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Cryopreservation of Boar Spermatozoa: An Important Role of Antioxidants

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1. Introduction

Artificial insemination (AI) is one of the first reproductive biotechnologies has been established and developed in the pig production system. In most case, liquid stored semen or fresh semen is used for AI in commercial swine herds (Wagner and Thibier, 2000). The use of FT boar semen for AI is limited due to the low fertility outcomes compared to extended fresh semen (Johnson et al., 2000; Wagner and Thibier, 2000). The first success of boar semen cryopreservation was reported in 1956 (Polge, 1956) and the first pregnancy was achieved with FT boar semen using surgical insemination in 1970 (Polge, 1970). Currently, the attempt to develop the boar semen cryopreservation technique is ongoing. Nevertheless, the success of boar semen cryopreservation is relatively variable because the factors responsible for the cryosurvival of boar spermatozoa have not been entirely elucidated.

Cryopreservation of boar semen is useful for preservation of genetic resources, improve the genetic progress and enhance the transportation of genetic material across countries (Almliid and Hofmo, 1996; Johnson, 1998). In addition, the frozen-thawed (FT) boar semen is also used with other reproductive technologies, such as in vitro fertilization (IVF), embryo transfer (ET) and sex pre-selection (Gerrits et al., 2005). Unfortunately, the advancement of sperm cryopreservation in pigs is slow, partly due to the pig producer is satisfied with the liquid stored semen and low conception rate and litter size remain the major problems when using FT boar semen (Eriksson et al., 2002.). Under field conditions, low fertility is still obtained even using FT boar semen with a sufficient motility and number of spermatozoa for insemination (Johnson et al., 2000, Eriksson et al., 2002).

The use of frozen-thawed (FT) boar semen has been developed for artificial insemination (AI) in pig long time ago in Europe and USA (Larsson and Einarsson, 1976). In Thailand, few studies on boar semen cryopreservation have been established (Buranaamnuay et al., 2006 ^{a,b}). However, a great variation on the survival rate of post-thawed spermatozoa are obtained, due to the lack of biological background concerning the cryopreservation technique (Buranaamnuay et al., 2006 ^{a,b}). During the recent years, studies on FT boar semen have dramatically improved boar semen cryopreservation technique, for instance, optimum freezing protocols (Eriksson and Rodriguez-Martinez, 2000), types of freezing package (Bwanga et al., 1991; Berger and Fisherleitner, 1992; Bwanga et al., 1991; Eriksson and Rodriguez-Martinez, 2000), semen centrifugation methods (Carvajal et al., 2004),

thawing process (Eriksson and Rodriguez-Martinez, 2000; Córdova-Izquierdo et al., 2006) and the supplement of some additives to the semen extender (Peña et al., 2003, Gadea et al., 2004; Roca et al., 2004, 2005).

Boar semen differs in several aspects from the semen of other domestic animals, for instance, the semen is produced in a large volume and highly sensitive to cold shock, the viability of the sperm cells is dramatically reduced when exposed to temperatures below 15 °C (Gilmore et al., 1996). Therefore, the manipulation of boar semen requires special consideration during cryopreservation process (Johnson et al., 2000). Many factors that should be concerned for the boar semen cryopreservation included composition of diluents, type and concentration of cryoprotective agent, equilibration time, cooling rate and thawing procedure.

The relatively low fertility of FT boar semen is associated with many factors including a highly sensitive plasma membrane of boar spermatozoa against the changing in temperature during cooling, freezing and thawing process (Holt, 2000; Watson, 2000). This problem is related to the lipid composition of the sperm plasma membrane. The plasma membrane of the boar spermatozoa contains a high level of polyunsaturated fatty acids (PUFAs) i.e., docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and had a low cholesterol to phospholipids ratio. DPA and DHA are dominant fatty acids in the plasma membrane of boar spermatozoa (Johnson et al., 1969).

During cryopreservation, PUFAs decrease dramatically due to lipid peroxidation. This is initiated when the spermatozoa is attacked by reactive oxygen species (ROS) (De Lamirande and Gagnon, 1992; Sikka et al., 1995). In mammals, the major sources of ROS formation include leucocyte, defective and dead spermatozoa (Aitken et al., 1994; Silva, 2006). The excessive ROS formation influence sperm motility, mid-piece abnormalities and sperm-oocyte fusion (Chatterjee et al., 2001; Agarwal et al., 2005).

The supplement of antioxidant compounds and some fatty acid to the semen extender, to minimize ROS formation and protect the plasma membrane function, have been used in many species (Peña et al., 2003; Gadea et al., 2004; Roca et al., 2004; 2005; Maldjian et al., 2005). It has been demonstrated that the proportion of DHA was significantly higher in the semen diluted with an extender supplemented with n-3 enriched hen egg yolks compared with the semen diluted with normal hen egg yolks (Maldjian et al., 2005). However, no study has been demonstrated clearly whether or not the supplement of DHA could improve the quality of the boar spermatozoa after cryopreservation. Rooke et al. (2001) found that DHA supplement in the boar feed increase progressive motility and normal acrosome and decrease abnormal spermatozoa. Recently, Kaeoket et al. (2008) reported that the supplement of DHA-enriched fish oil improved the FT boar semen quality. It has been shown that the supplement of cryoprotective agents (e.g., glycerol and Equex®), cholesterol analogue (Zeng and Terada, 2001) and antioxidants (e.g., Vitamin E, alpha-tocopherol, glutathione, taurine, cysteine, butylated hydroxytoluene, superoxide dismutase and catalase) in the semen extenders does improve the freezing ability of spermatozoa of many species such as stallion (Aurich et al., 1997; Ball et al., 2001), bull (Beconi et al., 1993; Bilodeau et al., 2001), ram (Uysal and Bucak, 2007; Bucak et al., 2007), avian (Donoghue and Donoghue, 1997), boar (Cerolini et al., 2000) and some wildlife (Leibo and Songsasen, 2000). Studies have demonstrated that the supplement of alpha-tocopherol (Peña et al., 2003), butylated hydroxytoluene (Roca et al., 2004), superoxide dismutase and catalase (Roca et al.,

2005) in the semen extenders reduces the ROS formation and improve post-thawed sperm motility and viability of FT boar semen. In addition, it was found that the supplement of extended boar semen with 5 mM of cysteine improved the viability and functional status of the chilled boar spermatozoa (Funahashi and Sano, 2005).

2. History of cryopreservation

Nowadays, there are 2 techniques for cryopreservation in boar semen, traditional nitrogen method and controlled rate freezing method. Verheyen (1993) reported a significantly better post-thaw sperm outcome when computer controlled rate freezing was used compared to non-controlled rate freezing. In human, it has been reported that controlled rate freezer method provided significant superior post-thaw sperm motility, viability, and cryosurvival rate, compared with traditional nitrogen method (Petyim and Choavaratana, 2006). However, the study of Thalchil (1981) did not confirm the different outcome of these 2 methods. Besides breed-specific fertility, data from field trials found that the mean motility of frozen-thawed semen between Norwegian Landrace and Duroc boars was difference. In the different breeds of boars found the differences in membrane lipid composition, can neither explain the major differences in post-thaw survival and fertility between breeds (Waterhouse et al., 2006).

Major limitation of frozen-thawed semen (FT-boar semen) have been observed, i.e. low conception rate and low litter size after AI (Johnson et al., 2000; Buranaamnuay et al., 2006). The relatively low fertility of FT-boar is associated with many factors. It has been reported that reactive oxygen species (ROS) generation, induced by the cryopreservation process, can be responsible for mammalian sperm damage (Griveau and Le Lannou, 1997) ROS production has been associated with reduction of sperm motility and decreased capacity for sperm-oocyte fusion. Spermatozoa are sensitive to lipid peroxidation due to their high content of polyunsaturated fatty acids, and are unable of resynthesizing their membrane components, although this may not be the sole mechanism by which sperm function is impaired by ROS. Many studies have shown that the supplementation of antioxidants in extenders improved the qualities of both fresh boar semen (Bamba and Cran, 1992; Funahashi and Sano, 2005) and frozen boar semen (Breininger et al., 2005; Gadea et al., 2005; Pena et al., 2003; Roca et al., 2004; Roca et al., 2005). Earlier studies showed that the supplementation of some antioxidant such as, water soluble Vitamin E 200 μ M to semen extenders for freezing boar spermatozoa reduced post-thaw ROS generation and improved sperm motility and viability (Pena et al., 2003). Funahashi and Sano (2005) reported that supplementation extended boar semen with glutathione or *L*-cysteine of 5 mM improved the viability and functional status of boar spermatozoa during liquid storage at 10 °C for at least 14 day. A recent report (Gadea et al., 2005) demonstrated that supplementation with 1 mM of reduced glutathione to freezing media resulted in a protective effect on sperm function.

3. Cryopreservation of animal spermatozoa

Cryopreservation of boar semen need to be developed for AI in the pig industry due to a number of reasons including preservation of a good genetic resource, increase genetic improvement, distribution of genetic lines across countries and reduce boar transportation

(Almlid and Hofmo, 1996; Johnson, 1998). The widespread exchange of genetic material between breeding populations with liquid stored semen is difficult because of the short life span of the spermatozoa (Wagner and Thibier, 2000; Johnson et al., 2000). The first FT boar spermatozoa have been reported since 1956 (Polge, 1956). Unfortunately, the FT spermatozoa has a very low fertilizing ability. In 1970, the first pregnancy was achieved with FT boar semen using a surgical insemination technique (Polge et al., 1970). In 1971, many studies have reported the pregnancies after intra-cervical insemination using FT boar semen in pig (Crabo et al., 1971; Pursel et al., 1971).

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

The freezing and thawing procedure have a significant impact on the survival rate of sperm after cryopreservation (Johnson et al., 2000). However, optimal freezing and thawing rates vary depending on the type and concentration of the cryoprotectant (Mazur et al., 1970; Fiser et al., 1993). Currently, the optimal rates for boar sperm freezing appear to be 30°C/min with 3% glycerol as cryoprotectant when freezing in 0.5 ml straws (Fiser et al., 1990) and 16°C/min with 3.3% glycerol in 5 ml straws (Pursel et al., 1985). For both these methods the optimal thawing rate is 1200 °C/min (Westerndorf et al., 1975; Fiser et al., 1993). Eriksson and Rodriguez-Martinez, (2000) found that the optimal freezing rate was 50 °C/min in 3% glycerol with a 900 °C/min thawing rate for flattened plastic bags (FlatPack®) container. A variety of cryoprotectants are used in the freezing extender of different in species. Glycerol, egg yolk and sodium dodecyl sulphate (SDS) (Equex STM or Orvus ES paste) is commonly used as cryoprotectants for the cryopreservation of boar semen (Westerndorf et al., 1975; Pursel et al., 1978; Holt, 2000b). The optimal concentration of glycerol was approximately 3 % in pig (Holt, 2000a). Egg yolk and SDS are non-permeable cryoprotectant used in freezing extender and provide protective effect to spermatozoa and improved post thawed sperm quality (Pursel et al., 1978). It has been suggested that SDS enhances the cryoprotective properties of the egg yolk to protect the sperm membrane from cryoinjuries (Buhr et al., 1996)

4. Boar semen cryopreservation methods

4.1 Semen collection

Three ejaculates from each boar are collected using gloved-hand method. During collection the semen is filtered through gauze and only sperm rich fractions are collected. Within 30 min after collection, semen volume, pH, sperm motility, concentration, percentage of live and dead sperm and morphology are determined. Only ejaculates with motility of ≥70% and ≥80 % morphologically normal are used for cryopreservation.

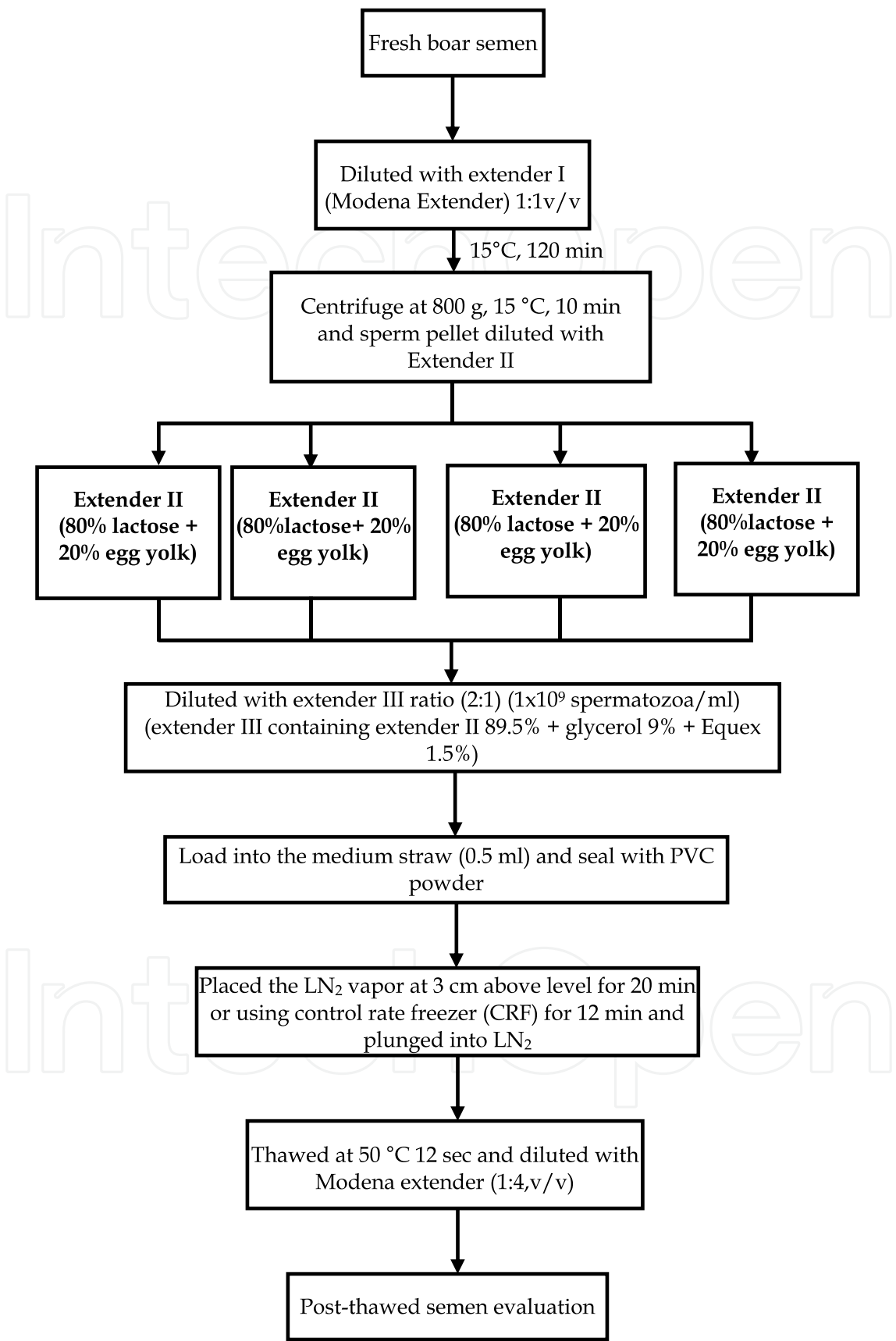


Fig. 1. Flow chart of the boar semen freezing processes, thawing and evaluation

4.2 Semen freezing and thawing procedures

Shortly after collection, the semen is diluted (1:1 v/v) with extender I (Modena™, Swine Genetics International, Ltd., Iowa, USA). The diluted semen is transferred to 50 ml centrifuge tubes, equilibrated at 15 °C for 120 min and centrifuged at 800x g for 10 min. The supernatant is discarded and the sperm pellet was re-suspended (about 1-2:1) with extender II (80 ml of 11% lactose solution and 20 ml egg yolk) to a concentration of 1.5×10^9 spermatozoa/ml. The diluted semen is cooled to 5 °C for 90 min. Then, two parts of the semen are mixed with one part of extender III (89.5% of extender II with 9% glycerol and 1.5% Equex-STM®). The final concentration of semen is approximately 1.0×10^9 spermatozoa/ml and contained 3% glycerol (modified after modified after Westerndorf et al., 1975 and Gadea et al., 2004). The processed semen is loaded into 0.5 ml straws (Bio-Vet, Z.I. Le Berdoulet, France). The straws are sealed with PVC powder before being placed in contact with nitrogen vapour about 3 cm above the liquid nitrogen level for 20 minutes in an expandable polystyrene box. Then the straws are plunged into liquid nitrogen (-196 °C) for storage. Thawing is achieved by immersing the straws in water at 50°C for 12 sec (Selles et al., 2003). Immediately after thawing, the semen is diluted (1:4) with a Modena™ extender. Post-thawed sperm qualities are evaluated after incubation in a 37°C water-bath for 15 min.

4.3 Semen extender

After incubation in extender I, the semen is divided into 4 groups according to the composition of extender II. Group I, extender II containing 80 ml of 11% lactose solution and 20 ml egg yolk. Group II, extender II is supplemented with 0.29 g of fish oil (Fish oil 1000; Blackmores LTD, New Southwell, Australia; containing DHA 120 mg/g fish oil) per gram of egg yolk. Normal egg yolk contains approximately 3.15 mg DHA per gram of egg yolk, as was analyzed at the Institute of Nutrition, Mahidol University (AOAC, 2007). Group III is supplemented with a combination of fish oil 0.29 g and L-cysteine 5 mM (Fluka Chemie GmbH, Sigma-Aldrich, Switzerland). Group IV is supplemented with a combination of fish oil 0.29 g and L-cysteine 10 mM.

5. Harmful effect of cryopreservation to spermatozoa

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

It is well documented that the boar spermatozoa are highly susceptible to temperatures below 15 °C. The viability of the spermatozoa are dramatically reduced within a few hours after expose to cooling below 15 °C, so call 'cold shock' (Gilmore et al., 1996). Cold shock caused the damage of plasma membranes and alterations in the metabolism of the spermatozoa. This caused by changes in the arrangement of plasma membrane compositions especially phospholipids (reviewed by Medeiros et al., 2002). The sperm damage cause by cold shock is characterized by an irreversible loss of motility and the loss of sperm permeability. Boar spermatozoa seem to acquire a cold shock resistance when the

semen is held at room temperature in seminal plasma for 1-5 hours (Pursel et al., 1972). It was found that viability and fertilizing ability of the boar spermatozoa was significantly improved when the fresh semen was held at 15 °C for over 3 hours before cryopreservation (Almlid and Johnson, 1988; Eriksson et al., 2001)

During the process of freezing, the decrease of temperature from -15 °C to -60 °C causes sperm damage (Mazur, 1985). This causes by the intracellular ice formation and cellular dehydration (osmotic stress). The subsequent physical events depend on the cooling rates. Intracellular ice formation occur during a rapid cooling when intracellular water does not leave the cell to maintain equilibration. If cooling is slow, the spermatozoa will lose water rapidly avoid to intracellular ice formation. However, if spermatozoa are cooled too slowly, they will expose to high concentration of solutes which caused intracellular water to diffuse out of the cell, dehydration both the cell and plasma membrane (also known as solution effects) (Mazur, 1970; Parks and Graham, 1992). Gilmore et al. (1996) demonstrated that the boar spermatozoa are sensitive to osmotic stress. Similar findings were also found in dog (Songsasen et al., 2002), cat (Pukazhenthi et al., 2000), ram (Curry and Watson, 1994), stallion (Ball and Vo, 2001) and bull (Liu and Foote, 1998).

Cellular damage due to intracellular ice formation and dehydration, oxidative stress is another important cause of sperm damage leading to abnormal sperm structure and function and subfertility. Currently, several studies have been reported oxidative stress affect the damage of sperm membrane, proteins and DNA in human (Agarwal et al., 2003), stallion (Baumber et al., 2000; 2003), bull (Bilodeau et al., 2001) and boar (Roca et al., 2004; 2005)(Fig. 2).

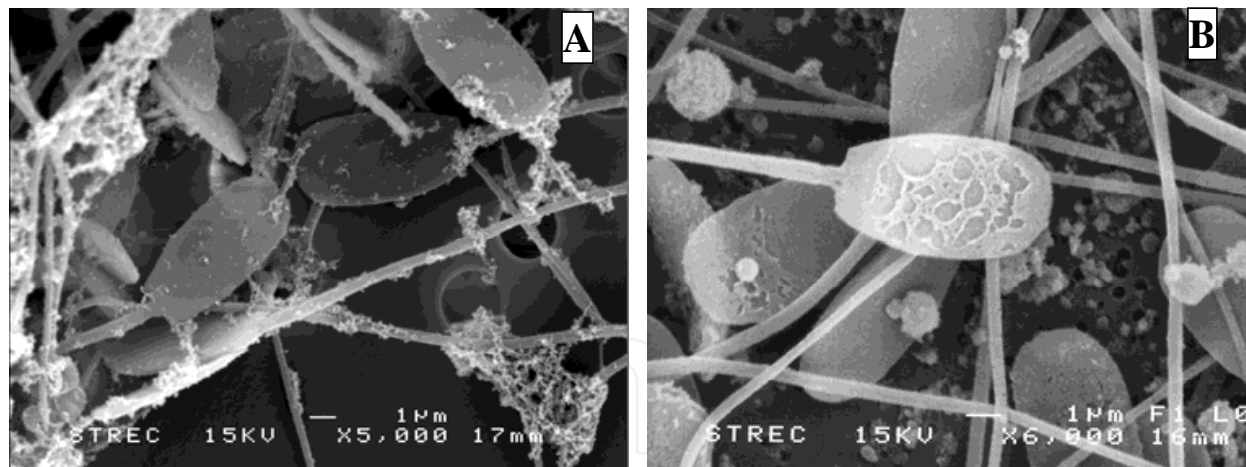


Fig. 2. Scanning electron microscopic (SEM) picture of fresh boar semen with normal plasma membrane (A) as compare with SEM picture of frozen boar semen with plasma membrane damage (B).

6. Capacitation like changes of the frozen-thawed boar semen and its influence on *in vivo* fertility

After ejaculation, the spermatozoa are not able to fertilize the oocyte. They undergo the activation process that called 'capacitation' and acrosome reaction which spermatozoa will able to reach the ampulla of the oviduct, penetrate the cumulus oophorus, bind to the zona

pellucida, activate the acrosome reaction and eventually fertilize the oocyte (Yanagimachi, 1994). Normally, capacitation *in vivo* occurs in the female reproductive tract, but capacitation can also be induced *in vitro* by the incubation in capacitating media which the most of media contain the bicarbonate, calcium and serum albumin (Yanagimachi, 1994). Furthermore, It has been demonstrated that the cooling and freezing process can also induced the capacitation like change which affect to the low fertilizing capacity of spermatozoa in boar and other mammalian species (Maxwell and Johnson, 1997; Green and Watson, 2001; Barrios et al., 2000). During cooling, freezing and rewarming process, it is hypothesized that change in low temperature cause the modification and destabilization of the lipid content in the sperm plasma membrane, reducing the selective permeability resulted in the cholesterol efflux and intracellular calcium uptake leading to the capacitation like change (Green and Watson, 2001; Tardif et al., 2001). To improve the the FT spermatozoa, there are some studies about the addition of cholesterol-loaded cyclodextrins increased the cryosurvival of boar, ram and bovine spermatozoa because cyclodextrins used to deliver cholesterol to the sperm plasma membrane which against cold shock (Purdy and Graham, 2004; Bailey et al., 2008; Mocé et al., 2009). In addition, the supplement of antioxidants such as vitamin E or alpha-tocopherol decreased the capacitation like change of cryopreserved boar spermatozoa (Satorre et al, 2007). Furthermore, the addition of seminal plasma to boar spermatozoa has been shown to reduce the capacitated spermatozoa in chilled and FT boar semen (Kaneto et al., 2002; Suzuki et al., 2002; Vadnais et al., 2005a,b; Okazaki et al., 2009, Garcia et al., 2009).

7. Laboratory method for semen quality assessment

7.1 Sperm concentration and progressive motility

Sperm concentration will be assessed by direct cell count using a Bürker haemocytometer (Boeco, Humburg, Germany) (Beardon and Fuquay, 1997). The visual progressive motility of both fresh and FT sperm is evaluated at 38°C under a phase contrast microscope at 200x and 400x magnification. The motility is assessed by the same person throughout the experiment.

7.2 Computer-assisted sperm analysis (CASA)

The motility patterns of diluted FT semen are assessed using the CASA system (Halminton Thorne Biosciences IVOS, Version 12 TOX IVOS, Beverly, USA). Each FT thawed semen samples is diluted with pre-warmed Modena extender (37°C) to obtain a final concentration of 50×10^6 spermatozoa/ml. A 5 µl of diluted semen is pipetted into the chamber and allowed the 1 min before analysis fore sample distribution and pre-warming (Iguer-Ouada and Verstegen, 2001). After the first assessment (T0), the diluted semen is evaluated after incubation at 37 °C for 30 min (T30) and 60 min (T60). The camera will recognize the position of the sperm heads in successive frames. Spermatozoa heads are marked with a different color to enable the observer and the analyzer to differentiate between the different motility patterns. Each semen sample is measured twice, 3 fields are evaluated and counted at least 1000 cells per analysis. Motility patterns including (1) Curvilinear velocity (VCL, µm/s), the average velocity measured in the progression line along the whole track of cell

path; (2) Average pathway velocity (VAP, $\mu\text{m/s}$), the average velocity of the smoothed cell path; (3) Straight line velocity (VSL), the average velocity measured in a straight line from the beginning to the end of the track ($\mu\text{m/s}$); (4) The amplitude of the lateral head displacement (ALH), the mean width of the head oscillation as the sperm cells swim (μm); (5) The beat cross-frequency (BCF, Hz), frequency of the sperm head crossing the average path in either direction; (6) The straightness (STR, %) = average value of the ratio VSL/VAP; (7) The Linearity (LIN, %) = average value of the ratio VCL/VAP.

7.3 Sperm viability

The percentages of sperm viability will be determined by 2 methods. The first one is eosin-nigrosin staining (Dott and Foster, 1972). The semen sample (50 μl) are mixed well with a drop of eosin-nigrosin dyes (Fluka Chemie GmbH, Sigma-Aldrich, Switzerland), and the mixture (10 μl) is smeared and dried on a glass slide. Evaluation is undertaken by counting 200 spermatozoa with 1000x magnification. Spermatozoa with an unstained head are regarded as live spermatozoa. The second method is evaluated by SYBR-14/Ethidiumhomodimer-1 (EthD-1) (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, The Netherlands). This technique is modified after Axnér et al. 2004 and Garner and Johnson, 1995). Ten μl of diluted semen are mixed with 2.7 μl of the user solution of SYBR-14 and 10 μl of EthD-1. The user solution is SYBR-14 diluted (1:100) in dimethyl sulfoxide (DMSO), fractionated and frozen in eppendorfs. After incubation at 37 °C for 20 min, two hundred spermatozoa will be assessed ($\times 1000$) under fluorescent microscope. The nuclei of the spermatozoa with an intact plasma membrane are stained green with SYBR-14, while those with damaged membranes stained red with EthD-1.

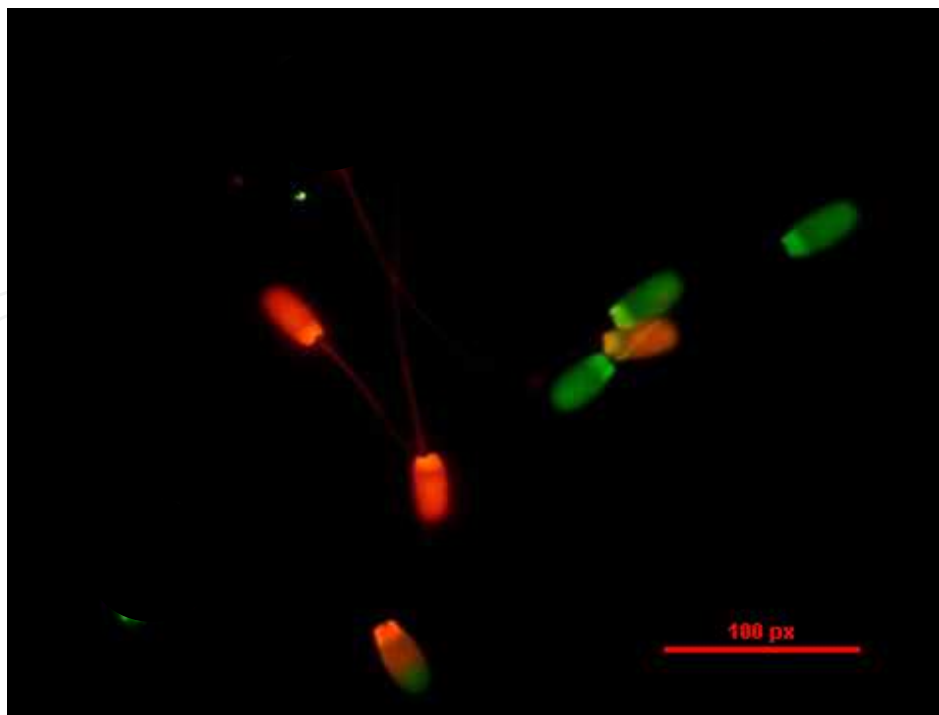


Fig. 3. Spermatozoa stained with SYBR-14/EthD-1 or PI: live spermatozoa stained green with SYBR-14 while dead spermatozoa stained red with EthD-1 or PI.

Spermatozoa are classified into three types; live spermatozoa stained green with SYBR-14, dead spermatozoa stained red with EthD-1 and moribund spermatozoa stained both green and red (Axnér et al. 2004; Garner and Johnson, 1995). The results are expressed as the percentage of live spermatozoa with intact plasma membranes.

7.4 Acrosome integrity

Acrosome integrity will be assessed using fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining. Ten μl of the diluted semen is mixed with 10 μl of Ethidiumhomodimer-1 and incubated at 37 °C for 15 min. Five μl of the mixture is smeared on a glass slide and fixed with 95 % ethanol for 30 second. Fifty μl Fit C-PNA (dilute Fit C-PNA with PBS 1:10 v/v) is spread over the slide and incubated in a moist chamber at 4 °C for 30 min. After incubation, it is rinsed with cold PBS and air dried. Two hundred spermatozoa are assessed under fluorescent microscope at 1000x magnification and classified as intact acrosome, damaged acrosome and missing acrosome (Cheng et al., 1996; Axner et al., 2004). The results are scored as the percentage of intact acrosome spermatozoa.

7.5 The functional integrity of the sperm plasma membrane

The functional integrity of the sperm plasma membrane will be assessed using a short hypo-osmotic swelling test (sHOST) (Perez-Llano et al., 2001). Spermatozoa are incubated, at 38 °C for 30 min, with 75 mOsm/kg a hypo-osmotic solution that consist of 0.368 % (w/v) Na-citrate and 0.675 % (w/v) fructose (Merck, Germany) in distilled water. Following this incubation time, 200 μl of the semen-hypo-osmotic solution is fixed in 1000 μl of a hypo-osmotic solution plus 5 % formaldehyde (Merck, Germany), for later evaluation. Two hundred spermatozoa are assessed under a phase contrast microscope at 400x magnification. The coiled tail (sHOST positive) spermatozoa found following incubation are functional intact plasma membrane.

7.6 DNA damage

DNA damage can be evaluated by Acridine orange (AO) staining or Halomax staining method (Fig. 4). The technique is modified after Thuwanut et al. (2008). Briefly, two smears from each sample were prepared on glass slide and air-dried. Each smear is fixed overnight in Carnoy's solution, freshly prepared with methanol and glacial acetic acid (3:1 v/v). The slide is removed from the fixative solution, air-dried, and then stained with 1% (100 mg/ml) AO (Sigma) in distilled water for 10 min. The AO staining solution is prepared by adding 10 ml of 1% AO in distilled water to 40 mL of 0.1 M citric acid (Merck, Darmstadt, Germany) and 2.5 ml of 0.3 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (Merck, Darmstadt, Germany) pH 2.5. The AO staining solution will be prepared daily and stored in the dark at room temperature until use. After staining, the slide is gently washed by distilled water and covered with the cover slip. One thousand spermatozoa are evaluated under the fluorescence microscope. The heads of the sperm cells with normal DNA (double-stranded) have green fluorescence, while those with damaged or single stranded DNA showed orange or red fluorescence. The results are expressed as the proportion of the damage/single stranded DNA per 1,000 counted spermatozoa.

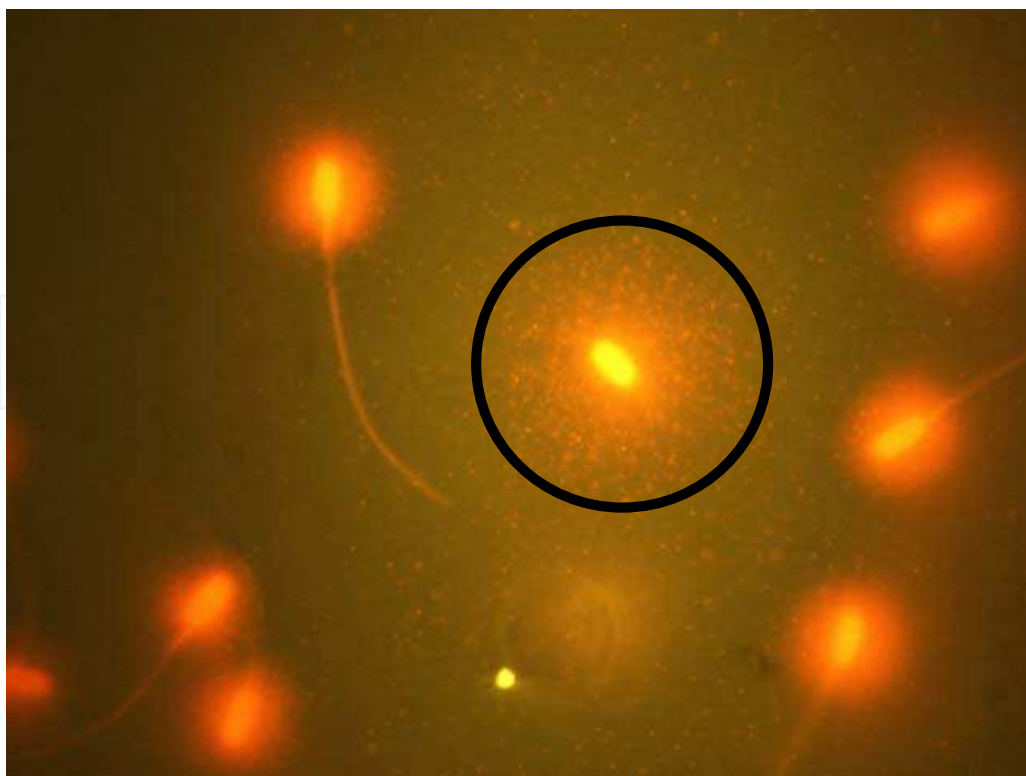


Fig. 4. Sperm DNA damage (black circle) stained with a commercial kit (Halomax staining)

7.7 Chlortetracycline (CTC) assay

The CTC assay is slightly modified from as described previously (Harayama et al., 2000). The CTC staining solution containing 750 μM CTC, 5 mM DL-cysteine, 130 mM NaCl and 20mM Tris (hydroxymethyl aminomethane) (pH 7.8) is prepared immediately before use. This solution is protected from light until analysis. Briefly, 50 μl of sperm suspension will be mixed with 50 μl of CTC staining solution for 30 sec, followed by the addition of 10 μl 12.5% paraformaldehyde in 0.5 M Tris-HCl (pH 7.4) as a fixative. Then, 10 μl of the sperm suspension is mixed well with equal volume of antifade solution (0.22 M of 1,4-diazabicyclo[2,2,2]octane ;DABCO) in glycerol:PBS (9:1) on the microscopic slide and gently compressed with coverslip. Two slides are prepared from each sample and stored in the dark at at 4 °C until evaluation. Two hundred spermatozoa per slide will be under a Nikon fluorescence microscope at 400x under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm). The spermatozoa are classified in to three staining patterns described by Fraser et al. (1995). F-pattern, fluorescence over the whole region of the sperm head are considered to be “non-capacitated spermatozoa”. B-pattern, fluorescence in the acrosomal region except post-acrosomal region are considered to be “capacitated spermatozoa”. AR-pattern, low or no fluorescence over the whole head except thin bright ban in the equatorial segment are considered to be “acrosome-reacted spermatozoa”.

7.8 Annexin-V/PI assay

Apoptosis will be evaluated by apoptosis detection kit ApopNexin™ (Chemicon Int., USA) using a fluorescent microscope. This assay will detect the phosphatidylserine translocation

from inner to outer leaflet of cell plasma membrane which is the hallmark of apoptosis during the degradation phase. Following manufacturer instructions, sperm cells are washed twice with PBS (pH 7.4) by centrifugation at 400 g for 5 min. Sperm pellet are resuspended with HEPES buffer (10mM HEPES/NaOH, pH7.4, 150mM NaCl, 5mM KCl, 1mM MgCl₂, 1.8mM CaCl₂, 2x10⁶ sperm/ml). One hundred μ l of sperm suspension are mixed well with 5 μ l annexinV-FITC conjugate and 3 μ l of Propidium iodide (PI;20 μ g/ml) and incubated for 15 min at room temperature in the dark. Two hundred spermatozoa are assessed under fluorescent microscope at 400x magnification. The apoptotic sperm cells will fluorescence green while necrotic sperm cells fluorescence red. Alternatively, flow cytometry analysis can also be used instead of fluorescent microscope (Fig. 5).

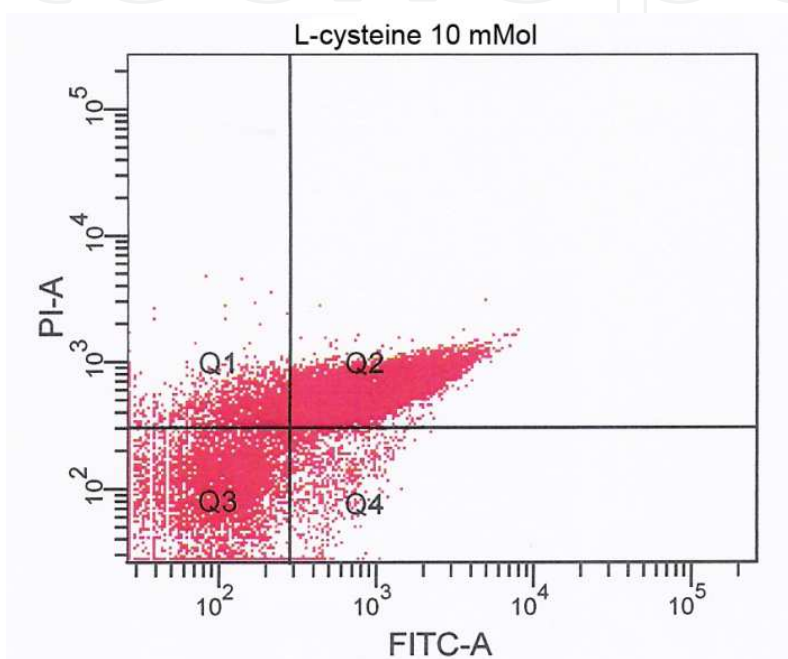


Fig. 5. FCS/SCC two-dimensional histogram, flow cytometry analysis of frozen-thawed boar spermatozoa (supplemented with L-cysteine) stained with Annexin-V/PI: Q3 represent the viable spermatozoa with intact plasma membrane, while Q2 represent dead spermatozoa with non-intact plasma membrane

8. Lipid composition of sperm plasma membrane

The lipid compositions of the plasma membrane of the mammalian spermatozoa are markedly different from those of somatic cells. In general, the sperm plasma membrane contains approximately 70% phospholipids, 25% neutral lipids, and 5 % glycolipids (Flesch and Gadella, 2000). All lipid components located in the sperm membranes responsible for the fluidity of membrane lipid bilayers, regulation of sperm maturation, spermatogenesis, capacitation, acrosome reaction and membrane fusion (Parks and Hammerstedt, 1985; Martinez and Morros, 1996; Sanocka and Kurpysz, 2004).

Sperm plasma membrane are made up of a phospholipids bilayer, with the major phospholipids were choline phosphoglycerides (CP), ethanolamine phosphoglycerides (EP) and sphingomyelin which their proportions differed between species. These phospholipids contain a high proportion of long chain, polyunsaturated docosapentanoyl (22:5) and

docosahexanoyl (22:6) groups which both lipids represent approximately 50 to 60 % of total phospholipids in boar and bull spermatozoa (Pursel and Graham, 1967; Johnson et al., 1969; Parks and Lynch, 1992). Cholesterol was the major sterol in sperm lipids of all species. Cholesterol to phospholipid molar ratios were 0.26, 0.30, 0.36, and 0.45 for sperm plasma membrane of the boar, rooster, stallion, and bull, respectively (Parks and Lynch, 1992). Glycolipids represented less than 10% of total polar lipids for all species.

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

As mentioned above the sperm plasma membrane has a very high amounts of polyunsaturated fatty acids (PUFAs) especially docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) (Johnson et al., 1969; Parks and Lynch, 1992). It has been suggested that the proportion of unsaturated fatty acid influence the properties of sperm plasma membrane (Miller et al., 2005). High levels of long chain PUFAs, DPA and DHA, are associated with an increased membrane fluidity (Quinn, 1985). During cryopreservation, the fluidity of the plasma membrane from boar spermatozoa is significantly decreased when compared to fresh spermatozoa which tend to restrict the post-thawed sperm quality (Buhr et al., 1994). In human, sperm with a high level of membrane fluidity had a higher post-thawed motility compared to sperm with a low level of membrane fluidity after cryopreservation (Giraud et al., 2000).

9. Docosahexaenoic acid (DHA)

Docosahexaenoic acid (commonly known as DHA; 22:6 (n-3)) is an omega-3 essential polyunsaturated fatty acid. DHA is most often found in cold water fatty fish (salmon fish, tuna fish) and in fish oil supplements, along with eicosapentaenoic acid (EPA). DHA is the main fatty acid composition of the spermatozoa as well as the brain and the retina (Neuringer et al., 1988). For the sperm plasma membrane, DHA play a major role in regulating membrane fluidity in sperm and in the regulation of spermatogenesis (Haidl and Oppel, 1997; Ollero et al., 2000). DHA content is significantly higher in immature spermatozoa than mature spermatozoa.

Studies have demonstrated that the supplement of PUFAs in the feed of the boar improve the quality of the boar spermatozoa (Paulenz et al., 1999; Rooke et al., 2001; Strezezek et al., 2004; Maldjian et al., 2005). In addition, Rooke et al. (2001) found that tuna oil supplemented in the boar diet increase viability, progressive motility and normal morphology. The supplementation of PUFAs also enhanced the survival rate of post-thawed boar spermatozoa (Strezezek et al., 2004). DHA improved the reproductive performance of the male turkey (Blesbois et al., 2004). Maldjian et al. (2005) found that the use of DHA-enriched

hen egg yolk for the semen extender and the supplement of 3% fish oil in the boar feed increased the DHA content of the boar spermatozoa post-thawed. However, the authors could not demonstrate the improvement of the quality of post-thawed spermatozoa.

10. Oxidative stress and sperm function

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

It is known longtime ago that ROS harm the spermatozoa (Macleod, 1943). Nowadays, studies have shown that the cryopreservation of spermatozoa induces the ROS formation and led to lipid peroxidation as well as DNA oxidation. These events attribute to the decrease of sperm function and infertility (Aitken et al., 1991; Alvarez and Storey, 1992; Agarwal, 2003). Nevertheless, spermatozoa normally produce a small amounts of ROS, needed for capacitation and acrosome reaction (Agarwal et al., 2005; De Lamirande and Gagnon, 1993).

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

LPO of sperm membranes is an autocatalytic self-reaction composed of 3 steps. Firstly, initiation step, this is the abstraction of a hydrogen atom from an unsaturated fatty acid. Secondly, propagation step, this is the formation of alkyl radical which followed by its rapid reaction with oxygen to form a peroxy radical is capable of abstracting a hydrogen atom from an unsaturated fatty acid with the concomitant formation of a lipid radical and lipid peroxide such as hydrogen peroxide (H_2O_2). Since the peroxy and alkyl radicals are regenerated, the cycle of propagation could continue indefinitely. Finally, the termination step, the substrates is consumed or stopped by the radical-radical reaction which produce a non-radical species (Sanocka and Kurpisz, 2004). LPO has been reported to affect the sperm dysfunction associated with decreased membrane fluidity, loss of membrane integrity and function of spermatozoa (Sanocka and Kurpisz, 2004). Furthermore, LPO also damage DNA and proteins resulted in an increased the susceptibility to be attacked by the macrophage (Aitken et al., 1994).

10.1 Effect of antioxidants on oxidative stress and sperm function

Antioxidants are compounds that suppress the formation of ROS and protect spermatozoa against ROS (Sikka, 1995). Studies have demonstrated that seminal plasma contains a

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

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

Studies have shown that the supplementation of antioxidants in extenders both chilled and frozen-thawed semen such as alpha-tocopherol, butylated hydroxytoluene, superoxide dismutase and catalase, cysteine or glutathione have been reported to improve the semen quality in boar (Pursel, 1979; Bamba and Cran, 1992; Brezezinska-Slebodzinska E, 1995; Cerolini et al., 2000; Penã et al., 2003; Gadea et al., 2004, Roca et al., 2004, 2005; Funahashi and Sano, 2005; Breininger et al., 2005; Satorre et al., 2007), bull (Bilodeau et al., 2001), turkey (Donoghue and Donoghue, 1997), stallion (Aurich et al., 1997; Ball et al., 2001) and ram (Uysal and Bucak, 2007).

10.2 Effect of L-Cysteine on frozen boar semen

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

During the past few years, many studies have been carried out by supplementation of various antioxidants (e.g. Vitamin E, Glutathione, Taurine) in the freezing extenders of frozen boar semen in order to minimize the detrimental effect of ROS which occurred during the freezing process (Pena et al., 2003; Roca et al., 2004; Breininger et al. 2005; Gadea et al., 2005). Funahashi and Sano (2005) demonstrated that supplement of L-cysteine (5 mM) could improve the viability and progressive motility in fresh boar semen, and also the same case found in frozen bovine semen (Bilodeau et al., 2001). This L-cysteine is also improve survival time of semen and sperm chromatin structure in fresh chilled boar semen at 15°C

(Szczesniak-Fabianczyk et al. 2003). In frozen dog semen, Micheal et al. (2007) reported that supplement of L-cysteine resulted in increased viability and rapid steady forward movement (RSF movement). Recently, Kaeoket et al. (2008b) also found that addition of 5 mM L-cysteine (the same concentration used for fresh boar semen preservation by Funahashi and Sano, 2005) has a tendency (not significant difference) to improve post-thawed semen quality when compare with the addition of glutathione and water-soluble vitamin E.

10.3 Effect of L-Cysteine x DHA on frozen boar semen

The characteristics of the sperm cryoinjury included the destabilization of lipid bilayer of the sperm plasma membrane, change in permeability of plasma membrane and a reduction of the viability of sperm. It is well documented that the boar sperm are highly susceptible to temperature below 15 °C mainly due to a relatively poor lipid composition and structure of plasma membrane compared to other domestic species. The boar sperm plasma membrane consisted of a high levels of polyunsaturated fatty acids (PUFAs) especially docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) and low level of cholesterol: phospholipids ratio. It has been shown that the level of PUFA content play an important role in the sperm membrane fluidity and cause sperm susceptible to lipid peroxidation (LPO). During frozen-thawed (FT) process, sperm are attacked by reactive oxygen species (ROS) owing to LPO and leading to significantly decrease in the PUFAs content of their plasma membrane. ROS were mainly produced by the defective or dead spermatozoa and result in a reduction of sperm motility, sperm viability and eventually fertilizing ability. In order to minimize the sperm cryoinjury, the supplement of antioxidant compounds and some fatty acid to the semen extender have been reported to minimize ROS formation and enhance the plasma membrane function in many species. *L*-cysteine, a precursor of intracellular glutathione, plays an important role in the protecting sperm from oxidative stress and act as capacitation inhibitor. Earlier studies have demonstrated that *L*-cysteine supplement in the semen extender improve the motility of FT bull semen, prolonged sperm survival time and reduced chromatin damage in FT boar sperm. In addition, the use of n-3-enriched hen egg yolk in the semen extender increased the proportion of DHA content in the boar sperm. Our previous study found that the addition of *L*-cysteine directly into lactose egg yolk (DHA-enriched) base extender significantly improves the sperm motility and intact acrosome of FT boar sperm. In addition, Kaeoket et al. (2010) found that the supplement of DHA (fish oil) improves the sperm motility, viability and acrosome integrity of the FT boar sperm.

10.4 Effect of seminal plasma on post-thawing semen quality and reproductive performance after artificial insemination

Seminal plasma is the liquid constituent of an ejaculate, comprising a combination of fluids secreted by the male accessory glands (i.e., mainly from the seminal vesicle in boars) during an ejaculation. There is evidence that seminal plasma is able to arrest or reverse cryoinjury and perhaps extend the longevity of the sperm by inhibiting or reversing capacitation and acrosome reactions, and also by its antioxidant activity (Brzezińska-Ślebodzińska et al. 1995; Strzezek et al. 1999; Suzuki et al. 2002; Vadnais and Roberts 2007; Bailey et al. 2008). During the cryopreservation process of boar semen, seminal plasma is normally not required, and discarded by the centrifugation at the beginning of semen preparation, which may result in a lack of a significant contribution (i.e., antioxidant property, inhibiting or reversing

capacitation, inhibiting and acrosome reaction and binding ability of its protein to the sperm plasma membrane) of seminal plasma in protecting sperm from cryoinjury. It has been demonstrated in rams that seminal plasma protein is able to revert the cold shock damage on the sperm membrane (Barrios et al. 2000). This effect has also been reported in boars, in that holding boar spermatozoa in its seminal plasma before cooling and freezing defends against the cold shock (Pursel et al. 1973). In addition, it has been shown that adding seminal plasma to the post-thawing solution increased the percentage of stallion sperm motility (Alghamdi et al. 2005). In dogs, it has been shown that frozen semen diluted with their prostatic fluid yielded a higher post-thawing motility (Rota et al. 2007). As a result, it seems likely that seminal plasma or prostatic fluid constituents have a positive effect on post-thawing sperm motility. In addition, semen with a high percentage of progressive motility illustrates their plasma membrane integrity and superb metabolism (Johnson et al., 2000) and also reflects their ability for fertilization (Vyt et al. 2004; Estienne et al. 2007). It can be hypothesized that the presence of supernatant (semen plasma plus semen extender) during the thawing process may improve frozen-thawed boar sperm motility.

Seminal plasma is the liquid constituent of an ejaculate, comprising a combination of fluids secreted by the male accessory glands (i.e. seminal vesicle) during an ejaculation. It is evidence that seminal plasma able to arrest or reverse cryoinjury and perhaps extend the longevity of the sperm by inhibiting or reversing capacitation and acrosome reaction (Suzuki et al., 2002). Besides, seminal plasma appears to play an important role in the female reproductive tract after insemination, e.g. attenuate the post insemination inflammatory response in the uterus of the sow which may influence the chances of conception (Rozeboom et al., 1999) and its component such as hormone estrogen may also resulted in a release of prostaglandins from the pigs endometrium to the utero-ovarian veins and lymphatic vessels which in turn decrease duration time from standing oestrus to ovulation in gilts (Clause et al., 1987, 1990; Weitze et al., 1990). Therefore, it seems likely that seminal plasma constituents (both the oestrogen and the protein fraction) have an effect on ovulation time in sows (Waberski et al., 1995; Kaeoket and Tummaruk, 2002b).

Generally, limitations to achieve a high reproductive performance in swine arise from a failure of sows to express estrus, failure to accurately determine onset of estrus for artificial inseminations and failure to determine the ovulation time after standing oestrus. The best predictor for time of ovulation is frequent detection of oestrus, because time of ovulation occurs approximately 38 to 48 h after onset of estrus (Anderson et al., 1993; Weitze et al., 1994; Soede et al., 1995). The effects of the timing of insemination relative to ovulation on fertilization rate has been study by Soede et al. (1995). Targeting insemination within 24 h before ovulation seems optimal to achieve a high farrowing rate and large litter size (Nissen et al., 1997). Insemination between 0 and 24 h before ovulation results in high fertilization rates and consequently, a low number of re-breeders and a slightly higher litter size (Kemp et al., 1996; Kaeoket et al., 2002a, 2005).

10.5 Effect of long term versus short term extenders as freezing extender I on quality of frozen boar semen

In pig industry, the boar semen used for artificial insemination is extended with semen extender and kept in cold storage at 18-20°C for few days before artificial insemination. It has been recently reported that using of long term extenders (i.e. Androstar®Plus,

Modena™, Vitasem LD) to preserve fresh semen for 7 days yield a superior fresh boar semen qualities compare with those using of short term extender (Kaeoket et al., 2010d). In addition, the different in extended fresh semen qualities were also found depending on each type of long term extender used. This indicated that some constituents in each long term extender may assist sperm to overcome cold shock during cold storage. Generally, the difference between the short term and long term extenders are the ingredients contained in the extenders. Long term extenders contain complex buffering agent (i.e. HEPES, Tris, TES and MOPs) and antioxidants (i.e., bovine serum albumin (BSA), beta-carotene, cysteine, taurine, vitamin E and ascorbic acid) (Alvarez and Storey, 1995; Gadea, 2003; Funahashi and Sano, 2005), which can maintain semen qualities during cold storage for a longer period than short term extender.

10.6 Effect of different sugars in LEY freezing extender on frozen boar semen quality

During cryopreservation, both physical and chemical factors including the rapid change in temperature or thermal stress, the intracellular ice formation, oxidative stress and osmotic stress led to the sperm plasma membrane damage (Meideros et al., 2000). Generally, the freezing extender consists of cryoprotectant, sugars, buffer, and some antibiotics (Johnson et al., 2000). Glycerol is the most common permeable cryoprotectant used for cryopreservation of boar semen (Holt, 2000). Egg yolk is a common non-permeable cryoprotectant. Different types of sugars, such as, trehalose, lactose, fructose, have been used in the freezing extender of boar semen (Purdy et al., 2006). Sugar is not only a source of energy but also protects the spermatozoa from dehydration and intracellular ice formation during the cryopreservation process (Watson, 2000). In general, lactose is the most common sugar used for the cryopreservation of boar semen (Johnson, 1985; Buranaamnuay et al., 2009; Chanapiwat et al., 2009; Chanapiwat et al., 2010; Kaeoket et al., 2010a; Kaeoket et al., 2010b; Kasettrut and Kaeoket, 2010). The effect of either type or concentration of sugar supplement in the freezing extender on the post-thawed semen qualities has been reported in dog (Yildiz et al., 2000; Yamashiro et al., 2007), ram (Aisen et al., 2002), bovine (Woelders, et al., 1997; Hu et al., 2010) and boar (Roca et al., 2008; Gutiérrez-Pérez et al., 2009; Malo et al., 2010; Mercado et al., 2010). For instance, the supplement of 55 mM glucose improved the motility pattern of the FT boar spermatozoa compared to 0 and 180 mM (Roca et al., 2008). Hu et al. (2009) found that the addition of 100 mM trehalose in the extender improved post-thawed boar sperm motility, viability and acrosome integrity compared to 0, 25, 50 and 200 mM. In addition, Malo et al. (2010) found that the trehalose-based freezing extender enhances the sperm survival rate and the fertilization rate by *in vitro* fertilization (IVF) compared to lactose and glucose based freezing extender. In order to improve the post-thawed boar sperm quality, it is important to investigate the influence of different sugars on FT boar sperm.

11. Phytosterol on frozen boar semen quality

During the past decade, several studies have focused on supplementation with a variety of antioxidants (e.g. vitamin E, vitamin C, L-cysteine, glutathione, taurine, pyruvate, SOD, catalase) in the freezing extenders of frozen boar semen with an attempt to minimize the detrimental effects of ROS, which occur during the freezing process.

Gamma-oryzanol, a phytosteryl ferulate mixture extracted from rice bran oil, has received a great deal of attention because of its significant various health-promoting functions such as

antioxidant activity, inhibition of lipoperoxidation by its scavenging activity, reduction in LDL cholesterol and induction of HDL cholesterol, inhibition of platelet aggregation [20], its potential implications as a UV-A filter in sunscreen cosmetics, treatment of type 2 diabetes mellitus and allergic reactions. These data suggest that gamma-oryzanol, especially, with its antioxidant and scavenging activities can be useful as an antioxidant and lipid peroxidation inhibitor (i.e., membrane stabilizing) during cryopreservation. Rice bran oil is widely used in salad dressing and cooking oil in Asian countries including China, India, Japan and Thailand. At present, it is becoming to gain acceptance in Western countries as well. However, no scientific information is available on its antioxidant and scavenging activities in minimizing the detrimental effects of ROS during the cryopreservation of semen.

12. Artificial insemination with frozen boar semen

In pigs, it is well-documented that the optimal insemination time for fresh semen to maximize the good fertilisation rate is within 24 h before ovulation. It has been shown that the fertile life span of the pig oocyte is limited to between 8-12 h after ovulation. At suboptimal times for artificial insemination (AI) leads to inferior FR and litter sizes results. In addition, Kaeoket et al. (2002; 2005) demonstrated that when sows were inseminated after ovulation, fertilised oocytes and developed embryos were observed up to Day 11 but no embryos were found at Day 19. Subsequently, these sows returned to oestrus with a prolonged interval.

It has been demonstrated that the duration of oestrus is related to the WOI, i.e., sows with a short WOI (3-4 days) on average have a long oestrus duration, which is associated with a longer time from onset of oestrus to ovulation. On the contrary, sows with a WOI of 5-6 days or longer, have a shorter time from onset of oestrus to ovulation, and therefore should be inseminated (with fresh semen) sooner after the onset of oestrus to ensure that the first insemination occurs before ovulation. This recommendation is in accordance with the observation that the average timing of ovulation varies between 64 and to 72% of the duration of oestrus.

For frozen boar semen, it is predictable that insemination with frozen-thawed semen will result in lower PR, low FR and litter sizes. During the last decade, most of the experiments with fertility tests (field trial) of frozen-thawed boar semen have been carried out by using deep intrauterine insemination (DIUI, dose ranged from 150 million to 1 billion spermatozoa). Nevertheless, for fresh semen, an intrauterine insemination (doses ranging from 1-3 billion) has been performed with a high fertility results (i.e., high PR, FR and litter sizes). Recently, it has been shown that a satisfactory fertility outcome was accomplished by performing IUI (doses ranging from 1.5-3 billion) together with fixed-time insemination (using a correlation of WOI-Oestrus duration-Ovulation time). This strategy may improve fertility of frozen boar semen when one performs insemination in a commercial pig farms (a field trial).

13. Conclusion

Based on above review, the conclusion can be drawn as follows: (I) some antioxidants, such as, Oryzanol, L-cysteine and its combination with DHA from fish oil, Vitamin E, Vitamin C, can be used in order to improve the quality of frozen boar semen" (II) the artificial

insemination (i.e. timing, dose and AI techniques) by using frozen boar semen on pig farm need further investigations.

14. References

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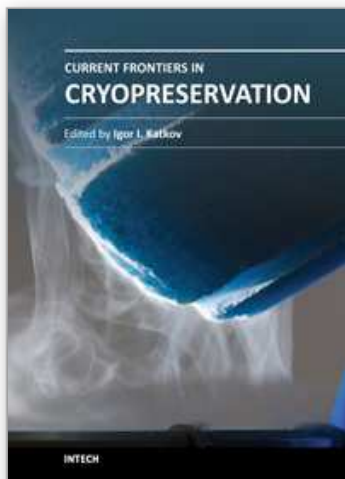
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Almost a decade has passed since the last textbook on the science of cryobiology, *Life in the Frozen State*, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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