Review Article

Cryopreservation of boar semen: where we are

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Abstract

The preservation of boar semen can be divided into two methods as follows: fresh boar semen and frozen boar semen. The first method is only to preserve the sperm for a short period, ranging from 3 to 10 days and depends on the semen extender used, however, its benefit is only in use for artificial insemination on a particular farm or for a short distant transportation. The latter method has been developed in order to keep the semen of superior genetic boars for long distant transportation or preservation of their genetics for future production or to set up a new herd in case of unforeseen outbreaks of a particular disease such as African Swine Fever and Highly Pathogenic Porcine Reproductive and Respiratory Syndrome. Nevertheless, the results of boar semen cryopreservation do not produce that high a yield, because during the cryopreservation process semen faced with several steps of temperature fluctuation, so called cold stress which leads to oxidation that can produce oxidative molecules (reactive oxygen species, ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^\bullet$) and the very reactive hydroxyl radicals (OH^-). These ROS have a detrimental effect on the sperm structure and result in low motility and fertilizing ability. This might be the reason that many studies have paid attention to diminishing the negative effect of ROS on sperm quality by supplementation of selective antioxidants (antioxidant enzymes, amino acids, vitamins, natural antioxidants etc.) into the freezing extender with the hope that those antioxidants will cope with the oxidative molecules by improving the in-vitro post-thawing semen quality and also the fertility test on farm. This review aims to provide and discuss the results of some studies on boar semen cryopreservation both with success or non-success and provide ongoing research for further development of boar semen cryopreservation.

Keywords: antioxidant, artificial insemination, boar, semen extender, sperm

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Introduction

Cryopreservation of boar semen needs to be developed for artificial insemination (AI) in the pig industry for many of the following reasons: including preservation of a superior genetic boar, increasing genetic improvement and the distribution of genetic lines within or across countries, creating a new herd after disease outbreak and reducing boar transportation (Almlid and Hofmo, 1996). The exchange of genetic material among pig breeding herds between countries with liquid stored semen is difficult because of the short life span of the spermatozoa, with a range from 3-7 days depending on the fresh semen extender used (Johnson *et al.*, 2000; Wagner and Thibier, 2000). The first frozen-thawed (FT) boar spermatozoa were reported after 1956 (Polge, 1956). Unfortunately, FT spermatozoa had a very low fertilizing ability at that time. It was not until 1970 that the first pregnancy was achieved with FT boar semen using a surgical insemination technique (Polge *et al.*, 1970). Thereafter, many studies have reported pregnancy after intra-cervical insemination using FT boar semen in pigs (Crabo and Einarsson, 1971; Pursel *et al.*, 1972; Larsson and Einarsson, 1976). In Thailand, studies on boar semen cryopreservation were established in 2006 (Buranaamnuay *et al.*, 2009). However, variations on post-thawing semen quality have been observed, due to the lack of biological background concerning cryopreservation techniques (Buranaamnuay *et al.*, 2009). Currently, there are 2 techniques for freezing boar semen, i.e. the traditional liquid nitrogen method and the controlled rate freezing method. It has been reported in humans that the controlled rate freezing method provides a significant superior post-thawed sperm motility, viability and survival rate, compared with the traditional liquid nitrogen method (Petyim and Choavaratana, 2006). In contrast, Thachil and Jewett (1981) did not show a different outcome for these 2 methods for human sperm banking. In our experience with boar semen, we found slightly higher success in post-thawing semen qualities for controlled rate freezer (CRF) but with a limitation on its freezing process that had spent significantly large volumes of liquid nitrogen (i.e. approximately 5 liters during freezing). The processes of freezing boar semen and the freezing curve of CRF are shown in Figures 1 and 2, respectively (Kaeoket *et al.*, 2008). It is well documented that the freezing and thawing procedures have a significant impact on the survival rate of sperm after cryopreservation (Johnson *et al.*, 2000). However, the optimal freezing and thawing rates vary depending on the type and concentration of the cryoprotectant (Mazur, 1970; Fiser *et al.*, 1993). Considering freezing rates, in the 19th century, 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 and Fairfull, 1990) and 16°C/min with 3.3% glycerol in 5 ml straws (Pursel and Park, 1987). For both these methods the optimal thawing rate is 1200 °C/min (Westerndorf *et al.*, 1975; Fiser *et al.*, 1993). Later, in the 20th century, Eriksson and Rodriguez-Martinez (2002) reported 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. The optimal freezing protocol using controlled rate freezing is not only dependent on the CRF used but also depends on the containers, e.g. mini straw (0.25 ml), medium straw (0.5 ml), maxi-straw (5 ml) or flattened plastic bags (5 ml).

Over the past decade, the formation of reactive oxygen species has occurred throughout the cryopreservation processes has been a major concern because ROS-induced oxidative damage can impair the post-thawing sperm qualities and functions. Boar sperm are highly susceptible to oxidative damage with regard to the high level of polyunsaturated fatty acids (PUFAs) in the plasma membrane and low scavenging activities in their cytoplasm (Waterhouse *et al.*, 2006). The spermatozoa are susceptible to oxidative damage when there is an imbalance between the high level of oxidative stress and a low protective level of antioxidant systems in boar seminal plasma. Many studies have reported that sperm damage can be diminished by both antioxidant supplementation in freezing extender and boar feed. Therefore, the adding of antioxidants to the freezing extenders is one of the several methods that is simple to perform and is able to protect spermatozoa during cryopreservation. Studies have shown the supplementation of antioxidants in semen extenders, of both chilled and frozen-thawed semen, such as alpha-tocopherol, BHT, SOD, catalase, L-cysteine and glutathione in many different species. This review gathered most of the published papers on supplementation of antioxidants during cryopreservation and the results of fertility tests after using frozen boar semen.

ROS and oxidative stress: 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 because they contain one or more unpaired electrons (Sikka *et al.*, 1995). ROS can be divided into different groups, i.e. superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^\bullet$) and the very reactive hydroxyl radicals (OH^-) (Figure 3). Two major resources of ROS in fresh semen are leukocytes and immature or defective spermatozoa (Silva and Gadella, 2006).

Oxidative stress is the consequence of an imbalance between ROS generation and scavenging activities (Sharma and Agarwal, 1996). Spermatozoa are sensitive to oxidative stress because of the low concentration of scavenging activities in the cytoplasm (de Lamirande and Gagnon, 1992; Saleh and Agarwal, 2002) and the plasma membranes containing a high level of PUFAs (Alvarez and Storey, 1995). ROS act as triggers in a chain reaction of lipid peroxidation (de Lamirande and Gagnon, 1992; Sikka *et al.*, 1995). Lipid peroxidation of the sperm plasma membrane is a significant mechanism of ROS-induced sperm damage (Alvarez and Storey, 1995) (Figure 4).

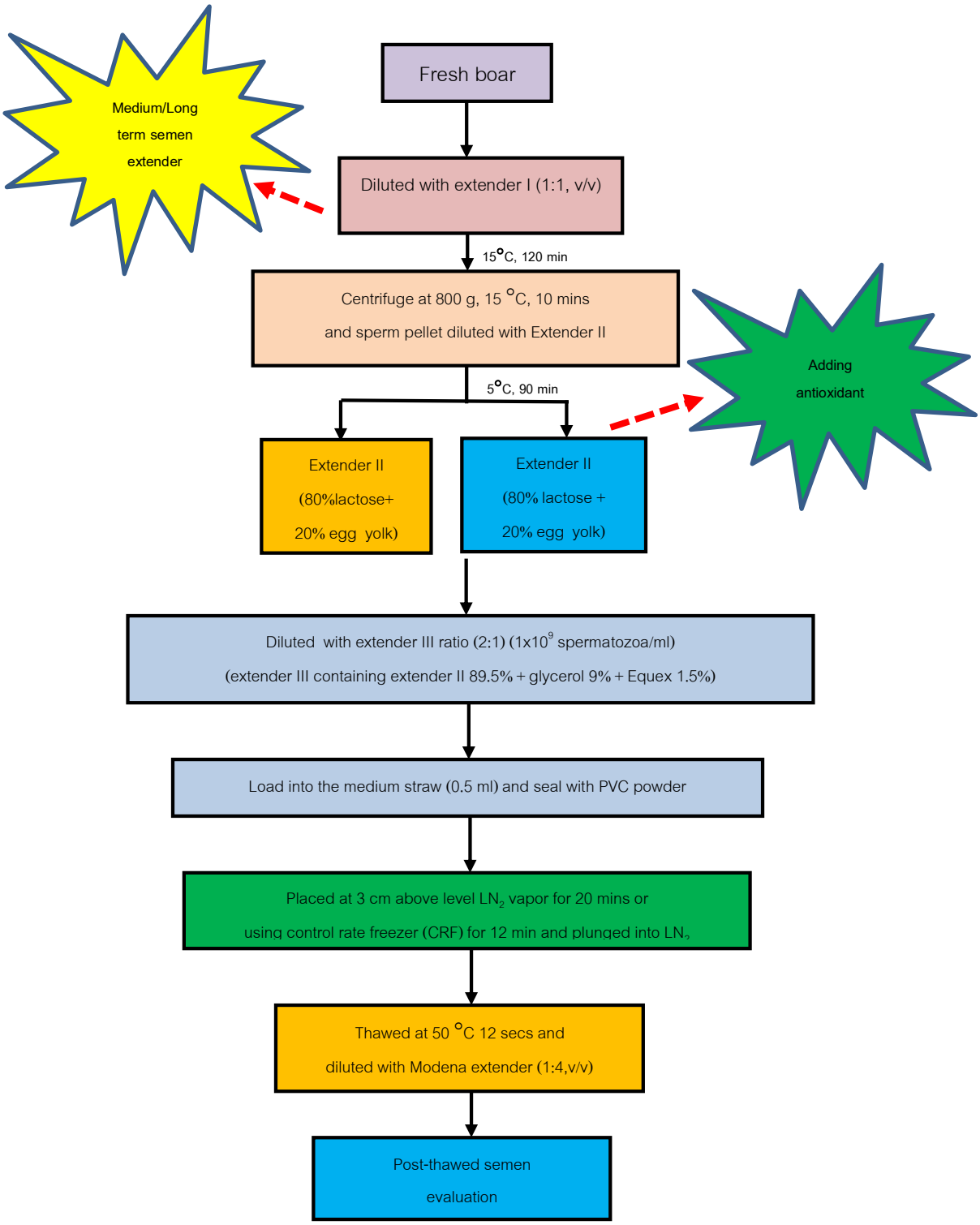

Figure 1 Flow chart of boar semen freezing processes, thawing and evaluation. Two stars represent 2 critical incubation periods in which spermatozoa are able to uptake nutrients and antioxidants from freezing extender I (yellow star) and freezing extender II (green star), respectively.

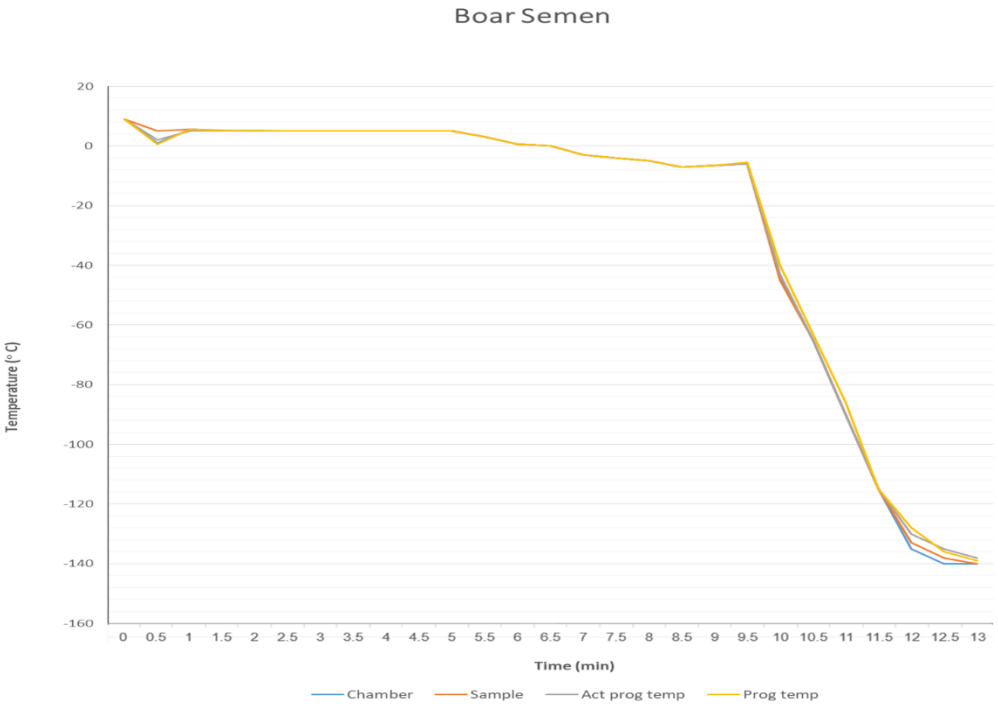

Figure 2 Freezing curve during semen cryopreservatio using control rate freezer (Sylab®, Austria), start freezing from 5 °C to -140 °C in 12 minutes at a freezing rate of -50 °C/min.

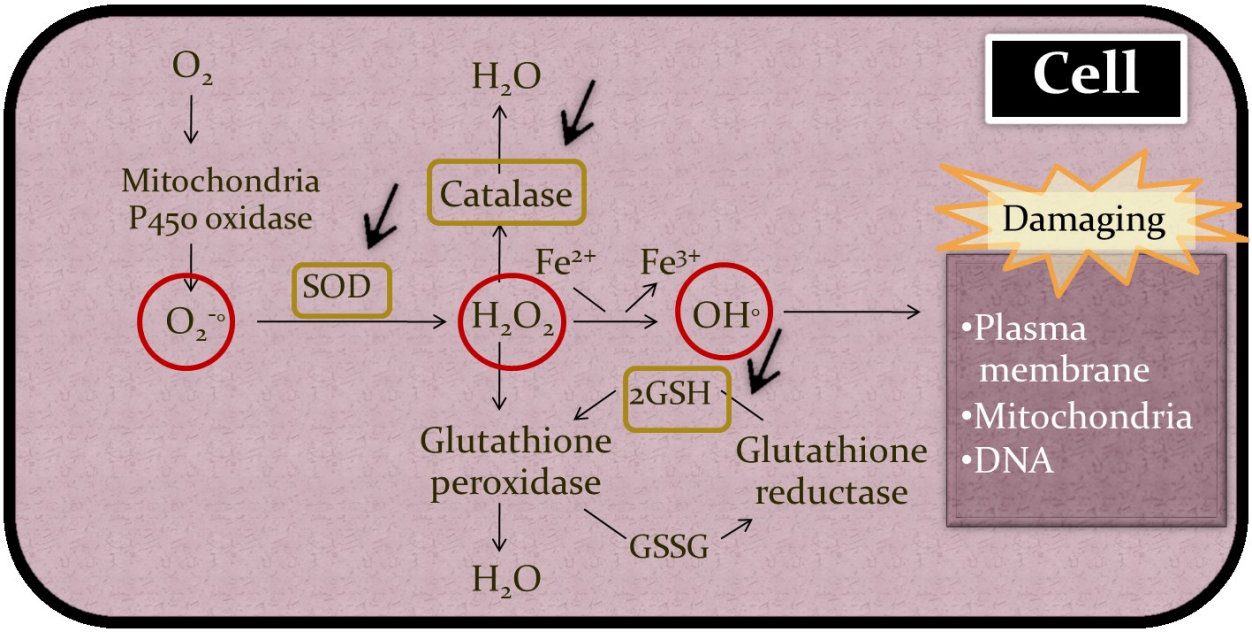

Figure 3 Interaction between different ROS and antioxidant enzymes (bold arrow) in seminal plasma milieu. This figure shows the ability of antioxidant enzymes (i.e. Catalase, SOD and GSH) to scavenge the ROS (i.e. H_2O_2, OH° and $O_2^{-\circ}$) which can minimize the sperm plasma membrane and DNA damage and maintain mitochrondrial function.

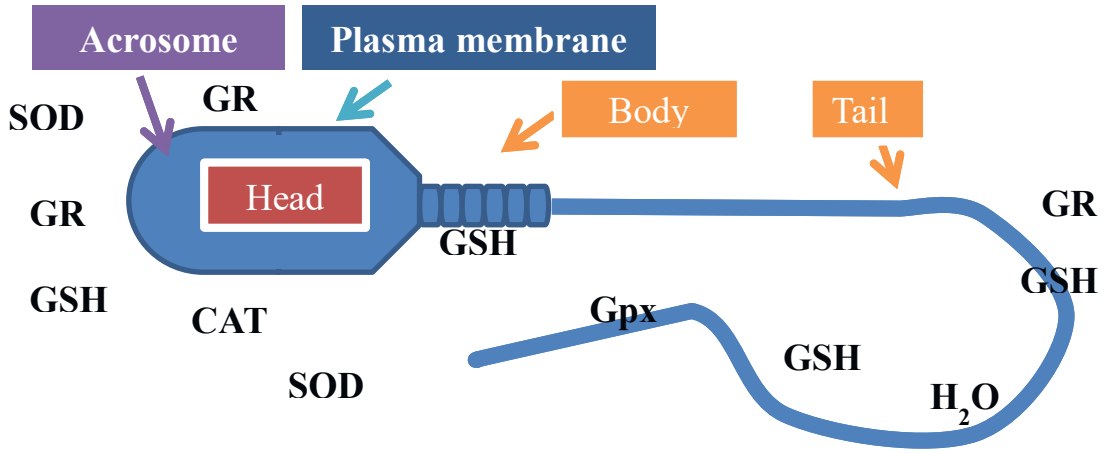

Figure 4 This figure shows that the sperm plasma membrane is protected by enzymatic antioxidants such as glutathione reductase (GR), glutathione peroxidases (Gpx), superoxide dismutase (SOD) and catalase (CAT), constituents in boar seminal plasma, which have the ability to reduce lipid peroxidation and consequently protect spermatozoa from ROS species.

Lipid peroxidation of sperm membranes is an autocatalytic self-reaction which is composed of 3 steps (Shi *et al.*, 1999). The initial step is the formation of lipid radicals from unsaturated fatty acids by ROS. Second, in the propagation step, lipid radicals rapidly react with oxygen molecules to form lipid peroxyl radicals. Lipid peroxyl radicals attack other unsaturated fatty acids and hydrogen atoms to produce lipid hydroperoxide with the concomitant formation of lipid radicals. The cycle of propagation, which can continue indefinitely, is a chain reaction. In the termination stage, the chain reaction is terminated when the lipid radical or lipid peroxyl radical is scavenged by antioxidants or stopped by a radical-radical reaction which produces a non-radical species (Sanocka and Kurpisz, 2004). It is well documented that lipid peroxidation causes sperm dysfunction associated with decreased membrane fluidity, loss of membrane integrity and the function of spermatozoa (Sanocka and Kurpisz, 2004). Besides, lipid peroxidation also damages DNA and proteins, consequently increasing susceptibility to scavenging by the macrophage (Aitken *et al.*, 1994).

However, the difference of lipid peroxidation among species is a topic of research interest. The underlying mechanism may be involved with the different characterization of lipid composition in plasma membranes and the variety of antioxidant systems in the seminal plasma of each species (Strzezek *et al.*, 1999). These discrepancies may affect the different susceptibility of spermatozoa to ROS damage during cryopreservation.

Boar semen cryopreservation and its steroid receptors: Over the past two decades, most of the steroid receptors have been studied in the female and male reproductive tracts, such as the sow uterus (Sukjumlong *et al.*, 2004) and the testis (Manee-in *et al.*, 2011). However, for the first report on steroid receptors in boar spermatozoa, Manee-in *et al.*, (2015) investigated the expressions of the estrogen receptor (ER) alpha, ER beta, and the progesterone receptor (PR) during semen cryopreservation and found that the expression of sex steroid receptors positively correlated with motility, an intact plasma membrane, acrosome integrity and non-capacitated spermatozoa, and also cryopreservation and thawing resulted in decreased expressions of ERα, ERβ and PR. However, in this study, adding L-cysteine at different concentrations during cryopreservation did not affect the expression of ERα, ERβ and PR.

The addition of antioxidants in freezing extender: During semen cryopreservation, the freezing and thawing protocol causes sperm damage as a result of many factors, such as dramatic changes in temperature from 5 °C to -196 °C, osmotic pressure from 300 mOsm to more than 1,000 mOsm and the increasing of oxidative stress which leads to abnormal sperm structure and function (Medeiros *et al.*, 2002). In general, spermatozoa are protected by various antioxidants, antioxidant enzymes and proteins in the seminal plasma by suppressing the production of ROS and thereby preventing oxidative damage (Bansal and Bilaspuri, 2011). Antioxidants are the compounds that break LPO reaction, consequently, reducing the oxidative stress and enhancing the sperm motility, viability, acrosome reaction and fertilizing capacity.

To minimize sperm cryoinjuries, different antioxidants are supplemented to freezing extenders. Following this, a variety of antioxidants have been reported to provide a cryoprotective effect on bull, ram, goat, boar, canine, feline and human sperm quality. Both non-enzymatic and enzymatic antioxidants include L-cysteine, ascorbic acid, alpha-tocopherol, butylated hydroxytoluene, phenolic compounds, gamma-oryzanol, glutathione, superoxide dismutase, catalase and glutathione peroxidase which has been proven to minimize the damaging effect of ROS on sperm quality after freezing and during storage.

The tale of L-cysteine as an antioxidant: L-cysteine, an amino acid containing a sulphydryl group, is a precursor of intracellular GSH biosynthesis. L-cysteine or N-Acetyl-Cysteine (NAC, a derivative of amino acid L-cysteine) and plays a role in the intracellular

protective mechanism against oxidative stress and as a membrane stabiliser and capacitation inhibitor (Johnson *et al*., 2000). It has been demonstrated that the supplementation of L-cysteine in the semen extender prevents loss of sperm motility by minimizing the H_2O_2 content of frozen semen in bulls (Bilodeau *et al*., 2001). Szczesniak-Fabianczyk *et al*., (2003) have shown that the addition of cysteine increases sperm survival time and reduces sperm chromatin damage. Funahashi and Sano (2005) found that the supplementing of L-cysteine at 5 mM improved the viability and functional status of boar spermatozoa during chilled storage at 10 °C.

The supplementation of L-cysteine alone or in combination with docosahexaenoic acid (DHA)-enriched hen egg yolk significantly increased subjective motility of frozen-thawed boar sperm (Chanapiwat *et al*., 2009). Furthermore, Kaeoket *et al*., (2010[a]) demonstrated that the addition of 5 or 10 mM of L-cysteine in freezing extender improved the post-thawed boar sperm quality. Recently, the supplementation of 1.0 N-acetyl-L-cysteine improved the ability of frozen boar sperm used during *in vitro* fertilization (Whitaker *et al*., 2012). Recently, it has been shown that adding L-cysteine into BTS base extender can preserve fresh boar semen qualities during storage at 17 °C for 7 days (Chanapiwat and Kaeoket, 2020[a]).

The tale of ascorbic acid as an antioxidant: Ascorbic acid, or vitamin C, a major chain-breaking antioxidant present in extracellular fluid, can neutralize hydroxyl, superoxide and hydrogen peroxide radicals (Saleh and Agarwal, 2002). In addition, it works along with vitamin E, a fat-soluble antioxidant, and the enzyme glutathione peroxidase to stop free radical chain reactions (Buettner, 1993). Vitamin C attributes to recycle vitamin E, thereby permitting it to function again as a free radical chain-breaking antioxidant (Buettner, 1993). The study has shown that ascorbic acid has protective effects on sperm membrane integrity in diluted stallion semen (Aurich *et al*., 1997). In humans, low or deficient vitamin C levels have been associated with low sperm counts, increased numbers of abnormal sperm, reduced motility and agglutination (Agarwal *et al*., 2005). In boar sperm, it is demonstrated that the addition of ascorbic acid 2-O-α-glucoside in freezing extender can improve the post-thaw qualities of Okinawan native pig sperm (Yoshimoto *et al*., 2008). Breininger and Beconi (2014) also reported that ascorbic acid decreased LPO and increased post-thawed boar sperm motility. Recently, the addition of 100 µM ascorbic acid in freezing medium influenced better boar sperm motility and normal apical ridge after freezing (Varo-Ghiuru *et al*., 2015). Furthermore, Giaretta *et al*., 2015) confirmed that the supplement of 100 µg ascorbic acid improved sperm quality compared with the control.

The tale of α-tocopherol as an antioxidant: α-tocopherol, or vitamin E, is a fat-soluble antioxidant in the cell membrane and inhibits LPO chain reaction by scavenging peroxyl and alkoxyl radicals (RO•). The oxidized α-tocopheroxyl radicals produced in LPO may be recycled back to the active form again by other antioxidants, such as ascorbate or ubiquinol (Wang and Quinn, 1999). In bovine cryopreserved sperm, vitamin E showed a protective effect on plasma membrane integrity during deep freezing (O'Flaherty *et al*., 1997).

Previous studies have demonstrated that the addition of 200 µg/ml α-tocopherol in freezing extender could be beneficial to protect boar spermatozoa against oxidative stress, improved sperm motility, sperm viability and decrease the capacitation-like state (Penã *et al*., 2003, Breininger *et al*., 2005, Satorre *et al*., 2007). In addition, α-tocopherol supplementation at 200 µM had a positive effect on post-thawed sperm survival and protected sperm by reducing lipid peroxidation and DNA fragmentation (Jeong *et al*., 2009). In chilled boar semen, Mendez *et al*., (2013) found that the addition of 400 µg/ml of vitamin E in diluted semen ensures higher sperm motility and reduced ROS production with regard to the control. Recently, the concentration of 200 µM vitamin E (Trolox®) added in the lactose-egg yolk extender provided high percentages of sperm motility after thawing and also positively influenced sperm motility and reduced DNA fragmentation when using a mixture of 200 µM vitamin C and 400 µM vitamin E (Giaretta *et al*., 2015). Vitamin E supplementation also significantly improved post-thaw sperm motility, progressive motility and membrane integrity, not only in pigs, but in frozen-thawed cat epididymal sperm (Thuwanut *et al*., 2008). In cooled equine semen, the supplement of 2mM α-tocopherol also provided a higher total sperm motility, as well as lower intracellular LPO level (Nogueira *et al*., 2015).

The tale of Glutathione as an antioxidant: Glutathione is the most powerful antioxidant which is the major non-protein sulphydryl compound in mammalian cells. Glutathione exists in two forms: the reduced form (GSH) and the oxidized form (GSSG). The protective mechanism of glutathione against ROS interacts with its associated enzymes, such as glutathione peroxidase and glutathione reductase. The scavenging function of GSH helps to attack ROS in sperm cells which results in protecting lipids, proteins and nucleic acids against oxidative DNA and membrane damage (Sikka *et al*., 1995) (Figures 1 and 2).

Bilodeau *et al*., (2001) found that GSH 5 mM prevented the loss of sperm motility in frozen-thawed bull semen. In agreement with Gadea *et al*., (2005), the addition of 5 mM GSH to freezing extender improved boar sperm motility and motion parameters, reduced the capacitated viable sperm and improved *in vitro* oocyte penetration ability. Previous studies have reported that 5 mM of GSH significantly decreased intracellular peroxide levels and increased sperm viability and acrosome integrity after freezing-thawing both in good and poor freezability boar ejaculate (Yeste *et al*., 2014). Furthermore, Giaretta *et al*., (2015) demonstrated that the supplement of 5 mM of GSH and 100 µg of ascorbic acid in freezing and thawing

medium had a combined improving effect on sperm parameters and intracellular ROS levels. In human sperm, the addition of GSH into freezing and thawing medium also decreases ROS levels and improves motility of human sperm (Gadea *et al.*, 2011).

The tale of enzymatic antioxidant: Many studies have already examined the effect of enzymatic antioxidants on semen storage. Spermatozoa predominantly possess three main enzymatic antioxidants as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase/glutathione reductase (GPx/GRD). Superoxide dismutase (SOD), a copper-containing antioxidant enzyme, removes the toxic superoxide radical by converting it to oxygen and hydrogen peroxide and then catalase converts hydrogen peroxide to oxygen and water, detoxifying ROS toxicity. In addition, catalase activates nitrous oxide (NO)-induced sperm capacitation, which is a complex mechanism involving hydrogen peroxide. Glutathione peroxidase, a selenium antioxidant enzyme and is related to the balance between glutathione disulfide (GSSG) and reduced glutathione (GSH) which neutralize hydroxyl radicals and eliminate the peroxides (Sharma and Agarwal, 1996). Therefore, glutathione peroxidase can protect sperm against oxidative stress.

It has been demonstrated that the addition of SOD has a positive effect on spermatozoa in humans (Kobayashi *et al.*, 1991) and bulls (Magnes and Li, 1980). In boars, the addition of SOD or in combination with catalase, to the freezing extender improves sperm motility, viability and in vitro fertilizing capacity (Roca *et al.*, 2005). In ram semen, the addition of catalase (100 and 200 U/ml) reduced the deleterious effects of cooling on total motility in ram sperm maintained at 5 °C for 24 h, although it did not affect the functionality of the sperm membranes. Maia *et al.*, (2010) evaluated the effect of Trolox and catalase by using them in Tris-egg yolk freezing extender to quantify lipid peroxidation and hydrogen peroxide generation after thawing. The results provide evidence that both treatments significantly reduced oxidative stress on ram sperm during cryopreservation process. Furthermore, Câmara *et al.*, (2011) and Forouzanfar *et al.*, (2013) supplemented MnTBAP, a superoxide-dismutase mimetic at 100 and 150 µM to Bioxcell extender and had a beneficial effect by increasing non-capacitated sperm compared to the control group and the harmful effects of cryodamage on the sperm plasma membrane were decreased, as well.

The tale of plant extracted antioxidants: Currently, natural (plant) antioxidants are gaining popularity for medical and cosmetic purposes because they are commercially available and have the slightest of side effects (Khopde *et al.*, 1999). There have been many investigations reported about the beneficial effect of adding antioxidants during the freezing process of boar semen cryopreservation on its post- thawing qualities, such as water soluble vitamin E, glutathione, fish oil containing DHA and L-cysteine (Breininger *et al.*, 2005, Gadea *et al.*, 2005, Kaeoket *et al.*, 2008, 2010[a,b]). Recently, Rice bran oil (RBO) which is composed of gamma-oryzanol (Shin *et al.*, 1997), an antioxidant, in which a high oxidation protection property has been used to improve frozen boar semen quality (Kaeoket *et al.*, 2012). However, the optimal concentration of this particular gamma- oryzanol for boar semen cryopreservation not only depends on the source of RBO but also the breed of boar (Chanapiwat and Kaeoket, 2015[a]). This is also supported by the study in stallions that feeding the stallions with commercial RBO elevated the total antioxidant potential of semen, sperm concentration, and motility (Arlas *et al.*, 2008). The proposed mechanism of gamma-oryzanol that can cope with hydroxyl and phenyl groups of ROS is shown in the Figure 5.

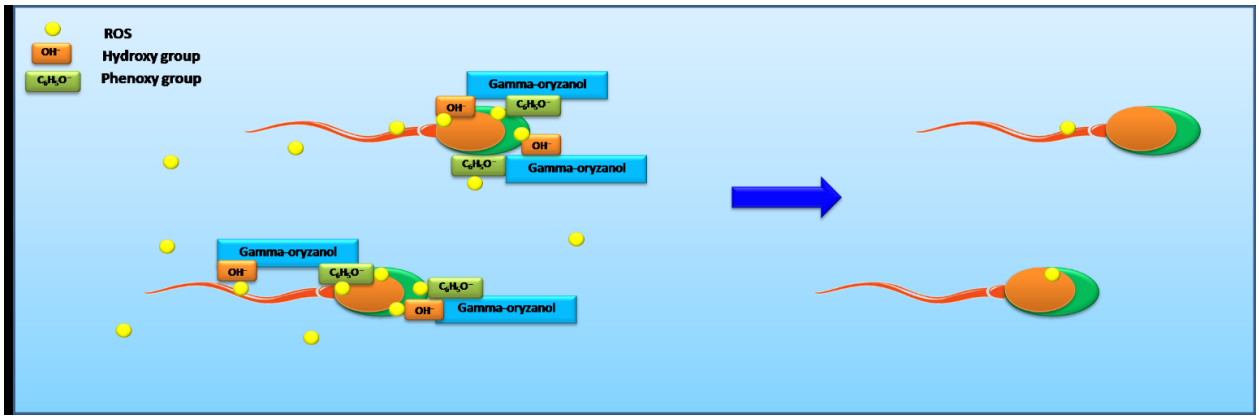

Figure 5 This figure shows the ability of gamma-oryzanol from rice bran oil with its high antioxidant activity of hydroxyl and phenyl groups to neutralize reactive oxygen species (ROS) that occur during the cryopreservation process, subsequently improving frozen-thawed boar semen quality.

In addition to gamma-oryzanol from RBO, there have been many herbs extracted in tropical countries, including Thailand, which can be used as an antioxidant. Curcumin, a yellow pigment which is extracted from turmeric (*Curcuma longa*), a polyphenolic compound that has been used not only in food ingredient but also in cosmetic and medical treatment for many years (Cousins *et al.*, 2007).

Curcumin is one of the natural antioxidants which act as anti-inflammatory and anticancer agents for medical treatment (Motterlini *et al.*, 2000, Jang *et al.*, 2009). Sreejayan and Rao (1994) proved that curcumin showed protective effects against cold shock and oxidative damage by inhibiting lipid peroxidation, as a result of its powerful scavenging activity against free radicals, such as superoxide anion, hydroxyl radicals and nitric oxide in in vitro assays (Ak and GÜlcin, 2008). With regard to its biological, pharmacological and antioxidant activities, curcumin has recently been shown to improve frozen boar semen quality (Chanapiwat and Kaeoket, 2015[b]) and also when supplemented in freezing extender for cryopreservation of goats (Bucak *et al.*, 2010) and bovines (Bucak *et al.*, 2012) semen. Recently, the study of *Rhodiola rosea* extracted 4-8 mg/L added to freezing medium demonstrated that *Rhodiola rosea* have a protective effect on boar sperm including sperm motility, mitochondrial activity, acrosomal integrity and plasma membrane integrity because *Rhodiola rosea* can inhibit lipid peroxidation and increase superoxide dismutase and glutathione peroxidase activity (Yang *et al.*, 2016). Besides the antioxidants from plants, interestingly, Mahad (*Artocarpus lakoocha*) or Lakoocha, one of the herbs, is a medicinal plant found in South Africa, Southeast Asia and South Asia, which can extract substances from the trunk, fruit and leaves such as flavonoids and oxyresveratrol (Poopyruchpong *et al.*, 1978; Saowakon *et al.*, 2009). Oxyresveratrol, an extracted substance from Mahad, has been shown to inhibit melanin production by inhibiting tyrosinase enzymes and thus it was famously used in whitening cosmetics (Likhitwitayawuid *et al.*, 2006), for medical purpose such as deworming internal parasites (Saowakon *et al.*, 2013) and as an anti-herpes virus (Chuanasa *et al.*, 2008). It also has the effect of an anti-inflammatory (Chung *et al.*, 2003) and the effect of an antioxidant (Lorenz *et al.*, 2003). Moreover, oxyresveratrol can reduce the incidence of apoptotic cells as neuroprotectants by its antioxidant activity (Andrabi *et al.*, 2004). Recently, it has been reported that oxyresveratrol has antioxidant activity two times higher than resveratrol (Povichit *et al.*, 2010). For the effect of oxyresveratrol on cryopreservation, Prommi *et al.*, (2018) reported that the optimal concentration of oxyresveratrol at 12.5 µM has been considered for improving the quality (i.e. motility and viability) of boar semen cryopreservation. In comparison to oxyresveratrol, the grape seed extract, namely resveratrol, has been added into freezing extender and found, at the concentration of 50 µM, to provide a superior post-thawing motility and viability (Chanapiwat and Kaeoket 2020[b]).

Chronicle of fertility test of cryopreserved boar semen: The fertility of frozen boar semen can generally be divided into two methods: in vitro test (laboratory testing), consisting of zona binding assay, in vitro fertilization test, in vitro maturation test and others (Funahashi, 2020); and *in vivo* testing (field trial in pig farm), it is expected that insemination with frozen semen or embryo transfer has resulted in a lower pregnancy rate, farrowing rate and total number of piglet born (Eriksson *et al.*, 2002). During the past decade, most of the experiments with fertility tests (field trial) of frozen-thawed boar semen have been carried out using deep intrauterine insemination (dose ranging from 150 million to 1 billion spermatozoa) (Roca *et al.*, 2003, Bathgate *et al.*, 2005, Wongtawan *et al.*, 2006). Nonetheless, an intrauterine insemination (doses ranging from 1-3 billion) using fresh semen has been performed with high fertility results (Kunavongkrit *et al.*, 2003). Recently, it has been shown that an acceptable 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) (Kasetrtut and Kaeoket, 2010) and not using this correlation (Kaeoket *et al.*, 2010[c], Chanapiwat *et al.*, 2014).

Considering artificial insemination techniques, it has been reported that supernatant (seminal plasma plus semen extender) of autologous and heterologous boars improved post-thawing sperm motility (Kaeoket *et al.*, 2011), subsequently increased fertility testing when performing IUI with fixed time, volume of artificial insemination (Kasetrtut and Kaeoket, 2010). This might be due to the fact 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 reaction (Suzuki *et al.*, 2002). Furthermore, seminal plasma appears to play an important role in the female reproductive tract after insemination, for example, it has been shown to diminish the post insemination inflammatory response in the sow endometrium which may influence the conception rate (Rozeboom *et al.*, 1999) and its hormone estrogen may also result in a release of prostaglandins from the endometrium to the utero-ovarian veins and lymphatic vessels which in turn shorten the duration time from standing oestrus to ovulation as shown in gilts (Claus, 1990, Weitze *et al.*, 1990). For ongoing research, it has been shown that inflammatory response occuring not only depends on the stages of the oestrus cycle and pregnancy but also following artificial insemination in the sow endometrium and oviduct (Kaeoket *et al.*, 2001[b,c]; Kaeoket *et al.*, 2003[a,b]; Dalin *et al.*, 2004; Jiwakanon *et al.*, 2005) and post-ovulatory artificial insemination also results in endometritis and vaginal discharge (Kaeoket *et al.*, 2003[c], Kaeoket *et al.*, 2005). Therefore, it is of interest to study the inflammatory response after artificial insemination with frozen boar semen since the freezing extender itself consists of egg yolk, glycerol, Equex paste STM and other components (Chanapiwat *et al.*, 2012; Kaeoket *et al.*, 2012) that might induce a high inflammatory response and consequently lower fertility found when compared with fresh semen.

In conclusion, in can be concluded that cryopreservation of boar semen not only benefits the transportation of superior genetic boars to other farms in the same country and overseas but also preserves genetics under the outbreak of contagious diseases, such as African Swine Fever (ASF) and Highly

Pathogenic Porcine Reproductive and Respiratory Syndrome (HP-PRRS). However, it is worth noting that the supplementation of antioxidants during cryopreservation is needed in order to achieve a superior result in *in vitro* tests and an optimal insemination protocol is also needed for a good fertility test on pig farms.

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