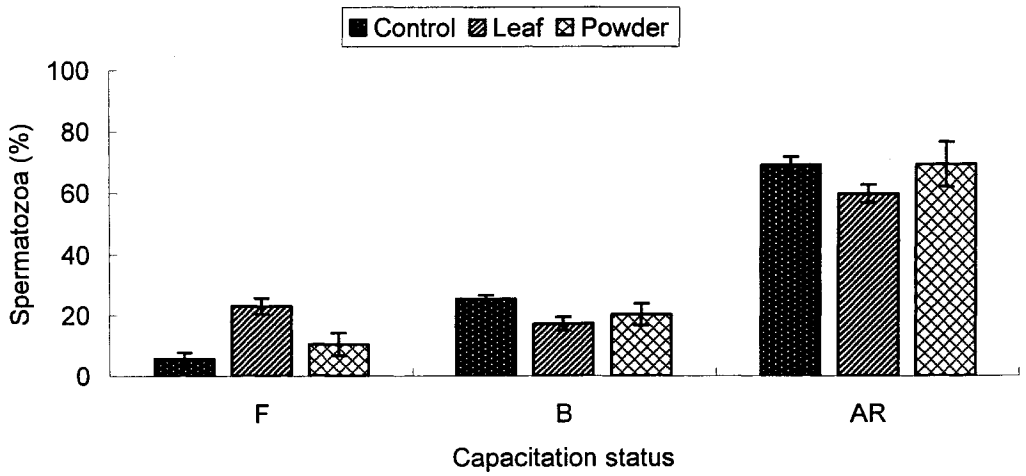
Appendix C-Chapter 5

Figure 4. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 5 °C in the absence of heparin and calcium ionophore after 4 h incubation (39 °C).



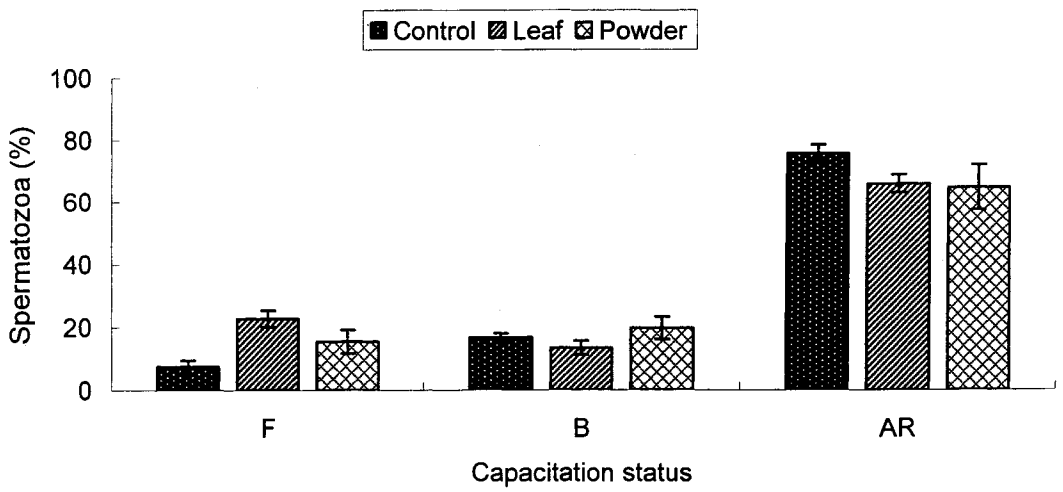
Appendix C-Chapter 5

Figure 5. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 16 °C in the absence of heparin and calcium ionophore after 4 h incubation (39 °C).



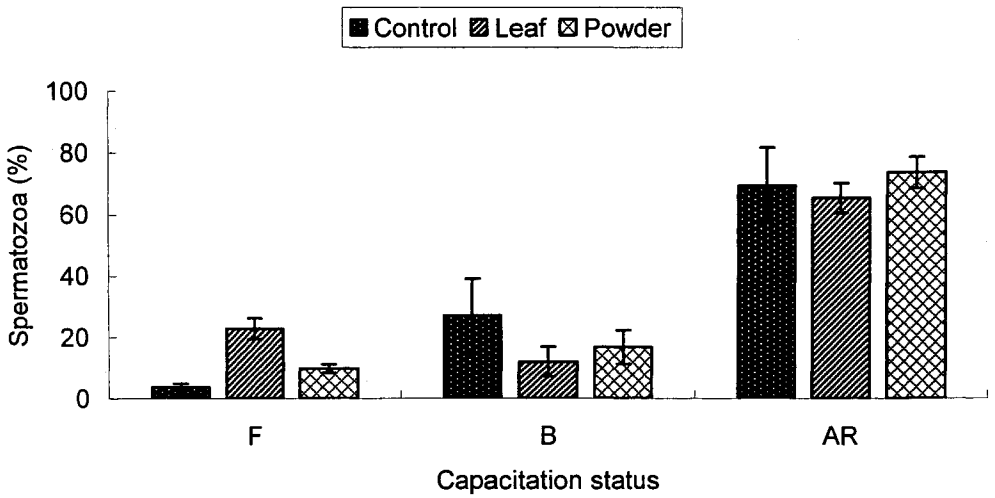
Appendix C-Chapter 5

Figure 6. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 22 °C in the absence of heparin and calcium ionophore after 4 h incubation (39 °C).



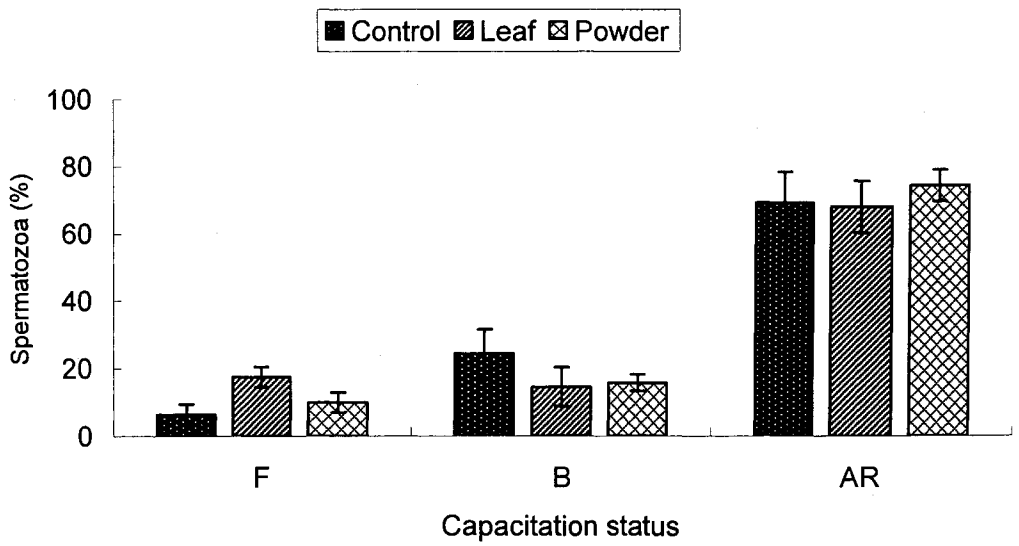
Appendix C-Chapter 5

Figure 7. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 5 °C in the presence of heparin but the absence of calcium ionophore after 4 h incubation (39 °C).



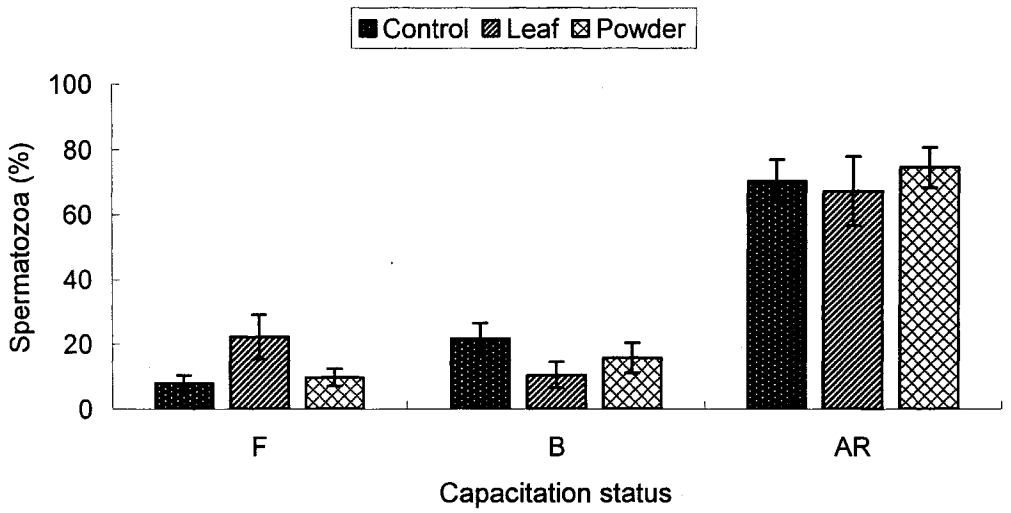
Appendix C-Chapter 5

Figure 8. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 16 °C in the presence of heparin but the absence of calcium ionophore after 4 h incubation (39 °C).



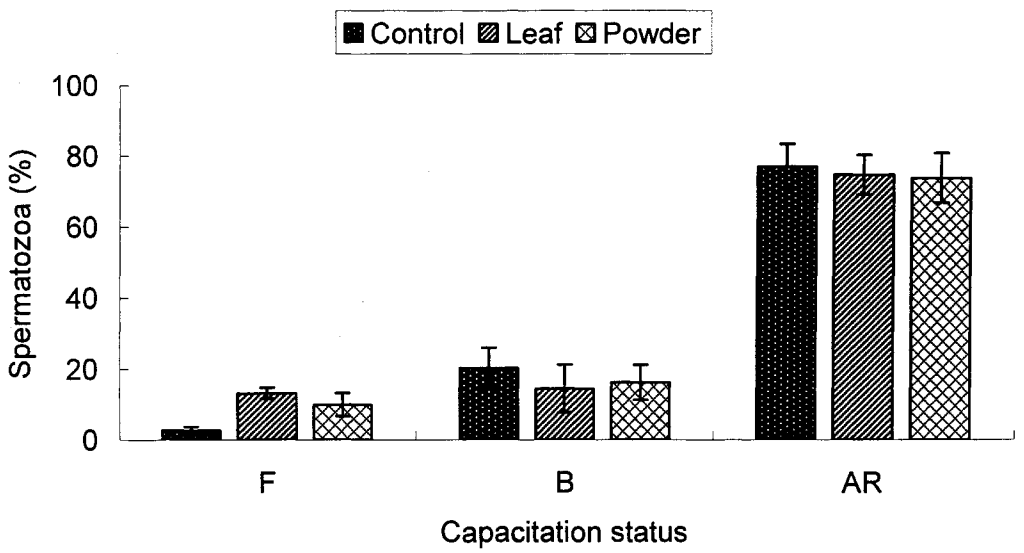
Appendix C-Chapter 5

Figure 9. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 22 °C in the presence of heparin but the absence of calcium ionophore after 4 h incubation (39 °C).



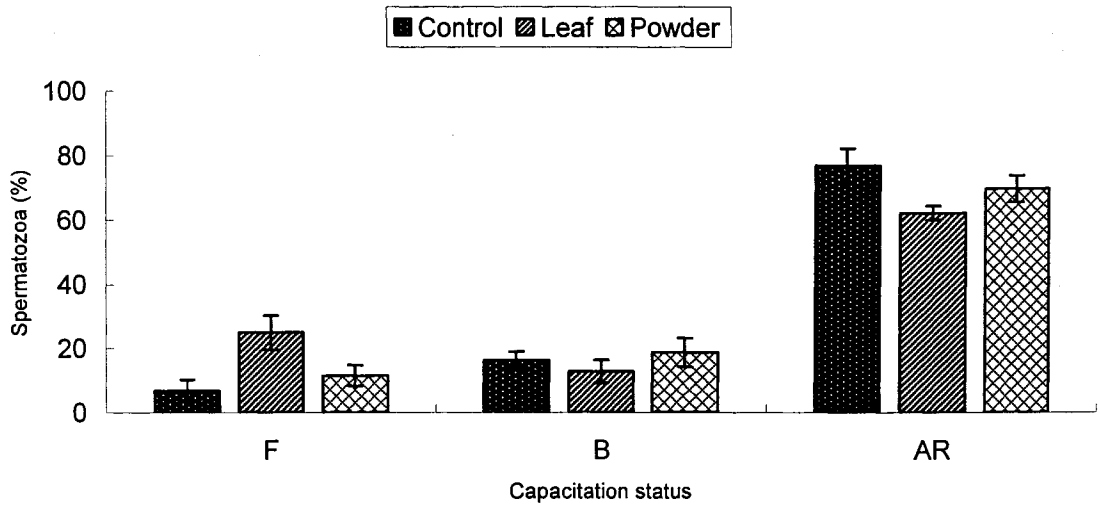
Appendix C-Chapter 5

Figure 10. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 5 °C in the absence of heparin but the presence of calcium ionophore after 4.5 h incubation (39 °C).



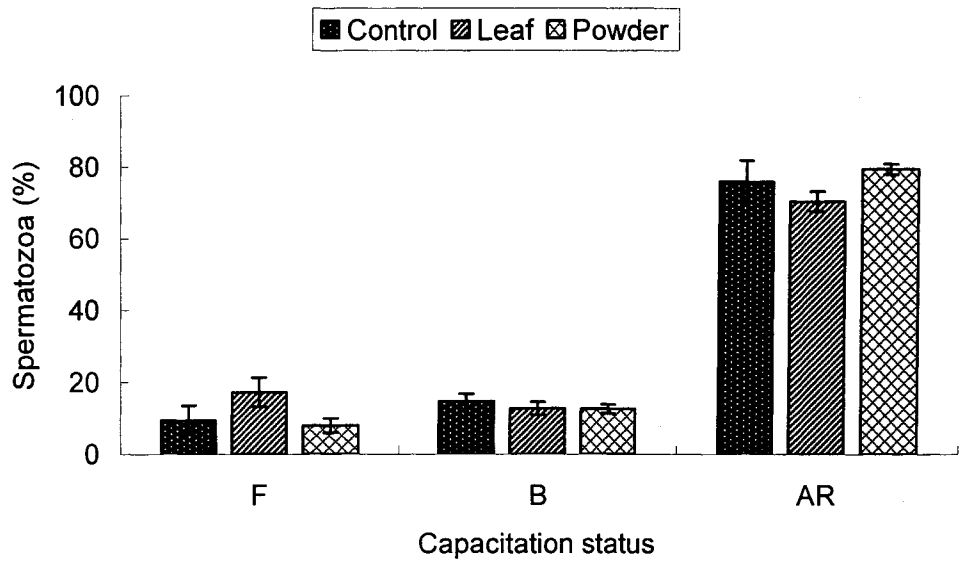
Appendix C-Chapter 5

Figure 11. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 16 °C in the absence of heparin but the presence of calcium ionophore after 4.5 h incubation (39 °C).



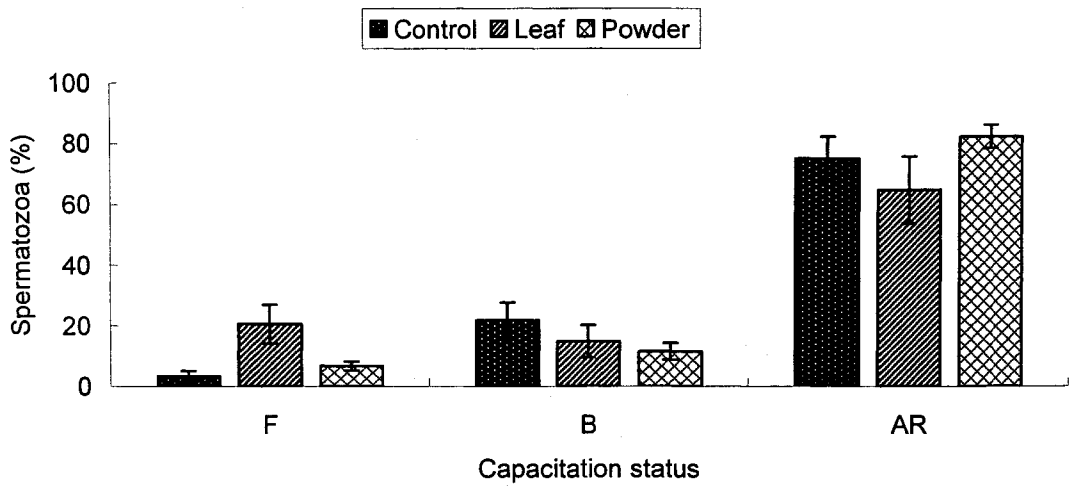
Appendix C-Chapter 5

Figure 12. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 22 °C in the absence of heparin but the presence of calcium ionophore after 4.5 h incubation (39 °C).



Appendix C-Chapter 5

Figure 13. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 5 °C in the presence of heparin and calcium ionophore after 4.5 h incubation (39 °C).



Appendix C-Chapter 5

Figure 14. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 16 °C in the presence of heparin and calcium ionophore after 4.5 h incubation (39 °C).

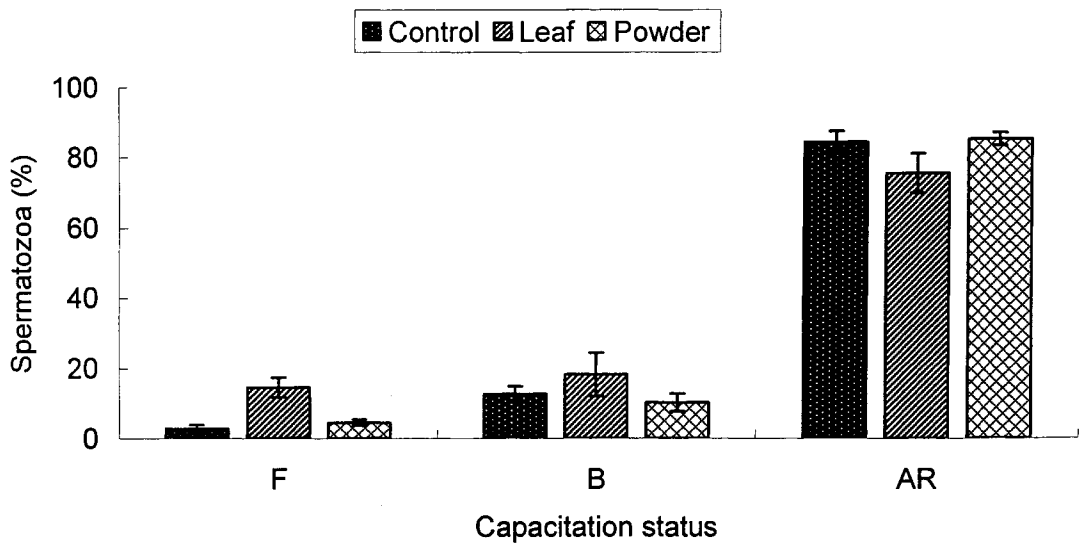


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Figure 15. Capacitation status (CTC staining) of boar spermatozoa stored in 3 extenders at 22 °C in the presence of heparin and calcium ionophore after 4.5 h incubation (39 °C).