Title of Thesis

CRYOPRESERVATION OF BOAR SPERMATOZOA USING TREHALOSE IN A GLYCEROL-FREE FREEZING EXTENDER

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Content

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Abstract

1. Effect of trehalose in glycerol-free freezing extenders on post-thaw survival of boar spermatozoa

Cryopreservation of boar semen is still considered suboptimal due to lower fertility as compared with fresh samples, when glycerol, a permeating cryoprotectant, is used. Trehalose is a non-permeable cryoprotectant and non-reducing disaccharide known to stabilize proteins and biologic membranes. The aim of this study was to evaluate the cryosurvival and the in vitro penetrability of boar spermatozoa when glycerol is replaced with trehalose in a freezing extender. Ejaculated Berkshire semen samples were diluted in egg yolk based freezing extender containing glycerol (100 mM) or trehalose (0,50, 100, 150, 200 and 250 mM) and cryopreserved using the straw freezing procedure (Exp. 1). Thawed samples were analyzed for motility, viability, mitochondrial membrane potential (MMP), and acrosome integrity. In Exp 2, penetrability of spermatozoa cryopreserved with 100 mM glycerol or trehalose was examined. The replacement of cryoprotectant glycerol (100mM) with trehalose was not effective in sperm viability, but replacing it with 100 mM trehalose improved motility, MMP and acrosome integrity significantly. Sperm motility and MMP were considerably higher in 100 mM trehalose, whereas the acrosome integrity was substantially higher in 100-250 mM trehalose. In vitro penetration rate was also significantly higher in spermatozoa cryopreserved with trehalose (61.3%) than glycerol (43.6%). In conclusion, non-permeable trehalose at 100 mM can be used to replace glycerol, a permeating cryoprotectant, to maintain better post-thaw quality of boar spermatozoa.

2. Effect of rapid thawing on post-thaw survival of boar spermatozoa cryopreserved with glycerol-free trehalose extenders

Thawing process is important in semen cryopreservation as it brings back the sperm cell to physiologic temperature reactivating the metabolism. Aims of the present study were to evaluate survival rate and in vitro penetrability of boar frozen spermatozoa after rapid and rapid transient thawing at a high temperature followed by warming procedure at 39°C. Ejaculated semen samples were diluted in an egg-yolk-based glycerol-free extender containing 100 mM trehalose and then cryopreserved in 0.5-mL straws according to a common protocol. In Exp. 1, when temperature inside the straws was monitored after thawing at 40, 60, 70 and 80°C, the average calculated warming rate in the straws from -196 to 15°C was much faster when thawed at 70 and 80°C than 40°C ($P<0.01$). Warming rate of temperature inside the straw was 7-12 folds higher during the first 2 sec than the second 2 sec after immersing in high temperatures. In Exp. 2, when frozen straws were thawed at 80°C for 9 s, the motility, viability and acrosomal integrity were significantly improved ($P<0.05$), as compared with controls. In Exp. 3, frozen straws were thawed at 39, 60, 70 and 80°C for 60, 10, 8 and 6 sec and then maintained at 39°C for 0, 50, 52 and 54 sec, respectively. Higher motility, viability, mitochondria membrane potential and acrosome integrity were observed ($P<0.05$) when frozen straws were thawed at 70°C for 8 sec and then maintained at 39°C for 52 sec as compared with control (39°C for 60 sec). In Exp. 4, in vitro penetrability of frozen spermatozoa thawed at 70°C for 8 sec and maintained at 39°C for 52 sec was higher than controls. In conclusion, the rapid transient thawing at 70°C for 8 sec followed by warming at 39°C for 52 sec maintained the motility, viability, mitochondria membrane potential,

acrosome integrity and in vitro penetrability of spermatozoa frozen in a glycerol-free trehalose extender and recommended as an optimum thawing conditions.

3. Effect of milk supplements in glycerol-free trehalose freezing extenders on cryosurvival of spermatozoa derived from low freezability boars.

Not all the boar ejaculates hold the capacity to withstand freeze thawing to the same level even though the fresh semen showed greater motility and viability. The aim of the present study was to evaluate the effect of milk supplementation in a glycerol-free trehalose extender on cruosurvival of spermatozoa of low freezability boars. Sperm samples were diluted in egg yolk based freezing extender containing 100 mM trehalose and 0.25% Equex STM supplemented with coconut milk (CM) or/and skim milk (SM, 2% and 5%) and cryopreserved using the straw freezing procedure. Thawed samples were analyzed for motility, viability, MMP, and acrosome damage. Motility and MMP was significantly higher in 2% SM supplemented extender than in control ($P < 0.05$). Addition of 2% CM + SM and SM alone significantly improve the post-thaw viability ($P < 0.05$). Acrosome damage was significantly lower when extender was supplemented with 2% CM ($P < 0.05$). In conclusion, 2% CM and SM can be used to supplement glycerol-free trehalose extenders to maintain good level of post-thaw survival in low freezability boar spermatozoa.

Declaration

This thesis contains no materials which has been accepted for the award of any other degree or diploma in any other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except for due references has been made in the text.

Signature:

Date:

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Poster presentations

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List of abbreviations

^{0}C	Degrees, Celsius
%	Percent(s)
µg	Microgram(s)
µL	Microliter(s)
µM	Micro molar
AI	Artificial Insemination
cAMP	cyclic Adenosine Monophosphate
CASA	Computer Assisted Semen Analysis
cm	Centimeter(s)
CM	Coconut Milk
COC	Cumulus Oocyte Complex
CTC	Chlortetracycline
eCG	equine Chorionic Gonadotropin
FITC-PNA	Fluorescein isothiocyanate peanut agglutinin
hCG	human Chorionic Gonadotropin
IVF	In Vitro Fertilization
LDL	Low Density Lipoproteins
LN_2	Liquid nitrogen
M	Molar
mg	Milligram(s)
min	Minutes
mL	Milliliter(s)
mM	Millimolar
mM199	modified Medium 199
MMP	Mitochondria membrane potential
mMS	modified Modena Solution
mPOM	modified Porcine Oocyte Medium
nm	Nanometer(s)
nM	Nanomolar
PI	Propodium Iodide
sec	Seconds
SM	Skim Milk
TL-HEPES	HEPES buffered Tyrodes Lactate solution
WR	Warming rate

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Chapter 1

General Introduction

1.1 Preface

Cryopreservation is the process of cooling and storing cells, tissues, or organs at very low temperatures, generally below the freezing point (-196^{o}C) to maintain their viability. Cryopreservation of spermatozoa is an important tool in preserve and transporting genetic material, livestock breeding and conservation. It is a complex process during which careful attention to sample volume, cooling rates and cryoprotectants are extremely important to ensure cell survival throughout the process. Successful preservation has been carried out on spermatozoa of a number of domestic animals. However, boar sperm cryosurvival is consistently low in comparison to other species mainly due to the low fertility and short viability levels. Glycerol is currently the most common cryoprotectant agent used in freezing livestock sperm. However, glycerol exhibits cytotoxicity and this complicates the cryopreservation process of boar spermatozoa as pig sperms react variably to glycerol. Consequently, there is an urgent need for investigating novel cryoprotectants, supplements and improved boar semen freezing and thawing method.

1.2 Review of literature

Domesticated ungulates play a major role in human lives than any others species in the kingdom Animalia. It is to an extent where people invest money in massive scale on the production, protection and survival of these eutherians. Pig is one of them, which plays an important role in farming, commercial industries (bones, hide, and bristles) and pet industry. Recently completed pig genomes analyses project found variants in 112 genes in the pig genome that were identical to variants implicated in human diseases, including aberrations associated with obesity, diabetes, dyslexia, Parkinson's disease and Alzheimer's disease (Groenen et al., 2012). This project further reveled that these porcine variants are of interest, as they will allow detailed characterization in an experimental model organism whose physiology is very similar to that of human.

1.2.1 Swine industry in Japan

Japan produces and consumes massive amounts of pork since it is the foundation of their food industry along with rice and vegetables (Oh and Whitley, 2011). In Japanese livestock production, dairy production is the highest at about 25.6% and swine production is second at about 21%, or approximately 6 billion dollars (Swine production report. 2009, JMAFF). Japan is the world's greatest pork importing country (World Markets and Trade. 2014. USDA) and the total value on imports of swine products in 2012-2013 reached $3.5 billion (The statistical yearbook, JMAFF).

1.2.2 Limitations to international trade

Biosecurity is a major concern in the swine industry when it comes to large herds. With great risk to a nation's swine herd health, animals are imported as a food source or for essential genetic improvement (Boender et al., 2007; Whiting, 2003). Disease control is of paramount importance since trading involves movement of animals nationally and globally. In addition, presence of an infectious disease in domestic herds can impose trade barriers between nations (Romero et al., 2001).

1.2.3 Importance of frozen-thawed semen

Natural breeding alone is not adequate to fulfill the demand for swine products in a country. There is a necessity for alternatives to produce boars in large scale to satisfy customer demand. Cryopreservation comes into action at this point as a safe tool in preserving and transporting germ cells and it has numerous impacts on the livestock breeding in the world. Exchange of genetic material between breeding populations across the world has become possible through cryopreservation. Semen cryopreservation greatly facilitates the distribution of agriculturally desirable genes resulting rapid increase in herd productivity. If a successful cryopreservation method for boar is discovered, it can be implemented commercially in large-scale pig farms. Use of frozen semen helps to control transmission of certain pathogens, thereby protecting the health status of the herd. At the same time, this would help to prevent transmission of zoonotic diseases caused by virus (Influenza virus H1N1; Olsen, 2002), bacteria (*Streptococcus suis;* Gottschalk et al., 2007) and parasites (*Toxoplasma gondii;* Evans, 1992). Moreover, a reserve of cryopreserved semen would minimize the post effects of a sudden outbreak of a contagious illness or a natural disaster.

Radiation-induced genomic changes, occurring in germ cells may have hereditary effects, including carcinogenesis, congenital malformation and growth retardation in offsprings (Yamashiro et al., 2014). After the Fukushima nuclear plant accident, cryopreservation of germ cells provide an essential resource to preserve the genetics and foetuses obtained by fertilization using the frozen sperm and oocytes of pigs for further studies on the effect of ionizing radiation on the next generations (Yamashiro et al., 2014).

More importantly the stability shown by frozen semen makes it an ultimate tool for export genetic materials (Johnson et al., 2000). Possibility of shipping in LN_2 is a less expensive alternative compared to importing live animals for adding new genetics to a herd (Eriksson et al., 2002). Ejaculates can be frozen and held for a determined period of time while a boar's health status is checked before its semen is shipped or used within a herd (Bailey et al., 2008). Moreover, frozen semen is useful to re-establish the paternal traits of valuable genetic lines after emergencies, such as a mass eradication of infected animals following a diseases outbreak such as foot and mouth disease and porcine epidemic diarrhea through gene banking (Hofmo and Grevel, 2000; Johnson et al., 2000).

The main limitation for utilizing frozen-thawed sperm for breeding purposes is the reduced farrowing rates and reduced litter sizes (Roca et al., 2006b; Watson, 2000). Shipping containers by themselves may pose a biosecurity threat if both in and outside surfaces of the container are not properly cleaned between shipments (Bielanski, 2005). Some organisms in the tank may survive the extremely low temperatures of LN_2 (Grout and Morris, 2009). Another issue is that producers are often unwilling to utilize frozen semen due to its high cost when compared to liquid semen (Gerrits et al., 2005).

1.2.4 History of cryopreservation

Cryoreservation is the process where cells, whole tissues, or any other substances susceptible to damage caused by chemical reactivity or time are preserved by cooling to sub-zero temperatures. How cells are able to survive exposure to extremely low temperatures and the processes by which they successfully traverse a wide range of temperature is poorly understood (Hammerstedt et al., 1990). Efforts to preserve sperm at low temperatures have been attempted since 1776 when Spallanazani experimented with frog, stallion, and human sperm in snow (Bwanga, 1991). The first instance of cryopreservation of sperm was in Russia

in 1937 involving freezing of the sperm of several species and the key to this success appeared to be the addition of 9.2% (v/v) glycerol to the media (Bernstein et al., 1937). Thurston et al., 2002 revealed that there could be a genetic predisposition for freezability of boar spermatozoa. The first freeze of boar sperm that resulted in reproducible post-thaw viability was reported in 1956 (Polge, 1956). Unfortunately, these spermatozoa had very low fertilizing ability. The first successful pregnancies resulted with AI with frozen bull (Bwanga et al., 1991a) and boar (Polge et al., 1970) sperm shed a light to the future of this technology.

1.2.5 Factors influencing cryopreservation boar spermatozoa

1.2.5.1 Packaging

Each species have different traits and require different ways of processing when it comes to handling. Swine semen were frozen in ampule or pellet form (Pursel and Johnson, 1975) in the past and more recently storing has switched to plastic straws and flatPacks of varying sizes ranging from 0.25 mL (mini) to 5 mL (maxi) (Bwanga et al., 1991a; Eriksson et al., 2001; Saravia et al., 2005). The smaller packaging types (0.5 to 0.25 mL) offer more surface area to volume ratio, allowing an even freeze or thaw throughout the package, thus limiting the damage through the use of a controlled freezing curve (Bwanga et al., 1991a). Low volume packaging types have a disadvantage from a practical stand point for insemination, as they contain obviously a less volume when compared to larger types. However, this disadvantage seems to be minor compared to the improvements in viability when the small package is used (Fiser and Fairfull, 1990). Packaging materials have been shown to be important in regulating rates of temperature change (Saravia et al., 2005).

1.2.5.2 Diluents

Semen extenders were developed to provide an acceptable buffering capacity and an energy source in the form of metabolizable substrates as spermatozoa are capable of both aerobic and anaerobic metabolism requiring energy for motility (Ford, 2006). It should also provide protection against bacterial growth, osmotic effects and cold shock damage. The discovery of significance of hen’s egg yolk in semen extender was a milestone in the cryopreservation history (Phillips, 1939). The protective action of egg yolk is recognized to be largely due to low density lipoproteins (LDL) and it has been suggested that LDL can adhere to cell membranes during the freezethaw process, thus preserving spermatozoa membranes

(Foulkes, 1977; Graham and Foote, 1987). LDLs are sphere-shaped with a liquid lipid core that is made of triglycerides and cholesterol esters. This core part is surrounded by one layer of phospholipids. Apoprotein and some cholesterol are incorporated into the phospholipid layer (Anton, 2007).

In general the diluents are composed of sugars, proteins, lipoproteins, buffers, additives and cryoprotective agents and can be divide into two categories, based on their constituents: diluents without buffers (egg yolk–glucose, egg yolk–lactose, egg yolk–saccharose–EDTA, Mg and Ca salts) and diluents with buffers (glycine–phosphate and glucose–phosphate, Beltsville F3_BF3, Beltsville F5_BF5, egg yolk–glucose– citrate, Tes-tris-fructose–citrate–egg yolk_TEST, Tes-NaK–glucose–egg yolk, egg yolk–glucose–citrate–EDTA–potassium–unitol–urea, Tris-fructose–EDTA–egg yolk, Tris-glucose–EDTA–egg yolk; reviewed by Johnson et al., 2000).

Freezing media comprised of lipids, specifically phospholipids, showed much success in limiting damage due to cryopreservation (Buhr et al., 2000; Jiang et al., 2007). Phospholipid based media have a protective effect on membranes as they have been suggested to replace membrane phospholipids that were damaged or lost during cryopreservation (Buhr et al., 2000; Maldjian et al., 2005). Membranes strengthened by phospholipids are able to regulate the flow of ions, proteins and ATP that are necessary for metabolism and motility (White, 1993). Although other lipid based extenders have been tested for use in swine (Einarsson et al., 1972; Salamon, 1973), egg yolk has proven to be the most effective because it contains many lipids similar to those found in sperm membranes (Buhr et al., 2000).

Egg yolk, which provides protection against cold shock to spermatozoa of different domestic animals, does not give the same level of protection to boar spermatozoa (Benson et al., 1967). Therefore, Ovrus ES Paste (OEP, new name is Equex STM), a surfactant act as a synthetic detergent and a wetting agent, is incorporated in the freezing medium to aid in the effectiveness of lipid based extenders. It is comprised of sodium and triethanolamine lauryl sulphate which breaks up accumulations of lipids and allowed to be absorbed by the sperm resulting in improved post-thaw survival (Almlid et al., 1987; Graham et al., 1971; Pettitt and Buhr, 1998; Pursel et al., 1978). For further improvement, adding antioxidants (Bathgate, 2011) or seminal plasma (Hernandez et al., 2007b; Okazaki et al., 2009) has been shown to be beneficial if added in proper proportions prior to freezing and/or during thawing.

Milk supplementation in the extenders is also common in semen cryopreservation. Skimmed milk is as efficient as whole milk in protecting sperm during semen storage at 4ºC or during cryopreservation (Almquist et al., 1954; Foote et al., 2002). The protective constituent of milk is most likely to be micelles of caseins, which are the major proteins of milk (Bergeron and Manjunath, 2006). Caseins are present in milk at ~27 g/L of total milk proteins and exist as heterogeneous colloidal particles (Amiot et al., 2002). Basically, casein micelles are constituted of a hydrophobic core of α and β caseins surrounded by κ caseins (Dalgleish, 1998). Other proteins, such as α-lactalbumin, β-lactoglobulin, albumin, and lactoferrin, are soluble in milk (~3.5 g/L) and are collectively called whey proteins (Amiot et al., 2002). Coconut milk is rich in amino acids, sugars, sugar alcohol, vitamins, nitrogenous compounds, organic acids, growth substances and electrolytes (George et al., 2008). Chemical composition of coconut milk shows very wide variations because of differences in factors such as variety, geographical location, cultural practices, maturity of the nut, method of extraction, and the degree of dilution with added water or liquid endosperm (Cancel, 1979).

1.2.5.3 Cryoprotective agents

Cryoprotectants aid in the freezing process by building a hypertonic cellular environment and by modifying the sperm membrane to enhance removing of water from the cell (Corcuera et al., 2007; Zeng et al., 2001). Depending on the rate of freezing and thawing, and the type of cell, addition of cryoprotectants to the freezing medium minimizes cryoinjury. Unfortunately, most cryoprotectants exhibit cytoxicity and this complicate the cryopreservation process, as the cryoprotectant must often be removed during the thawing process (Capicciotti et al., 2013). Cryoprotectants are mainly of 2 types, permeating and non-permeating. The most important characteristics that these cryoproctectants possess are an affinity for water and the ability to act as solvents for the solutes that precipitate, as the water freezes (Nash, 1966; Mazur, 1966).

Discovery of glycerol as a cryoprotectant and its minimal harmful effects on freezing of rooster spermatozoa (Polge et al., 1949) was a major breakthrough in the semen cryopreservation history, allowing it to use in a wide range of animals. Glycerol is currently the most common permeating cryoprotectant used in freezing livestock sperm and it eliminates eutectic phase changes of the extender, making it a very suitable CPA when added at < 3% rates (Holt, 2000a; Han et al., 2004). While such amounts does not affect cryosurvival in “good-freezer” boars, those considered moderate or bad freezers benefit from

a minimum of 3% glycerol (Hernandez et al., 2007a). However, concentrations greater than this cause decreased survivability post-thaw and compromised acrosomes, resulting in reduced fertility (Fiser and Fairfull, 1990; Holt, 2000b). Glycerol has osmotic effects and appears to have a direct effect on the plasma membrane, apparently the binding of glycerol to the membrane phospholipids of the cells (Anchordoguy et al., 1987). Although the key advantageous effect of glycerol is extracellular, it enters and resides in the cell membrane and cytoplasm of the sperm cells (Amann and Pickett, 1987).

Many cryoprotective agents have been tested, but none have proved better for preserving boar spermatozoa than glycerol (Watson, 1995). From the other compounds tested, only exythriol, xylitol, adonitol, acetamide and DMSO, in relatively low concentrations, improved the post-thaw motility of boar spermatozoa, but the proportion of sperm cells with intact acrosomes decreased (Paquignon, 1985). However, boar spermatozoa show greater sensitivity to glycerol than other domestic animals and react variably. Why some species tolerate, or even require higher glycerol concentrations than others is still unknown (Holt, 2000a).

1.2.5.3.1 Limitations in use of glycerol

It has been shown that glycerol, despite its cryoprotective value, is metabolically toxic to sperm cells depending on the concentration and the temperature at which it is added (Fahy 1986). Moreover, non-glycolytic metabolism of glycerol results in the accumulation of toxic metabolites such as methylglyoxal in the cytoplasm (Riddle and Lorenz 1973). The methylglyoxal-mediated activation of phospholipases and proteases causes irreversible damage to the cell (Riddle and Lorenz 1973; Jones et al., 1992).

Glycerol also interacts with membrane proteins and glycoproteins and causes clustering of intramembranous particles (Niedermeyer et al., 1977; Armitage, 1986). The addition of glycerol to cells reduces the membrane capacitance indicating large scale rearrangements of membrane structure. This is due to the induced gap-junction-like structures in the plasma membrane of cells (Rudenko et al., 1984; Kachar and Reese, 1985). Glycerol induces interdigitation of the two bilayer leaflets, altering membrane fluidity by increasing the order of the interior fatty acyl chains (Boggs and Rangaraj, 1985). It also induces changes in the microtubular structure of the cytoskeleton, changing the interaction of microtubule-associated

proteins with the cytoskeleton, thus altering membrane signal transduction or disrupting membrane domains (Keates, 1980).

Replacing glycerol with amides (formamide; methyl- or dimethylformamide, acetamide; methyl- or dimethylacetamide) at ~5% concentration has proven beneficial for cryosusceptibility of boars, due to the effective permeability of amide through the plasma membrane than glycerol, thus causing less osmotic damage during thawing (Bianchi et al., 2008). A broad range of other solutes mostly alcohols, sugars, diols and amides have also been tested for CPA capacity (Fuller 2004). Since, alcohols and diols can induce membrane blebbing, monosacharides and disacharides such as the lactose, fructose, glucose, sorbitol, sucrose, raffinose or trehalose which increase viscosity and stabilize the membrane by interacting with phospholipids, have also been studied (Chanapiwat et al., 2012;Malo et al., 2010;Hu et al., 2009).

1.2.5.3.2 Trehalose

Trehalose, also known as mycose or tremalose, is a natural alpha-linked disaccharide formed by an α,α-1,1-glucoside bond connecting the reducing ends of two glucosyl residues, eliminating its reducing power (Fig 1.1). Trehalose is a white, odorless powder with relative sweetness, possessing a melting point of 203^{o}C and freezing temperature of (100 mg mL^{-1} water) -197^{o}C (Jain and Roy, 2009). This naturally occurring disaccharide is ubiquitous in distribution from fungai, plants to animals. Trehalose has been known for many years with the first discovery in 1832, in ergot of rye (reviewed in Elbein, 1974). Extracting trehalose was once a difficult and costly process, but the Hayashibara Company (Okayama, Japan) confirmed an inexpensive extraction technology from starch for mass production by using a bacterial strain belonging to the genus *Arthrobacter* sp (Higashiyama, 2002). Because of the inherent properties of trehalose, it has proved quite useful in a number of industries including food processing, cosmetics and pharmaceutics.

1.2.5.3.3 Trehalose as a cryoprotectant

Trehalose is a good candidate for cryoprotectant because it functions as; an energy and carbon reserve, a stabilizer and protectant of proteins and membranes, a sensing compound and/or growth regulator and also, it provides protection against dehydration, heat and cold shock, and damage by oxygen radicals (reviewed by Elbein et al., 2003). Use of disaccharides

such as sucrose, lactose, and trehalose as cryoprotectants and stabilizers during dehydration has been practiced widely since recent time. Trehalose is a nonpermeant cryoprotectant known to protect the sperm membrane structure from oxidative and cold shock damage during the freeze-thaw process. Trehalose has a protective action related to the osmotic effect. Moreover, trehalose bind to the surface of ice and facilitate a localized freezing point depression and induces a change in the ice crystal habit (Capicciotti et al.,2013). It irreversibly binds to specific planes of a growing ice crystal, preferential on the prism faces of ice, thus inhibiting ice growth along the *a*-axis (Wilson et al., 1993). The beneficial effects of the addition of trehalose to the extender on the post-thaw viability of mammalian sperm cells (other than boar) have been reported in many studies (Ram- Molinia et al., 1994a; Aisen et al., 2000; Tonieto et al., 2010, Bull- Foote et al., 1993; dog- Yildiz et al., 2000, Mouse: An et al., 2000; Sztein et al., 2001; Goat: Aboagla et al., 2003; Naing et al., 2010, Rabbit: Dalimata and Graham, 1997).

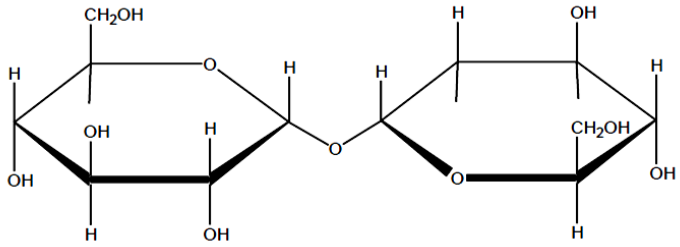

Fig. 1.1 Molecular structure of trehalose (Patist and Zoerb, 2005)

1.2.5.3.4 Perseveration mechanism of Trehalose

The mechanism by which trehalose protects cells subjected to dehydration or freezing involve its stabilizing effects on both cellular proteins and membranes; however, the nature of this mechanism remains unclear. Disaccharides (along with many other sugars, including polysaccharides such as hydroxyethyl starch and dextran) have the ability to form glasses, which have very high viscosity and low mobility, leading to increased stability of the preserved material (Patist and Zoerb, 2005). Damage to biological systems resulting from freeze drying can be attributed to two primary causes: changes in the physical state of membrane lipids and changes in the structure of sensitive proteins. Removal of hydrogen-bonded water from the head group region of phospholipid bilayers increases the head group packing and forces the alkyl chains together, thereby increasing the probability of

van der Waals interactions (Crowe et al., 1985). As a result, the lipid may undergo a transition from lamellar to gel phase (Crowe et al., 1998). As the membrane passes through this phase transition, there are regions with packing defects upon rehydration, making the membranes leaky (Block et al., 1975).

Water replacement hypothesis of Crowe et al., (1998) suggested that trehalose shows a direct interaction with the head groups during drying, reducing the van der waals interactions among the hydrocarbon chains and the membrane integrity remains intact upon rehydration. Lambruschini et al., (2000) proved that trehalose is participating in the network of hydrogen bonds between the phospholipid polar heads, thus replacing the water of hydration at the membrane-fluid interface and maintaining the headgroups at their hydrated position. In addition trehalose has been shown to preserve both structure and functionality of isolated proteins during drying by forming hydrogen bonds with the proteins when water is removed, thus preventing protein denaturing (Patist and Zoerb, 2005).

The relative effectiveness for preserving biological membranes connected with the magnitude of the effect of the sugar on disrupting the tetrahedral hydrogen bond network of water and the reduction of the amount of freezable water (Branca et al., 1999; Hancer, 2000). In addition, trehalose considered to be a good water structure breaker (Patist and Zoerb, 2005). The heats of solution (enthalpy) of sugars (ΔH_{sol}Trehalose= 19.1 kJ/mol) correlate with their effectiveness in cryogenic preservation, suggesting that ability of trehalose to disrupt the water structure contributes to its superior cryoprotecting properties with respect to other disaccharides (Miller, 2000; Patist and Zoerb, 2005).

Good glass formers undergo glass transitions at higher temperatures. Another reason behind trehalose being good at preserving biosystems compared to other sugars is its higher glass transition temperature (T_g) in the anhydrous state (trehalose -,m 115^{o}C, maltose – 84^{o}C and sucrose – 60^{o}C: Crowe, 1996; Patist and Zoerb, 2005). In vivo and in vitro studies showed that trehalose protects cells from heat by stabilizing proteins at high temperatures. Using two different temperature sensitive reporter proteins, these investigators showed that enzymes are better able to retain activity during heat shock in cells that are producing trehalose (Singer and Lindquist, 1998). These studies showed an additional and important role of trehalose, that is, the ability to suppress aggregation of proteins that have already been denatured.

1.2.5.4 Freezing and thawing rates

Challenge for the cells undergoing cryopreservation is the lethality of an intermediate zone of temperature, which they must traverse twice during the cryogenic cycle, during both during cooling/freezing and thawing rather than their ability to tolerate very low temperatures at storage (Gao, 2000). According to the two-factor theory proposed by Mazur et a1 (1972), cell damage or cell death can result as a consequence of either prolong exposure to high concentrations of solutes , also known as the "solute effect", occurring when the freezing rate is too low, or the formation of lethal intracellular ice, when the freezing rate is too high. Large internal ice crystals caused by slow freezing rates result in compromised organelles and further stress to the membrane (Hammerstedt et al., 1990). For every cell type there is an optimal cooling and warming rate that is determined by the permeability of the cell membrane to water and the cryoprotectant. Hence, cryopreservation is performed with either slow or fast cooling rates depending on cell type (Fig. 1.2).

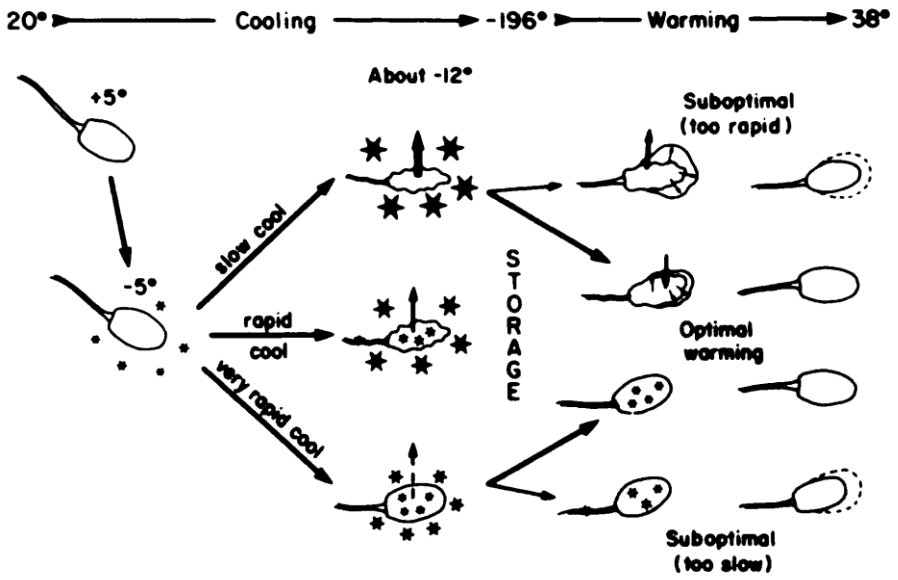

Fig 1.2 Schematic diagram showing putative changes between the sperm and extender during freezing and thawing. The effect of various cooling and freezing rates on formation of ice crystals and microcrystals (large and small stars) and the movement of solvents and penetrating solutes (heavy and light arrows) are shown (Hammerstedt, et al., 1990).

Generally, semen is frozen by keeping over liquid nitrogen vapor at rates of ~1 ºC/min, however since the introduction of the computer controlled freezing chamber, many studies have been conducted to determine the outcomes of multiple freezing rates, or curves (Almlid and Johnson, 1988; Hernandez et al., 2007a; Thurston et al., 2003). Optimal freezing rates in the range of 30–50ºC/min for 0.5 ml straw (Fiser and Fairfull, 1990; Thurston, 2003) have been theoretically predicted (Devireddy, 2004; Woelders, 2004) and confirmed by use of novel procedures, such as equilibrium freezing (Woelders, 2005). During the cooling phase, the proper rate of cooling allows sperm to adjust to temperature changes thus minimizing membrane damage due to phase transitions (Pursel et al., 1973; Pursel and Park, 1985). However, some studies suggested that boar semen tolerates a range of freezing rates around an optimum (Watson, 1979).

During thawing the sperm is once again exposed to all of the damaging effects of the cooling and freezing phases, however in reverse order (Holt, 2000b). Therefore a rapid rate of thawing has been shown to be most effective (1200 to 1800 ºC/min), thus limiting the cell's exposure to these harsh conditions and regrowth of ice crystals (Fiser et al., 1993; Thurston et al., 2001). General belief is that, conflict between cooling and thawing rates might affect adversely on cryosurvival of spermatozoa. When rapidly cooled cells are re-warmed at a slow rate, small ice crystals aggregate and form larger crystals that disrupt internal organelles and rupture membranes (Mazur, 1984). Conversely, cells that are frozen slowly and thawed rapidly will undergo swelling because of osmotic changes in the extracellular medium (Mazur, 1977).

Koshimoto et al., (2002) reported that slow warming is deleterious even when the sperm were cooled at an optimal rate, at which rate intracellular freezing almost not occur. Moreover, Almquist and Wiggin (1973 a, b) and Robbins et al., (1973) concluded that freezing rates are relatively unimportant in affecting post-thaw motility or acrosomal integrity if the semen thawed rapidly. In contrast, Rodriguez et al., (1975) found that a slow rate of freezing resulted in low sperm survival independently of the thawing rate. Nevertheless, according to Morris et al., (2012) rapid rates of cooling damage spermatozoa because of an osmotic imbalance encountered during thawing, rather than intracellular ice formation.

The thawing rate has an influence on post-thaw sperm survival and several studies have shown that increasing thawing rate generally improves motility and acrosomal integrity of

domestic species (Review by Watson, 1990). The effect of thawing rate on post-thaw survival has been reported for ram (Nicolae et al., 2014), bovine (Muino et al., 2008; Nur et al., 2003), canine (Minter and DeLiberto, 2005; Nothling and Shuttleworth, 2005), equine (Pugliesi et al., 2014) and avain (Blanco et al., 2012) spermatozoa. However, duration of exposure to high temperatures more than body temperature must be carefully timed and monitored especially at temperatures above in which protein denaturation occurs, because overexposure to theses temperatures will result in death of the spermatozoa and inevitable loss of fertility (Senger et al., 1980). Enhanced viability, fewer abnormal acrosome and greater motility was observed when frozen spermatozoa of animals apart from agricultural species, such as dog (Nothling and Shuttleworth, 2005), giant panda (Aitken-Palmer et al., 2007) and collared peccary (Silva et al., 2013), thawed at 70-75^{o}C. It was thought to be beneficial for boar spermatozoa in different aspects (Pursel and Johnson, 1976; Fiser et al., 1993; Eriksson and Rodriguez-Martinez, 2000; Cordova et al., 2006, Tomas et al., 2014).

Warming rate can be changed with the packaging used for cryopreservation, such as 0.5 mL/ 0.25mL straws, maxi straws or flatPacks as surface-to-volume ratio is different in various packing systems. Senger et al., (1980) reported that the rate of increase of the temperature within the unit was much more rapid for straws than for the ampule. Nothling and Shuttleworth (2005) observed that, 0.5-mL straws were superior to 0.25-mL straws when thawed at 70^{o}C as compared with 37^{o}C for dog semen. Moreover, pronounced difference in the temperature (3-4 folds) between the center and the periphery of the maxi-straws has also been reported (Weitze et al., 1987; Eriksson and Rodriguez-Martinez, 2000). Optimum temperature to thaw frozen pellets were recognized as 50^{o}C (Salamon et al., 1973; Pursel and Johnson, 1975). Pregnancy rate of 85% and fertilization rate of 87% in gilts with fertilized ova after inseminating with boar spermatozoa frozen in pellets, thawed in room temperature and 50^{o}C thawing solution has been reported (Pursel and Johnson (1975). Moreover, boar spermatozoa frozen in FlatPacks and thawed at 50 and 70^{o}C, yielded spermatozoa with higher velocity and lateral head displacement, with more linear motile spermatozoa and fewer circular motile ones (Eriksson and Rodriguez-Martinez, 2000).)However, Dhami et al., (1992) reported that thawing temperature of 60^{o}C for 15 seconds was found to be optimal over 80^{o}C for 5 seconds for cattle and buffalo semen frozen in straws.

1.2.6 Effect of cryopreservation on spermatozoa

During cryopreservation, ice is formed in the aqueous extender medium surrounding the spermatozoa and as ice crystals grow in the free water that builds the bulk of this extracellular milieu, the amount of solvent decreases while the solute becomes more and more concentrated. Spermatozoa loose intracellular water in order to compensate for this effective osmotic stress, leading to freeze dehydration of the cells. Eventually, when temperatures are below ~ −80°C, the highly concentrated, highly viscous solution within and outside the spermatozoa turns into a metastable glassy matrix, which is basically maintained when spermatozoa are stored at −196°C (Rodriguez-Martinez et al., 2011).

Several aspects of frozen-thawed sperm functional activity are different from that of fresh sperm because of the cryopreservation process. Even under the best cryopreservation protocols, about half of the initially motile population does not survive thawing (Medeiros et al., 2002). Decreased fertility following AI and cell damage in the form of reduced motility seems to be common across species (Watson, 2000). Indeed, poorly motile sperms are less likely to arrive at the site of fertilization in vivo or penetrate the oocyte vestments. However variations in the fertility across species have been reported and are thought to be due to differences in cell membrane structure, permeability to water, cell activation energy requirements and differences in sperm transport within female reproductive tracts (Drobnis et al., 1993; Holt, 2000a; White, 1993).

The dramatic swing in temperature and osmotic pressure during the cooling and freezing phases of cryopreservation cause severe damages and alterater of the function of the plasma membrane, flagellum, and intracellular components of sperm, thus resulting in decreased lifespan, viability, and fertility post-thaw (Corcuera et al., 2007). It has been suggested that membranes are compromised due to reordering of membrane lipids during cooling and rewarming, thereby disturbing the lipid-lipid and lipid-protein associations required for normal membrane function (Poulos et al., 1973; Hammerstedt et al., 1990).

Sperm subjected to cryopreservation are exposed to the combined conditions of seminal plasma dilution, low temperature and hypertonic environment leading to loss and reorganization of sperm plasma membrane components similar to those observed in capacitation (Medeiros et al., 2002). These membrane alterations, when sublethal, result in

decreased sperm life span and ability to interact with the female reproductive tract environment. Several important factors in the freeze-thaw process influence the post-thaw quality of the sperm including the internal factors such as inherent characteristics of spermatozoa and inter and intra boar variations and external factors like packaging in which the spermatozoa are frozen, composition of diluents, type and concentration of cryoprotective agent and the freezing and thawing rates (Johnson et al., 2000).

1.2.7 Damage due to cryopreservation.

A majority of the cells that are cryopreserved have reduced post-thaw survival due to the severe cellular membrane restructuring and osmotic stressors that occur during the dramatic changes in temperature. Primary factors causing these damages are phase transitions and separations in the sperm membrane, as well as intra and extra cellular ice crystal formation (Hammerstedt et al., 1990; Parks, 1997) resulting decrease post-thaw survival (Watson, 2000). During the cooling phase, temperature fluctuations change membrane compositions and integrity resulting alterations in the functionality. Challenges that sperm must cope with changing osmotic pressures and the formation of ice crystals in and around the cell further alter the sperm cell's ability in interact with the egg (Hammerstedt et al., 1990). Damages from these steps combine to cause often irreversible and lethal alterations to membranes, acrosome function, and DNA (Holt, 2000a).

1.2.7.1 Damage associated with cooling and cold shock.

Cold shock injury is the damage to cell structure and function resulted from a sudden change in temperature during cryopreservation protocol. Cellular components including lipids, proteins and ions lose from the cells due to the damaging of selective permeability of the membrane (Salisbury et al., 1978). The sperm membrane is composed of multiple classes of lipids in a heterogeneous arrangement some of which assist with maintenance of the bilayer structure (Quinn, 1985). Sperm membrane with low sterol concentrations and high polyunsaturated fatty acid concentrations are more susceptible to cold shock damage (Darin-Bennett et al., 1973; White, 1976). This classification distinguished fowl and human spermatozoa as more cold-shock resistant than bull and ram spermatozoa (Holt, 2000b). Due to the composition in phospholipids and cholesterol of the plasma membrane, boar

spermatozoa show the highest sensitivity to cold shock compared with spermatozoa of domestic animals (Watson and Plummer 1985).

Much of the damage associated with cold shock of a boar sperm cell is a result of the phase transitions or rapid structural re-organizations that take place as membranes change from a liquid crystalline to gel state. The phase change occurs approximately at 5-15ºC (Drobnis et al., 1993) and it could be the prime range for the temperature dependent injury (Watson, 2000). Sperm phospholipid based (>70%) membrane is affected negatively by cold shock or damaged from extensive stresses due to the lowered temperatures prior to freezing (Holt, 2000a; Komarek et al., 1965; Watson, 2000). Many aspects of cold shock associated with membrane damage can be controlled to some extent by proper freezing mediums and cooling rates but difficult to eliminated (Hammerstedt et al., 1990). Incubating the spermatozoa at a lower extension rates for 1-5 hr at ambient temperatures is believe to produce a resistance to cold shock (Pursel et a1 1972, Pursel et a1 1973). Based on these findings, most freezing protocols for boar semen include a holding time (HT) of a few hours, at or above 15°C. Proportion of spermatozoa surviving cold shock increases noticeably when the incubation time is increased to 16 h (Tamuli and Watson 1994) or 24 h (Zom 1987, Weber 1989) before decreasing the temperature to below 15ºC. The beneficial effect of an extended holding time of 20 h instead of 4 h, during cooling on post-thaw sperm viability (Kotzias-Bandeira 1997) and on *in vivo* fertility (Simmet 1993) has been reported. Often, irreversible damage persistent due to cold shock is a direct result of the composition of the boar sperm membrane. Therefore it is essential that sperm are allowed to stabilize in a temperature controlled environment and a suitable medium prior to freezing.

1.2.7.2 Damage due to ice formation and osmotic stress

As the cryoprotectant penetrates the sperm, the environment becomes hypertonic and water is forced out to prevent formation of ice crystals in the cell (Hammerstedt et al., 1990). The cells ability to regulate the exit of water is in part determined by the level of damage sustained by membrane during cooling (Hernandez et al., 2007a). This dehydration results in additional osmotic stress, as well as decreased membrane and organelle functionality (Corcuera et al., 2007; Courtens and Paquignon, 1985; Muldrew and McGann, 1994). In addition, sperms are rapidly re-hydrated during thawing and exposed to the majority of the osmotic stressors endured during the freezing and cooling phases in reverse order. Thus,

sperms are subject to severe changes in osmotic pressure and the formation of ice crystals (Bailey et al., 2000; Fiser et al., 1993; Hammerstedt et al., 1990).

Hypertonic solutions formed because of ice crystal development, injure the cell membrane due to removal of membrane proteins during this phase of the cryopreservation process if it is too prolonged (Holt, 2000b). Moreover, volume of sperm is loss due to the osmotic pressure as ice crystals form in the freezing medium resulting additional stress to the membrane (Corcuera et al., 2007; Holt, 2000a). Optimal freezing rates must be identified in order to achieve suitable dehydration as the cell adjusts to the osmotic changes caused by the cryoprotectant, while avoiding lethal ice crystal formations. Recent research has shown that fairly rapid freezing rates (~30 ºC/min), compared to slower rates (~1 ºC/min), are most effective in meeting these requirements for boar sperm (Devireddy et al., 2004; Hernandez et al., 2007a). Additional care must be taken during freezing as it has been suggested that release of the specific heat of water can raise the temperature of sperm during the freezing process, thus altering formation of ice crystals (Pursel and Park, 1985). The rate of freezing does have an impact on the most effective rate of thawing (Hammerstedt et al., 1990).

1.2.7.3 Damages associate with cryocapacitation and capacitation like changes

The spermatozoa have to undergo an activation process that called ‘capacitation’ after which they will acquire the ability 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). Induction of in vitro capacitation involves partial stripping of loosely associated extracellular coating material from ejaculated spermatozoa and removal of decapacitation factors and stabilizing seminal plasma proteins (Caballero et al., 2009). Substances such as caffeine are used for this induction and it stimulates capacitation and spontaneous acrosome reaction of boar spermatozoa in vitro (Funahashi and Nagai, 2001).

It has been demonstrated that the cooling and freezing process can also induced the capacitation like change which cause low fertilizing capacity of spermatozoa in boar and other mammalian species (Maxwell and Johnson, 1997; Green and Watson, 2001; Barrios et al., 2000). Cryopreservation causes a hiatus in sperm development and when it is continued upon rewarming, the spermatozoa emerge in an advanced state of maturation, apparently having bypassed the need for capacitation (Watson 1995). It is believed that the expression of these

capacitation-like changes follows a different pathway to that of true capacitation (Green and Watson 2001).

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). This has been associated with a decreased lifespan, and irregular interactions with the female reproductive tract thus limiting the sperm's ability to fertilize an egg (Bailey et al., 2000; Green and Watson, 2001; Wang et al., 1995).

Markedly, aggregation of rafts at the apical ridge area of the sperm head surface, stable docking of the acrosome by SNARE proteins, enhanced affinity for the zona pellucida and the generation of hyperactivated motility; as impairment of these processes probably relates to the poor fertility of frozen–thawed boar spermatozoa following artificial insemination (Guthrie and Welch 2005). Moreover, lateral phase separation of lipids in frozen–thawed spermatozoa is not reversibly restored upon thawing (Gadella et al., 2008)

To improve 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, 2004a; Bailey et al., 2008; Mocé et al., 2010). In addition, 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; Okazaki et al., 2009, Vadnais et al., 2005). Furthermore, the supplement of antioxidants such as vitamin E or alpha-tocopherol decreased the capacitation like change of cryopreserved boar spermatozoa (Satorre et al., 2007).

1.2.7.4 Damage due to Reactive Oxygen Species (ROS)

In a healthy cell, 1–2% of the oxygen is converted to a reactive oxygen intermediate, referred to as reactive oxygen species (ROS), belonging to the class of free-radicals containing one or more unpaired electrons. These ROS include the superoxide anion (O_2^-), hydrogen peroxide (H2O2), the hydroxyl radical (·OH), nitric oxide (NO) and peroxynitrite

($ONOO^-$) (Bathgate, 2011). Two main sources of ROS in semen include leukocytes and immature or defective spermatozoa (Aitken et al., 1992; Silva, 2006).

ROS play a physiological role as well as a pathological role in spermatozoa (Kothari, 2010). Spermatozoa normally produce small amounts of ROS, needed for capacitation and acrosome reaction (Agarwal et al., 2005; De Lamirande and Gagnon, 1993). Freezing-thawing processes including the storage of frozen sperm in liquid nitrogen however, lead to the generation of ROS that impair sperm motility, membrane integrity, and fertilizing potential (Alvarez and Storey 1992; Upreti et al., 1998; Chatterjee et al., 2001). Sperm with compromised membranes are also susceptible to lipid peroxidation by naturally occurring ROS which are particularly detrimental to DNA integrity (Bailey et al., 2000; White, 1993). Imbalance between ROS generating and scavenging activities result in oxidative stress (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 polyunsaturated fatty acids(Alvarez and Storey, 1995).

Antioxidants are compounds that act as free radical scavengers and help to keep free radicals at homeostatic levels in order to maintain physiologic function and prevent pathological effects due to development of oxidative stress (Agarwal, 2004). It can be either enzymatic (superoxide dismutase) or non-enzymatic (reduced glutathione and a-Tocopherol) in constitution (Bathgate, 2011). Seminal plasma has antioxidant like properties, aids membrane function, and reverses capacitation (Vadnais et al., 2005), and protects DNA integrity (Fraser and Strzezek, 2007a). Supplementing antioxidants in freezing medium or thawing solution has been proved to improve the post-thaw survival and/or in vitro fertility of spermatozoa of domestic animals (Aisen et al., 2005; Bucak et al., 2007; Woelders, 1996; Roca, 2004).

1.3 Conclusion

Cryopresevation of boar spermatozoa are remain suboptimal due to low level of post-thaw survival and fertility. Boar spermatozoa are more susceptible to cryodamage mainly due to its structure and composition. Cryoprotectant toxicity and membrane damages due to osmotic stress, intracellular and extra cellular ice formation, lipid peroxidation due to ROS and

cryocapaciation and variations present within and between boars can be recognized as the main reasons for suboptimal post-thaw survival of boar spermatozoa. Regulation of ability of the cell to control intracellular osmotic pressure associated with ions, water, and heat may eventually allow the cell to survive the freezing process. Use of proper concentrations of cryoprotectant and freezing rates could limit the damage sustained during the freezing phase of the cryopreservation process. Therefore, addressing these areas is of paramount importance to improve boar spermatozoa cryopreservation.

1.4 Aims of the study

The aims of the present study were to;

1. Evaluate the effect of trehalose as a substitute cryoprotecting agent for glycerol, on the cryosurvival and in vitro penetrability of boar spermatozoa.
2. Evaluate the effect of rapid thawing and warming in water at 60, 70 and 80^{o}C on post-thaw normality and in vitro penetrability of boar spermatozoa extended in glycerol-free trehalose medium.
3. Evaluate the effects of milk supplements in the glycerol-free trehalose freezing medium to improve cryosurvival of boar spermatozoa.

Chapter 2

Materials and methods

2.1 Chemicals and extenders

Unless specified, all the chemicals were purchased from Sigma Aldrich Japan K.K. (Tokyo, Japan). The basic diluents used in the experiments was modified Modena Solution (mMS; Funahashi and Sano, 2005, Appendix 1). Egg yolk based extender (20% hen's egg yolk in mMS) was used as the cooling extender (Appendix 2). Freezing extender consisted of cooling extender + 0.25% Equex STM™ (Nova chemical sales, Inc, Massachusetts, USA) + Cryoprotectant and supplementation depending on the experiment (Glycerol/Trehalose/Skim milk/Coconut milk).

The medium used for washing and manipulating the cumulus-oocyte complexes (COCs) and the frozen-thawed spermatozoa for in vitro fertilization experiments was TL-HEPES-PVA solution (Funahashi and Nagai, 2001, Appendix 3). The medium used for oocyte maturation was modified Porcine Oocyte Medium (Yoshioka et al., 2008) containing 50μM β-mercaptoethanol (mPOM, Appendix 4) (Akaki et al., 2009). The basic fertilization medium used was modified Medium199 (mM199, Appendix 5) (Funahashi et al., 2000).

Stock solutions of milk were prepared by dissolving 1 g of coconut milk powder (MAGGI coconut milk powder, Nestle, Colombo, Sri Lanka) / skim milk powder (Wako Pure Chemical Industries, Ltd, Osaka, Japan) in 10 mL of preheated mMS. Coconut milk solution was filtered using a cheesecloth and centrifuged at 700 x *g* for 15 min at room temperature. Coconut milk was carefully sucked up from below the upper oily layer using a new needle and syringe and stored for further use.

2.2 Animals

Animals used in the experiment were 1-3 years old Berkshire boars in the Okayama prefectural Center for Animal Husbandry & Research. These animals are used commercially for AI process having proven fertility.

2.3 Semen collection and processing

The sperm rich fraction from individual ejaculates was collected to a pre-warmed tube once a week by using gloved-hand technique (Fig 2.1). At least 4 ejaculates were collected from each boar. The fraction was diluted with mMS (1:4) before transported to the laboratory. Considering the heat loss on the way and body temperature of the boar, semen samples were kept in a Styrofoam box with warm packs (39°C) and transported within 1.5 hours. At the laboratory, the samples were assessed for sperm concentration (hemacytometer), viability (SYBR/PI) and motility (CASA). Sperm samples with >70% of motility and viability were used in the current experiments.

Diluted semen samples were centrifuged (450 x *g*, 5 min, room temperature) and adjusted the concentration to 1 x 10^8 cells/ml with 20% (v/v) seminal plasma in mMS. Then it was cooled to 15°C in 4 h using a thermoblock (ThermoStat plus, Eppendorf, Hamburg, Germany). After incubation at 15°C overnight, sperm samples were washed with mMS three times by centrifugation (620 x *g*, 5 min, 15°C) to remove the seminal plasma. Afterwards, concentration of the sperm sample was readjusted to 1 x 10^9 cells/ml with mMS before cryopreservation.

2.4 Cryopreservation of spermatozoa

Sperm samples were suspended in the cooling extender (1:4) at 15°C and cooled down to 5°C in 2 h (ThermoStat plus, Eppendorf, Hamburg, Germany). Then it was resuspended in the freezing extender (1:1) and loaded into pre-cooled 0.5-ml straws (Fujihira Kogyo Inc., Tokyo, Japan) while keeping on ice. The straws were frozen by keeping them 4.5 cm above the level of a liquid nitrogen bath, approximately at -160°C for 15 min, after sealing the free end. Finally, the straws were plunged into liquid nitrogen (-196°C) and stored for 2-3 days until thawing.

2.5 Oocytes collection and in vitro maturation

Oocytes were obtained from prepuberal gilt ovaries from a local slaughterhouse and transported to the laboratory within 1 h in 0.9% NaCl solution supplemented with antibiotic-antimycotic (GIBCO, Life Technologies Japan, Tokyo). COCs were aspirated from

3-6 mm follicles in diameter and processed for washing and in vitro maturation according to our laboratory protocol (Akaki, Y. et al., 2009). After the sample was washed three times with TL-HEPES-PVA, and groups of 50 COCs with an uniform ooplasma surrounded by a clear and compact cumulus cell mass were cultured in 500 µl of mPOM containing 10 IU/ml eCG, 10 IU/ml hCG and 1 mM dibutyryl cAMP in an atmosphere of 5% CO_2 in air at 39°C for 20 h. Then it was continued to culture in a fresh mPOM medium without those supplements for another 24 h period (Funahashi et al., 1997).

2.6 In vitro fertilization and the penetrability assessment

After in-vitro maturation of oocyte, groups of 30-50 oocytes were denuded from cumulus cells with 0.1% hyaluronidase by pipetting. Then the oocytes were processed for in vitro fertilization according to our laboratory protocol (Funahashi et al., 2000). Briefly, 30-50 oocytes were co-cultured with frozen-thawed spermatozoa (5 x 10^5 cells/ml) in mM199 (100 µl) containing 5 mM caffeine-benzaite for 8 h at 39°C in an atmosphere of 5% CO_2 in air. After culture for in vitro fertilization, the oocytes were fixed in 25% (v/v) acidic alcohol at room temperature for at least 3 days, stained with 1% (w/v) orcein in 45% (v/v) acetic acid for 3 min, mounted on a glass slide and sperm penetration and pronuclear formation were observed under a phase-contrast microscope (x400) (Funahashi and day, 1993).

2.7 Evaluation of post-thaw spermatozoa quality

2.7.1 *Motility*

The percentage of total motile spermatozoa was determined using a computer-assisted semen analysis system (CASA, with the Sperm Motility Analysis System software, Digital Image Technology, Tokyo, Japan) with 60 FPS and 125 shutter. For each sample, three sub-samples were analyzed, and 2 µl for each sub-sample was placed on an objective micrometer (Fujihira Kogyo Inc., Tokyo, Japan) and a minimum of 300 sperms per sub-sample was analyzed.

2.7.2 *Viability*

Viability was evaluated according to the protocol of LIVE/DEAD sperm viability kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). One microliter of SYBR Safe DNA in

DMSO (1:9), 5 µL of PI (1 mg/mL) and 2.5 µL of sperm cells were added to 491.5 µL of mMS. The mixture was kept in the dark for 1 min and 30 sec. Then, 16 µl of the mixture were placed on a Thoma glass slide (0.1 mm deep, Erma Inc., Tokyo, Japan) and observed under a fluorescence microscope (Eclipse 80i, Nikon Inc., Tokyo, Japan). Green cells indicated live sperms while red cells were considered as dead sperms. A total of 400 sperm cells was counted and the percentage of live cells was calculated (Fig.2.3).

2.7.3 *Acrosome integrity*

Acrosome integrity (intactness of the acrosome membrane) was evaluated by Chlortetracycline (CTC) assay as described before (Funahashi and Nagai, 2009; Funahashi et al., 2000). Briefly, the suspended sperm cells were stained with 4 µl of 20 mg/ml bisbenzimide in TL-HEPES. After keeping it in the dark for 3 min, it was transferred to 4 ml of 3% PVP -PBS solution. The suspension was centrifuged at 850 x *g* for 5 min. The sperm cells in the pellet were mixed thoroughly with 45 µl of freshly prepared CTC solution (1:1). The CTC solution contained 750 µM CTC in a buffer of 130 mM NaCl, 5 mM cysteine, and 20 mM Tris-HCl. The solution was wrapped in a foil to prevent the entry of light and stored at 4°C until use. Sperm cells were then fixed by adding 8 µl of 12.5% (w/v) paraformaldehyde in 0.5 M Tris-HCl buffer (final pH 7.4). Slides were prepared by placing 10 µl of this suspension on a clean slide. One drop of 0.22 M l,4-diazabicyclo[2.2.2]octane in glycerol was mixed to retard fading of fluorescence. A cover slip was placed and the slide was gently but firmly compressed between tissues to remove excess fluid. Only living sperm cells (Hoechst negative) were examined for CTC staining. Two hundred live sperm cells were then examined under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm) and classified according to CTC staining patterns (Fig. 2.4). The three fluorescent staining patterns identified were as follows: F, with uniform fluorescence over the whole sperm head (acrosome intact cells); B, with a fluorescence-free band in the post-acrosome region (capacitated cells); AR, with almost no fluorescence over the sperm head except for a thin band of fluorescence in the equatorial segment (acrosome-reacted cells) (Funahashi et al., 2000)

2.7.4 *Mitochondrial membrane potential (MMP)*

Two microliters of JC-1 (153 µm, Invitrogen, Molecular Probes, 5,5',6,6'–tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl-carbocyanine iodide) and 3 µl of PI (1 mg/mL) were added to a 150 µl of sperm sample and incubated at 39°C for 8 min in the dark. Then, 8 µl of the mixture were placed on a glass slide and observed under a fluorescence microscope (x1000, Eclipse 80i, Nikon Inc., Tokyo, Japan). In mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange wavelength of 590 nm, when excited at 488 nm and with low membrane potential, JC-1 forms monomers, which emits in the green wavelength (525–530 nm) when excited at 488 nm (Pena et al., 2003). Green sperm head with an orange- yellow midpiece indicated viable spermatozoa with high MMP (Fig 2.5). Total of 300 sperm cells were counted and the percentage of sperm cells with high MMP was calculated.

2.7.5 *Fluorescence multiple staining procedure*

This was performed using PI/JC-1/FITC-PNA in the experiment in chapter 5. Briefly, 3 µl of PI (1 mg/mL), 30 µl of FITC-PNA (200 µg/mL) and 2 µl of JC-1 (153) were added to 150 µl aliquot of spermatozoa. Next, samples were incubated at 38 ^{0} C for 8 min in the dark. Then, 8 µl of the mixture was placed on a glass slide and observed under a fluorescence microscope (X1000, Eclipse 80i, Nikon Inc., Tokyo, Japan). A total of 300 sperm cells were counted and the percentage of sperm cells with viability (green head), high MMP (orange-yellow mid piece) and acrosome damage (green acrosome cap) were calculated (Fig. 2.6).

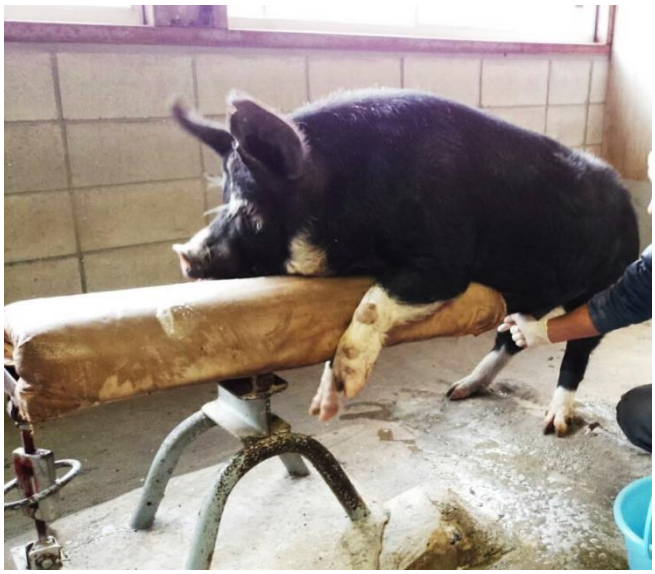

Fig 2.1 Semen collection using gloved-hand technique.

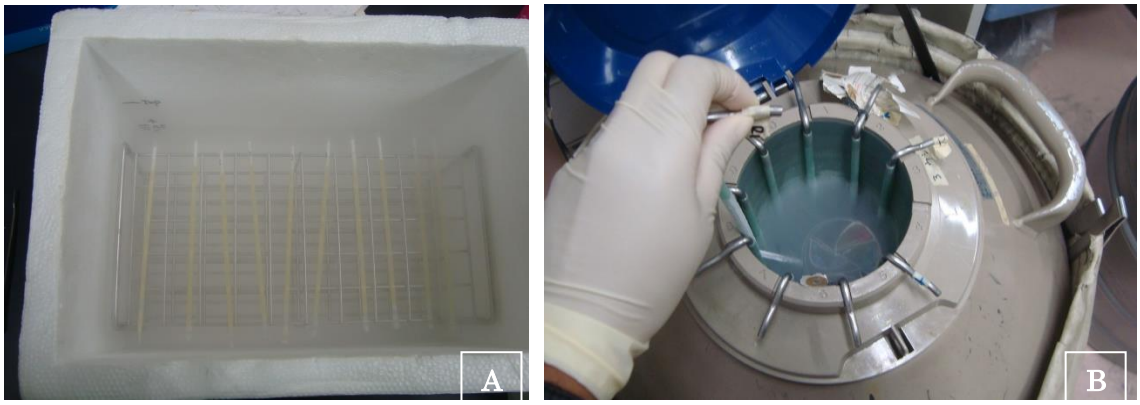

Fig 2.2 Freezing spermatozoa packed into straws (A) Freezing straws in LN_2 vapor by keeping them on 4.5 cm above the LN_2 level. (B) Plunging the straws into LN_2.

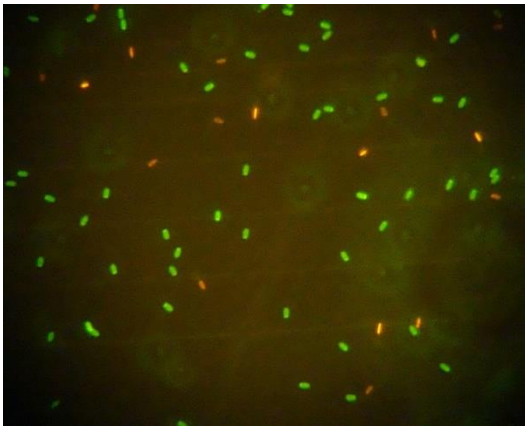

Fig 2.3 Spermatozoa stained with PI/SYBR indicating viability. Green cells showing live sperms while red cells showing dead sperms.

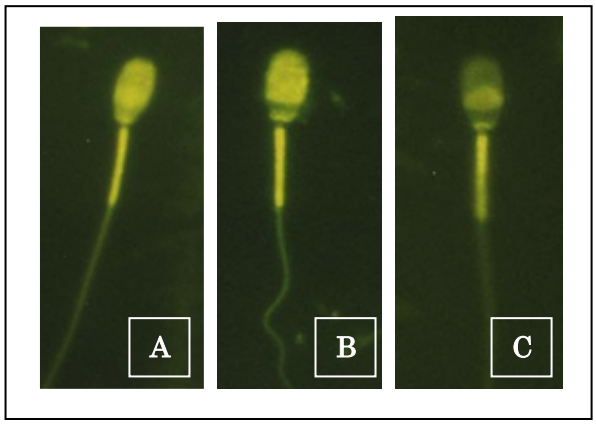

Fig 2.4 Spermatozoa stained with CTC indicating acrosome status. (A) F, with uniform fluorescence over the whole sperm head showing acrosome intact cells, (B) B, with a fluorescence-free band in the post-acrosome region shoeing capacitated cells, (C) AR, with almost no fluorescence over the sperm head except for a thin band of fluorescence in the equatorial segment showing acrosome-reacted cells

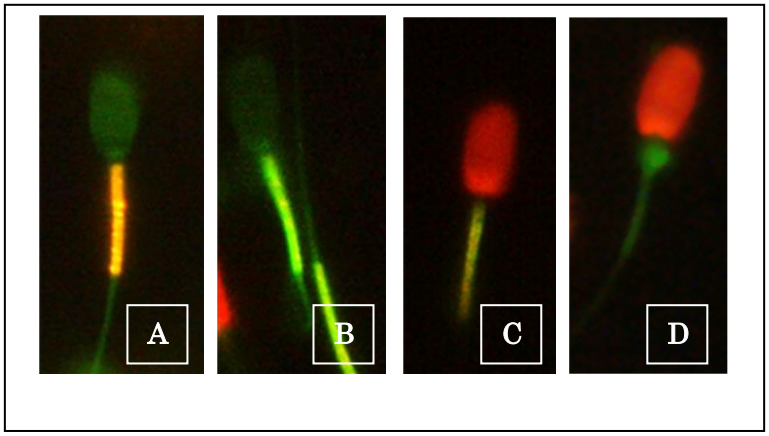

Fig 2.5 Spermatozoa stained with JC-1/PI indicating mitochondria membrane potential. (A) Live high MMP, (B) Live low MMP, (C) Dead high MMP, (D) Dead low MMP

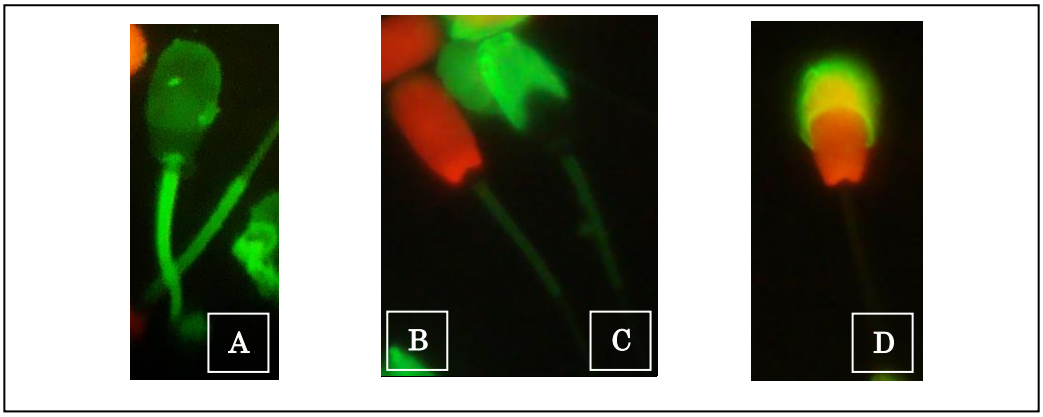

Fig 2.6 Spermatozoa stained with JC-1/PI/FITC-PNA. (A) Live intact acrosome (B) Dead intact acrosome (C) Live damaged acrosome (D) Dead acrosome damaged

Chapter 3

Effect of trehalose in glycerol-free freezing extender on post-thaw survival of boar spermatozoa

3.1 Introduction

Cryopreservation of boar spermatozoa is important in the preservation of excellent genetic resources and useful for exchange of genetic material between breeding populations across the world. However, the commercial use of cryopreserved boar spermatozoa is still not extended due to its disappointingly low cryo-survivability and the high level of cryoinjuries in the biological membranes, which result in low conception rates and smaller litters after artificial insemination (Johnson et al., 2000; Roca et al., 2006a; Rodriguez-Martinez and Wallgren, 2011). Cryoinjuries also lead to cryo-capacitation after thawing, thus shortening the life span of spermatozoa (Breininger et al, 2005; Cerolini et al., 2000; Maxwell and Johnson, 1997). Therefore, further improvements in the fertility of frozen-thawed boar spermatozoa will make commercial use more efficient and definite.

Glycerol is the most common permeating cryoprotectant for the livestock sperm, and optimum concentration added during the cryopreservation process is 1% - 3% due to its potential toxicity, but boar spermatozoa react variably (Hold, 2000). The increasing viscosity of glycerol inhibits ice crystal growth before achieving the glassy matrix state (Fuller, 2004). The presence of glycerol also appears to ameliorate the rise in salts to a critical damaging concentration at temperatures below the ice transition (Lovelock, 1954). However, fertility of cryopreserved boar spermatozoa has consistently been low due to the osmotic shock sensitivity, oxidative stress, and low-temperature cryoprotectant intoxication (reviewed by Rath et al., 2009). Glycerol molecules can be inserted into the membrane lipid bilayer, altering the stability of the membrane; thereby it influences the ability of sperm to undergo capacitation and the acrosome reaction, or even results in cell death (Leibo et al., 1978). Therefore, glycerol may not be the most effective cryoprotectant for boar spermatozoa. Thus, more studies using non-permeating cryoprotectants such as carbohydrates including monosaccharides, disaccharides, and polysaccharides have been performed resulting in an improved sperm survival rate after freeze-thawing (Sztein et al, 2001; Molinia et al, 1994;

Malo et al., 2010). Non-permeable disaccharides, including trehalose, also have a high cryoprotectant ability and kinetically inhibit ice crystal growth due to high viscosity [8]. Trehalose is known to help in resisting dehydration or freezing in a number of plants and animals including freeze-resistant insect species (Westh and Ramlov, 1991). Disaccharides such as trehalose and sucrose are also known to stabilize the membrane by interacting with the polar head groups of phospholipids and increasing its fluidity (Crowe et al., 1985). Trehalose also induces a decline in the membrane phase transition temperature of dry lipids to form a glass and it can reduce the concentration of original cryoprotective agent to achieve a glassy state at a given critical cooling rate (Sutton, 1992; Sutton, 1991). Some studies have reported combined beneficial effects of trehalose on the cryosurvival of boar spermatozoa in a glycerol based extender (Malo et al., 2010; Hu et al., 2009; Gutierrez-Perez et al., 2009).

Since the presence of highly permeable glycerol cannot avoid biological toxicity, application of non-permeable cryoprotectants like trehalose for the cryopreservation of boar spermatozoa is worth examining. In this study, we hypothesized that the use of non-permeable cryoprotectant trehalose for the cryopreservation of boar spermatozoa could increase its post-thaw survival, motility and/or penetrability even in a glycerol-free extender. Objectives of the current study were to evaluate the effect of trehalose as a substitute cryoprotecting agent for glycerol, on the cryosurvival and in vitro penetrability of boar spermatozoa.

3.2 Experimental design

3.2.1 Exp 1: Effect of trehalose on spermatozoa post-thaw survival

Ejaculates of 3 boars were used in this experiment. Sperm samples were frozen with glycerol (100 mM) or trehalose (50, 100, 150. 200 and 250 mM) with 0.25% Equex STM™ as the freezing extender. Negative control consisted of 0 mM of either glycerol or trehalose. Sperm cells were packed into 0.5-ml straws after cooling and cryopreserved in LN_2. Samples were thawed at 39°C for 30 sec and tested for post-thaw sperm qualities after storing for 2-3 days.

3.2.2 Exp 2: Effect of the presence of trehalose versus glycerol during cryopreservation on the in vitro penetrability of boar spermatozoa after thawing

In vitro penetrability of spermatozoa frozen with 100 mM glycerol or trehalose was compared. A total of 187 mature oocytes from four replicates were used in the experiment.

3.2.3 Statistical analysis

Statistical analyses of results replicated 4-6 times were used for treatment comparisons and carried out by a one-way ANOVA followed by Tukey's multiple range test (Exp. 1) or Chi-square test (Exp.2) using GraphPad Prism 6 Statistical Software (GraphPad Software Inc., California, USA). Interrelation of concentration and post-thaw variables were assessed by Pearson correlation. All the percentage data were subjected to arcsine transformation before statistical analysis if the percentage data contained values less than 10% and/or more than 90%. All data were expressed as mean ± SEM. Differences were considered significant at $P < 0.05$.

3.3 Results

3.3.1 Exp 1: Effect of trehalose on boar spermatozoa post-thaw survival

The effect of glycerol and trehalose added to the freezing extender, on the motility, viability, acrosome integrity and mitochondrial membrane potential of boar spermatozoa after freezing–thawing was evaluated. All the treated samples showed a significant increase in the motility compared to the negative control (Fig. 3.1). Motility was significantly higher in spermatozoa extended in 100 mM trehalose when compared with 100 mM glycerol ($P < 0.05$) and 250 mM trehalose ($P < 0.01$). It was not considerably different among 50, 150, 200 and 250 mM trehalose extenders ($P > 0.05$). Post-thaw viability was significantly higher in all the treated samples when compared to the negative control ($P < 0.01$, Fig.3.2). There were no significant differences among treatments between 100 mM glycerol and 50-250 mM trehalose extenders. When the acrosome status was evaluated by CTC assay, spermatozoa in the trehalose extenders containing 100 mM or more, exhibited significantly higher acrosomal integrity compared to the extenders containing 0 or 50 mM trehalose and 100 mM glycerol ($P < 0.01$, Fig. 3.3).

Acrosome integrity was not significantly different in extenders supplemented with trehalose from 100-250 mM concentrations. Percentage of viable spermatozoa with HMMP was significantly higher in all the treated extenders compared to the negative control ($P < 0.01$, Fig. 3.4). Spermatozoa extended in 100 mM trehalose exhibited a significantly high percentage of high MMP when compared to 100 mM glycerol ($P < 0.05$). There were no

significant differences between 50, 150, 200 and 250 mM trehalose extenders ($P > 0.05$). Motility and acrosome integrity showed a significant relationship with the trehalose concentration (Fig. 3.5). Motility was negatively correlated with the trehalose concentration ($r = - 0.49$, $P < 0.01$), whereas acrosome integrity was positively correlated ($r = 0.58$, $P < 0.005$). Motility showed a strong negative correlation with trehalose concentration after 100 mM ($r=-0.95$, $P < 0.05$). Viability and mitochondrial membrane potential were not significantly correlated with trehalose concentration ($P > 0.05$).

3.3.2 Exp 1: Effect of the presence of trehalose versus glycerol during cryopreservation on the in vitro penetrability of boar spermatozoa after thawing

In vitro penetrability frozen-thawed of spermatozoa cryopreserved with glycerol or trehalose is shown in Table 3.1. The extenders containing trehalose considerably preserved the in vitro penetrability of spermatozoa as compared with glycerol ($P < 0.01$). However, the incidence of monospermic oocytes was higher when the spermatozoa were frozen with glycerol ($P < 0.01$). The percentage of oocytes with pronucclear formation between the two groups (trehalose vs. glycerol) 8 hours after IVF, however, was not significantly different.

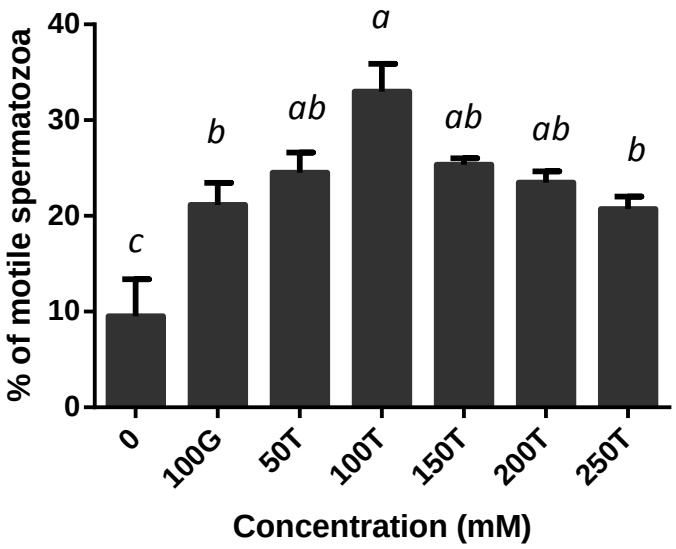

Fig. 3.1 Post-thaw motility in boar spermatozoa frozen in extenders supplemented with glycerol or trehalose. Error bars represent SEM. $^{a,b,c}P < 0.05$, n = 6.

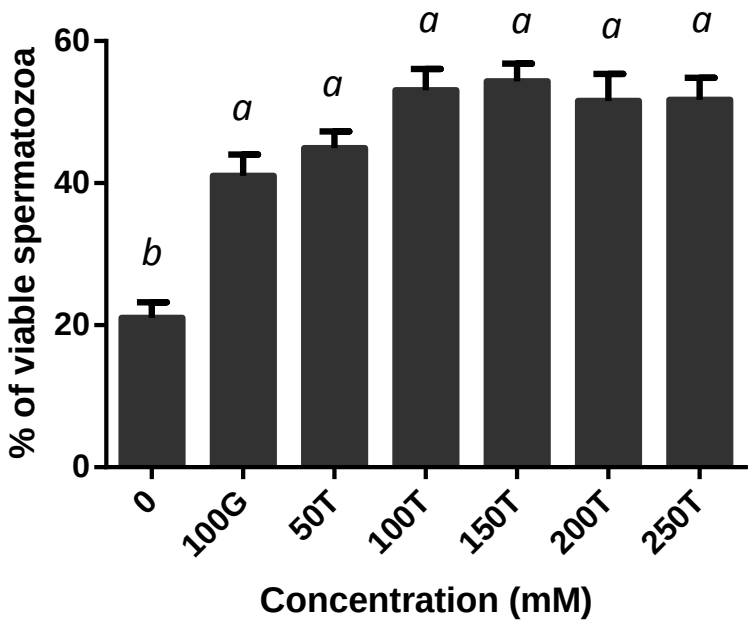

Fig. 3.2 Post-thaw viability in boar spermatozoa frozen in extenders supplemented with glycerol or trehalose. Error bars represent SEM. $^{a,b}P < 0.05$, n = 10.

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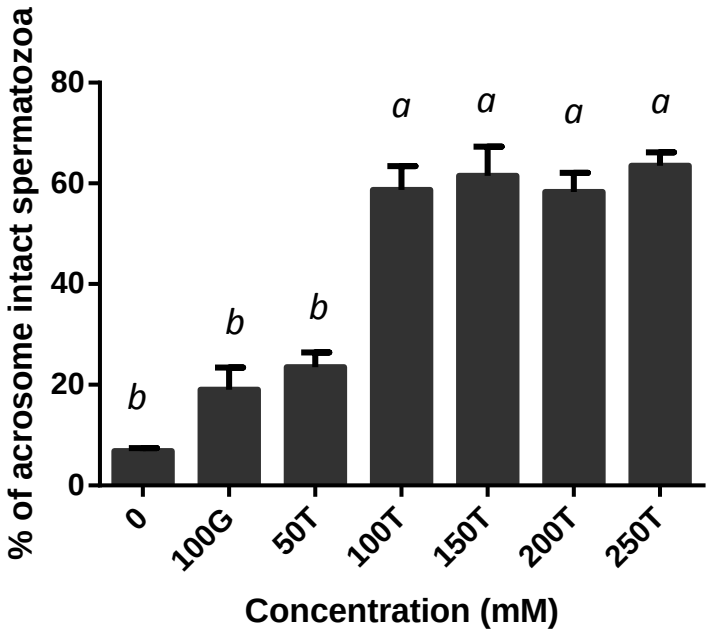

Fig. 3.3 Post-thaw acrosome integrity in boar spermatozoa frozen in extenders supplemented with glycerol or trehalose. Error bars represent SEM. $^{a,b}P < 0.05$, n = 6.

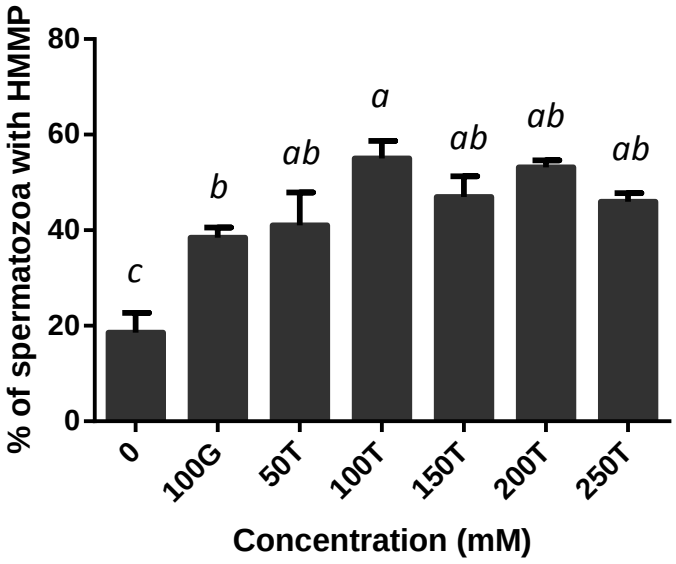

Fig. 3.4 Percentage of spermatozoa with high mitochondria membrane potential in boar spermatozoa frozen in extenders supplemented with glycerol or trehalose. Error bars represent SEM. [a,b,c] $P < 0.05$, n = 6.

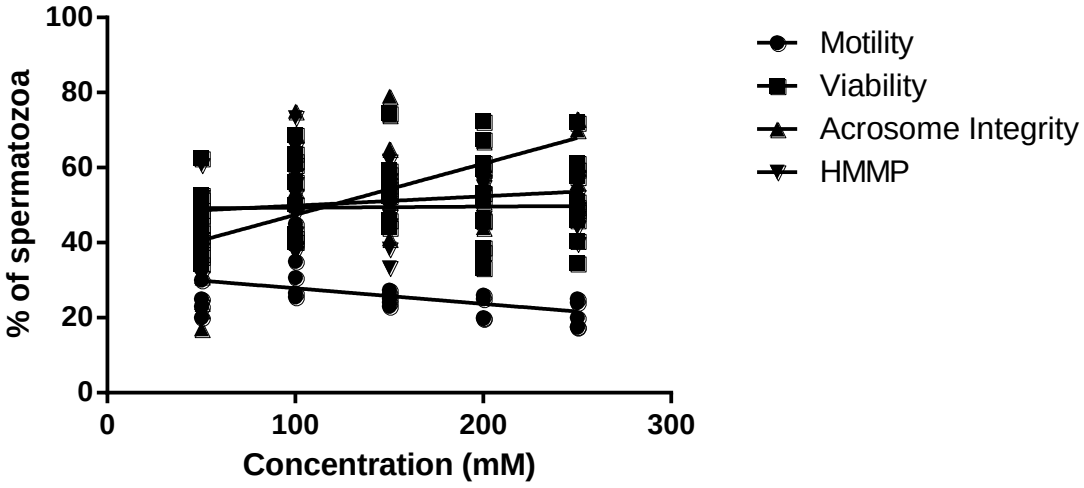

Fig. 3.5 Correlation between trehalose concentration and post-thaw variables.

Table 3.1 Effect of glycerol and trehalose in an extender on penetrability of boar spermatozoa following freezing and thawing.

Treatment	No. of oocytes examined	No. (% mean ± SEM) of oocytes		
		Penetrated[1]	Monospermy[2]	Formed pronuclei[2]
Glycerol	82	34 (43.6 ± 11.9) [a]	29 (89.6 ± 7.9)[a]	19 (50.0 ± 14.9)
Trehalose	105	66 (61.3 ± 9.5) [b]	47 (76.5 ± 9.1)[b]	43 (59.2 ± 9.6)

[1]Percentage to the number of oocytes examined.

[2]Percentage to the number of oocytes penetrated.

[a,b]$P < 0.05$, n = 4.

3.4 Discussion

Results of the present study show that the replacement of cryoprotectant from glycerol to trehalose significantly improves motility, viability, acrosome integrity, MMP and in vitro penetrability of post-thaw boar spermatozoa. Effects of trehalose on cryosurvival of spermatozoa were reported in mammalian species other than boar, such as ram (Bucak et al., 2007; Jafaroghli et al., 2011; Tonieto et al., 2010), goat (Aboagla et al., 2003; Khalili et al., 2009; Naing et al., 2010), bull (Chhillar et al., 2012) and gazelle (Garde et al., 2008), when the extenders containing glycerol were supplemented with trehalose. Several investigators have found that the addition of trehalose in glycerol-based cryopreservation extenders protects boar spermatozoa against freeze damage. According to Gutierrez-Perez et al., (2009), almost double the sperms were alive, motile and intact in the presence of 250 mM trehalose and 1% (v/v) glycerol than in 4% glycerol alone. Moreover, Malo et al., (2010) stated that the addition of trehalose in the first medium before extended with 3% glycerol significantly improved the freezability of boar spermatozoa, achieving higher sperm survival and fertilization rates. These results were obtained in the presence of glycerol at a relatively low concentration and trehalose probably helped to produce glassy-forming state Sutton (1991, 1992). Our findings clearly demonstrate that the beneficial effect of trehalose is effective even in a glycerol-free condition. Although a dehydration effect is expected even in the presence of trehalose alone, due to a non- permeability of trehalose, biological toxicity in boar spermatozoa appears to be reduced than in the presence of glycerol alone. Chemical toxicity cannot be ignored in application of glycerol to cryopreservation of boar spermatozoa despite the benificial effects, since the boar spermatozoa may be very sensitive to the toxicity. However, some studies reported that supplementing the extender cotaining glycerol with trehalose has no significant effect on cryopreservation of spermatozoa (Molinia et al., 1994; Squires et al., 2004).

In the present study, in fact, the motility, MMP, and acrosome integrity were well maintained when the spermatozoa were frozen with trehalose when compared to glycerol. Since similar results have been observed in previous studies where the extenders were supplemented with 3-5% glycerol with 100 mM trehalose (Hu et al., 2009; Jafaroghli et al., 2011). The beneficial effects of trehalose on biological membranes probably active regardless of the presence of glycerol. Our results revealed that the motility decreased in the presence of 150 mM trehalose or higher concentration in a glycerol-free extender. Since the motility of frozen-thawed spermatozoa was also reduced in the presence of both trehalose (> 200 mM),

and 3% glycerol (Aisen et al., 2002), this reduction in motility may be due to the concentration-dependent effect of trehalose. Even though the motility is negatively correlated with trehalose concentration, MMP and viability were not affected by the high concentrations. This fact suggests that high trehalose concentrations are not detrimental to spermatozoa membranes despite the low motility. In addition, although the osmotic effect is high, trehalose may not involve in organelle membrane damages, which occur due to influx, and efflux of cryoprotectant during freezing and thawing as it is non-penetrative. Viscosity of the medium is greater in the presence of a higher concentration of trehalose, making it difficult for sperm to move. Alternatively, the friction in the sperm tail is increased due to the loss of intracellular free water. This causes an inhibition of sliding of the microtubule filaments or other structural elements in the flagellum (Hu et al., 2009) and consequently, the motility is reduced. According to Rutllant et al., (2008), trehalose broadened the boar spermatozoa osmotolerance by protecting the cell membrane and the mitochondrial function but affected negatively on motility. However, a study of Bama miniature pig semen has demonstrated significantly high motility in 200 mM trehalose integrated with 9% LDL and 2% glycerol (Kong et al., 2012).

Furthermore, results of the present work also showed that the addition of a concentration of 100-250 mM trehalose to a glycerol-free extender preserves the acrosome integrity and the percentage of acrosome intact spermatozoa were positively correlated with the trehalose concentration. According to Hu et al., (2009), addition of 100-200 mM trehalose to an extender containing 3% glycerol preserves acrosome integrity of boar spermatozoa. This result is also consistent with our current result that acrosome integrity was significantly maintained in the presence of 100 mM trehalose rather than 100 mM glycerol. However, higher concentrations of trehalose in the presence of glycerol have also been reported to have a detrimental effect on the acrosome integrity of frozen-thawed spermatozoa (Aisen et al., 2002). It has been suggested that the main cryoprotective effect of trehalose is the preservation of the membrane structure. Lambruschini et al., (2000) stated that trehalose participate in the network of hydrogen bonds between the phospholipid polar heads, thus replacing the water of hydration at the membrane-fluid interface and maintaining the head groups at their hydrated position. In addition, the osmotic effect of trehalose decreases the intracellular freezable water, hence the formation of ice crystals inside the cells (Fuller, 2004). Therefore, using of trehalose at 100 mM as a cryoprotectant in the extender for boar spermatozoa reduces the injuries in membranes and consequently improves the motility, MMP and acrosome integrity of frozen-thawed spermatozoa.

As trehalose preserves membrane fluidity during cryopreservation, it would eventually affect the fertility as well. However, a limited number of studies have been carried out to evaluate the fertility of spermatozoa frozen-thawed with trehalose. Results of the current study clearly demonstrated that replacement of glycerol in an extender for cryopreservation with trehalose improved the in vitro penetrability of frozen-thawed boar spermatozoa. Malo et al., (2010) has shown that 57.5% of in vitro penetrability was achieved when boar spermatozoa were cryopreserved with trehalose in glycerol-based extender. Supplementation of an extender including 5% glycerol with 100 mM trehalose has also been reported to improve the fertility of frozen-thawed ram spermatozoa following artificial insemination with ewes (Jafaroghli et al., 2011). In a recent report in mice (Sztein et al., 2001), frozen/thawed spermatozoa cryopreserved with 300 mM trehalose retained significantly better fertility (79%) than the sperm frozen in 0.3 M glycerol (11%). Presence of trehalose as a cryoprotectant in an extender improved the penetrability of frozen-thawed boar spermatozoa, rather than combining with glycerol or using glycerol alone as the cryoprotectant, probably due to its favorable effects on the biological membranes.

3.5 Conclusion

Trehalose, a non-permeable sugar was capable of maintaining good levels of motility, viability, acrosome integrity, mitochondrial membrane potential and in-vitro penetrability of boar spermatozoa after cryopreservation in a glycerol-free freezing extender. We also recommend applying 100 mM trehalose as the optimum concentration to a glycerol-free freezing extender for cryopreservation of boar spermatozoa.

Chapter 4

Effect of rapid thawing on post-thaw survival of boar spermatozoa cryopreserved with glycerol-free trehalose extenders

4.1 Introduction

Scientists are still seeking for a proper cooling and thawing protocol to cryopreserve boar spermatozoa even though there are many different procedures are currently in operation. Artificial insemination with frozen boar spermatozoa results in a lower conception rate and smaller litter size, as compared with fresh semen (Johnson et al., 2000). Despite documented efforts to reach acceptable fertility and prolificacy after AI, overall boar spermatozoa cryosurvival is consistently lower in comparison to other species, owing to physical damage during freezing and thawing process (Rodriguez-Martinez et al., 2011). To obtain a favorable survival rate of spermatozoa, thawing procedure is just as important as freezing method.

The appropriate thawing rate is thought to be influenced by numerous other factors of the cryopreservation procedure, such as type of extender, cryoprotectant concentration, packaging method and freezing rate (Rodriguez et al., 1975; Robbins et al., 1976). Thawing procedure brings back the sperm cell to physiological temperature reactivating the metabolism; hence, it should be done with care to avoid any possible damages. For this to happen, it is important that the sperm sample get warm enough, but not exceed the temperature limit during thawing. Therefore, immersing the straws in the water for exact duration is necessary.

It has raised the interest regarding the most adequate speed of the thawing process, which result the highest possible percentage of viable boar sperm cells in egg yolk- and glycerol-based extenders (Bamba and Cran, 1985; Eriksson and Rodriguez-Martinez, 2000; Cordova et al., 2006). Various studies have been conducted to determine the optimum temperature and duration of thawing on spermatozoa of other species as well (Correa et al., 1996; Muino et al., 2008; Silve et al., 2013). According to Hammerstedt (1990), very rapid thawing in a glycerol-based extender results in unbalanced rates of efflux of glycerol and influx of water, while a very slow thawing rate results in recrystallization of micro crystals of intracellular ice and subsequent damage to subcellular organelles.

Furthermore, boar spermatozoa are susceptible to cold shock as quick cooling below 15^{o}C (Pursel et al., 1973), whereas the spermatozoa acquire a gradual resistance to cold shock by cooling slowly or preincubation before decreasing temperature below 15^{o}C (Johnson et al., 2000). However, the passage state between the melting point and the 15^{o}C during thawing process and its effect on the damage on frozen thawed boar spermatozoa is still unclear. In contrast to cooling process, spermatozoa probably required to pass through the limits of temperature below 15^{o}C very rapidly before the metabolism in the spermatozoa are reactivated.

In our previous study, we have demonstrated that non-permeating sugar trehalose can maintain motility, viability, acrosome integrity, mitochondria membrane potential and in vitro penetrability when boar spermatozoa were frozen and thawed in a glycerol-free freezing extender. In the glycerol-free extender, rapid thawing may not cause unbalanced rates of cryoprotectant efflux and the water influx in the spermatozoa. Therefore, the current study was undertaken to evaluate the effect of rapid thawing in water at 60, 70 and 80^{o}C on post-thaw normality of boar spermatozoa extended in glycerol-free trehalose extender.

4.2 Experimental design

4.2.1 Exp. 1: Monitoring temperature during thawing

Two-channels digital record thermometer (TASCO TNA- 140, Japan) was used to measure the temperature inside the straws Appendix 6). Straws were filled with freezing extender and one sensor wire of the thermometer (T1) was inserted into the straw before freeze in LN_2. Second sensor wire (T2) was kept in the water bath. When the T2 reached desired temperature (40, 60, 70 and 80^{o}C), the straws were removed from LN_2 and immersed in the water bath. The time taken by the extender inside the straw to reach 39^{o}C was recorded using the inbuilt digital recorder every 2 seconds intervals (the maximum frequents of the recorder). Two thawing methods were adopted based on the results of the experiment 1.

4.2.2 Exp. 2: Effect of rapid thawing at 60, 70 and 80^{o}C

Ejaculates from 5 individual boars were used in this experiment. Sperm samples were frozen in an egg yolk-trehalose-Equex STM (glycerol-free) freezing medium. Spermatozoa were packed into 0.5 mL straws and stored in LN_2 for 2-3 days. The straws containing

spermatozoa were thawed in water at 40, 60, 70 and 80ºC for 36 (Control), 13, 11 and 9 s, respectively, Thawed sperm suspension was diluted with mMS (1:2) at 39ºC and washed once by centrifugation (700 x *g*, 3 min, 39°C). The precipitate resuspended in 1 mL of mMS was evaluated after incubating at 39°C for 5 min. Post-thawed spermatozoa were analyzed for motility, viability, acrosome integrity, MMP and penetrability in vitro.

4.2.3 Exp. 3: Effect of rapid transient thawing at 60, 70 and 80ºC followed by maintaining procedure at 39ºC

Straws with frozen spermatozoa were immersed in 60, 70 and 80ºC water for 10, 8 and 6 sec and maintained at 39ºC for 50, 52 and 54 s, respectively. Spermatozoa thawed at 39ºC for 60 sec were considered as the control. Then the thawed samples were diluted with mMS (1:2) at 39ºC and washed once by centrifugation (700 x *g*, 3 min, 39°C). The re-suspension in 1 mL of mMS was evaluated after incubating at 39°C for 5 min. Post-thawed spermatozoa were analyzed for the same parameters in Experiment 2.

4.2.4 Exp. 4: Effect of the thawing temperature on the in vitro penetrability of frozen boar spermatozoa.

In vitro penetrability of frozen spermatozoa thawed at 70ºC for 8 sec and maintained at 39ºC for 52 sec was compared with spermatozoa thawed at 39ºC for 60 s. A total of 228 mature oocytes from 4 replicates were used in the experiment.

4.2.5 Statistical analysis

Statistical analyses were carried out by one-way ANOVA followed by Tukey's multiple range test (Exp. 1, 2 and 3) or Chi-square test (Exp.4) using GraphPad Prism 6 Statistical Software (GraphPad Software Inc., California, USA). Interrelation of thawing temperature and post-thaw variables were assessed by Pearson correlation. All percentage data were subjected to arcsine transformation before statistical analysis, if the percentage data contained values less than 10% and/or more than 90%. All data were expressed as mean ± SEM. Differences were considered significant at $P<0.05$.

4.3 Results

4.3.1 Exp. 1: Monitoring temperature during thawing

There were two phases in the change of temperature inside the straw during thawing; rapid at the beginig of the course of thawing and relatively slower warming at the liquid-phase (Fig. 4.1). When the change of temperature in the straws of frozen semen was monitored for every 2 seconds (a minimun recording interval of the digital thermometer), the changing rate did not detect any drastic differences among 4 groups (40, 60, 70 and 80^{o}C), until 6 sec after the start of warming (Table 4.1). However, the rate was significantly higher after 6 - 10 sec and 12 sec after the frozen straws were exposed to 80^{o}C ($P < 0.05$) and 70^{o}C ($P<0.01$) than 40^{o}C, respectively. Assuming a linear relationship between the nearest two points above and below the 0 and 15^{o}C, calculated time the straws took from -196^{o}C to 0^{o}C was 6.5, 5.9, 5.6 and 4.6 sec when the straws were thawed in water at 40, 60, 70 and 80^{o}C, whereas the time to 15^{o}C was 13.8, 9.7, 9.2 and 6.9 s, respectively. However, according to the expected curves in the graph, 7.5, 6.0, 5.0 and 4.5 sec were taken to pass 0^{o}C and 13.2, 9.5, 8.0 and 6.5 sec were consumed to traves 15^{o}C. Average warming rates during the first 2 sec were 4770, 4878, 4938 and 5214^{o}C /min when the straws were exposed to water at 40, 60, 70 and 80^{o}C, respectively, whereas the average rate from -196 to 15^{o}C were 917.4, 1305.2, 1376.1 and 1834.8^{o}C/min according to the calculated time.

4.3.2 Exp. 2: Effect of rapid thawing at 60, 70 and 80^{o}C

The effect of rapid thawing by immersing the straws in a high temperature water bath (60^{o}C for 13 s, 70^{o}C for 11 sec or 80^{o}C for 9 s) on the motility, viability, acrosome integrity and MMP of frozen boar spermatozoa was evaluated. As shown in Table 4.2, motility was higher when the straws of frozen semen were thawed in 80^{o}C for 9 sec as compared with controls (40^{o}C for 36 s, $P < 0.05$). Viability of spermatozoa was considerably higher when straws of frozen spermatozoa were thawed in water at 60, 70 or 80^{o}C ($P < 0.05$), as compared with 40^{o}C. CTC evaluations demonstrated an increased incidence of spermatozoa with intact acrosome when thawed in water at 60, 70 or 80^{o}C ($P < 0.05$), in comparison with 40^{o}C water. When microscopic evaluation of spermatozoa was carried out using JC-1/PI staining, no differences in MMP of sperm cells were found between 4 groups thawed in water at different temperatures.

Motility, viability and acrosome integrity of spermatozoa were positively correlated with temperature when the straws were thawed rapidly ($r^2 = 0.21$, $P < 0.05$; $r^2 = 0.24$, $P < 0.05$ and $r^2 = 0.40$, $P < 0.001$, respectively, Fig.4. 2), whereas, MMP was not significant ($P = 0.93$).

4.3.3 Exp. 3: Effect of rapid transient thawing at 60, 70 and 80ºC followed by maintaining procedure at 39ºC.

In this experiment, frozen straws were thawed at 60, 70 or 80ºC for 10, 8 or 6 sec and then maintained at 39ºC for 50, 52 or 54 sec before washing with mMS. Effect of maintaining procedure at 39ºC after thawing at 60, 70 or 80ºC was examined. As shown in Table 3, a significant difference ($P < 0.05$) in motility was observed only when frozen spermatozoa were thawed at 70ºC for 8 sec and maintained at 39ºC for 52 s. As well as Exp. 2, viabilities of spermatozoa thawed at 60, 70 or 80ºC for 10, 8 or 6 sec and then maintained at 39ºC for 50, 52 or 54 s, respectively were higher ($P < 0.01$) than controls (thawed and warmed at 39ºC for 60 s). Percentage of spermatozoa with high MMP was remarkably higher ($P < 0.05$) when the straws were thawed at 70 or 80ºC for 8 or 6 sec and then maintained at 39ºC for 52 or 54 s, respectively than controls. Acrosome integrity of spermatozoa was higher ($P < 0.05$) when the straws were thawed at 60 or 70ºC for 10 or 8 sec and then maintained at 39ºC for 50 or 52 s, respectively, as compared with controls.

Thawing temperature and parameters associated with sperm quality showed a substantial relationship when the spermatozoa were maintained at 39ºC after transient thawing at a high temperature (Fig.4.3). Significant positive correlations were observed between thawing temperature and motility ($r^2 = 0.25$, $P < 0.05$), viability ($r^2 = 0.54$, $P < 0.01$), MMP ($r^2 = 0.41$, $P < 0.01$) and acrosome integrity ($r^2 = 0.23$, $P < 0.05$).

4.3.4 Exp. 4: Effect of the thawing temperature on the in vitro penetrability of frozen boar spermatozoa.

Since thawing straws at 70ºC for 8 sec and then maintained at 39ºC for 52 sec resulted in a beter post-thawing qualities, straws thawed at above conditions were examined for penetrability in vitro and straws thawed at 39ºC for 60 sec were taken as the controls (Table 4.5). The penitrability in vitro of frozen spermatozoa thawed at 70ºC for 8 sec and then maintained at 39ºC for 52 sec (70.7 ± 9.5%) was significantly higher than that of the controls ($P < 0.01$). The incidences of monospermy and oocytes formed both male and female proncuclei were not significantly different between 2 groups 8 hours after insemination.

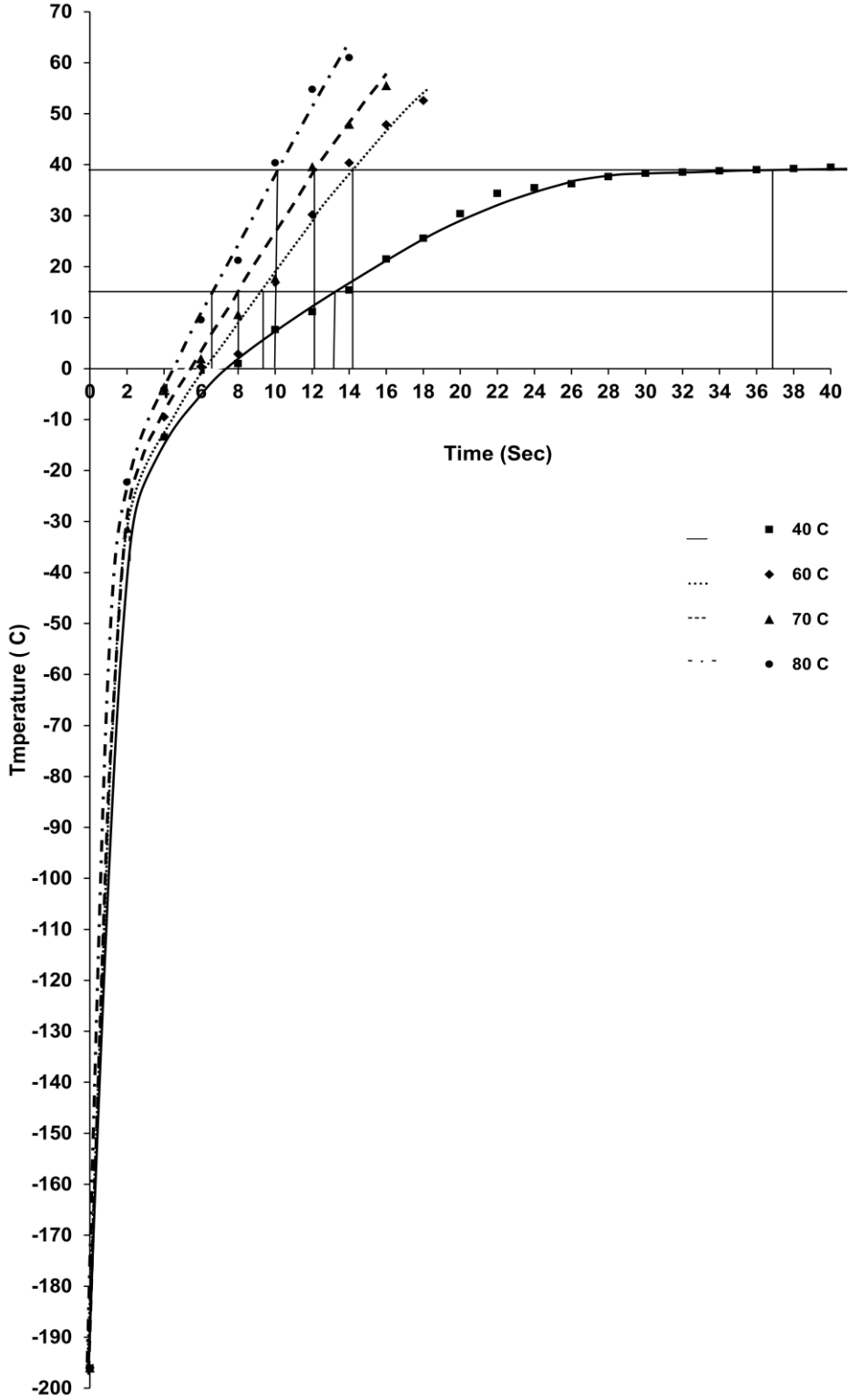

Fig 4.1 Change of temperature inside the straws during thawing in a water bath with different temperatures (40, 60, 70, and 80^{o}C). Temperature was recorded at every 2 seconds and the smooth curves were drawn manually from mean values of 8 replicates.

Table 4.1 Average temperature and warming rate inside the straw during thawing in water at different temparetures

Time (sec) after immerse in thawing water	Temperature (°C) of water in which the straws of frozen semen were thawed							
	40		60		70		80	
	Temp (°C) in the straw	Warming rate (°C /sec)	Temp (°C) in the straw	Warming rate (°C /sec)	Temp (°C) in the straw	Warming rate (°C /sec)	Temp (°C) in the straw	Warming rate (°C /sec)
0	-196.00	-	-196.00	-	-196.00	-	-196.00	-
2	-37.0 ± 6.5	79.5 ± 3.6	-33.3 ± 7.0	81.3 ± 3.5	-31.3 ± 10.8	82.3 ± 5.4	-22.3 ± 3.2	86.9 ± 1.6
4	-13.4 ± 8.0	12.1 ± 2.6	-9.5 ± 7.5	11.9 ± 3.1	-6.8 ± 3.5	12.2 ± 6.4	-4.1 ± 2.0	9.1 ± 2.4
6	-0.3 ± 0.4 ^{a}	6.5 ± 3.9	0.4 ± 2.3 ^{a}	4.9 ± 3.6	1.9 ± 2.1 ^{a}	4.2 ± 1.6	9.6 ± 2.0 ^{b}	6.8 ± 1.5
8	1.0 ± 1.0 ^{a}	0.7 ± 0.4 ^{A}	2.9 ± 1.8 ^{ab}	1.2 ± 1.2 ^{AB}	10.6 ± 2.9 ^{b}	4.3 ± 0.5 ^{AB}	21.2 ± 2.9 ^{c}	5.8 ± 1.8^{B}
10	7.6 ± 1.1^{a}	3.3 ± 0.3 ^{A}	16.9 ± 2.5^{ab}	7.0 ± 1.1 ^{AB}	17.7 ± 2.8^{b}	3.5 ± 1.8 ^{A}	40.4 ± 2.9^{c}	9.6 ± 2.0 ^{B}
12	11.2 ± 1.3^{a}	1.8 ± 0.6 ^{A}	30.2 ± 3.1^{b}	6.7 ± 1.1^{B}	39.6 ± 1.6^{c}	11.0 ± 0.7 ^{C}	-	-
14	15.4 ± 1.2^{a}	2.1 ± 0.7	40.4 ± 2.4^{b}	5.1 ± 1.3	-	-	-	-

Different small and capital superscripts indicate a significant difference in temperature and the warming rate within the same row, respectively ($P < 0.05$, n=8).

Table 4.2 Post-thaw quality parameters of boar spermatozoa thawed by using rapid thawing

Thawing temperature (°C)	Thawing period (sec)	Percentage of spermatozoa (mean + SEM)				
		Motility	Viability	HMMP	Acrosome Integrity	
40	36	12.0 ± 0.7^{a}	39.3 ± 1.6^{a}	38.9 ± 2.7	52.5 ± 1.5^{a}	
60	13	16.2 ± 1.8^{ab}	53.0 ± 1.5^{b}	42.0 ± 4.0	64.5 ± 2.5^{b}	
70	11	18.5 ± 1.8^{ab}	48.9 ± 2.5^{b}	39.8 ± 3.7	66.3 ± 2.1^{b}	
80	9	20.0 ± 2.8^{b}	51.0 ± 3.5^{b}	41.2 ± 3.9	66.2 ± 4.0^{b}	

P ^{a,b} < 0.05, HMMP: High Mitochondria Membrane Potential, n= 6

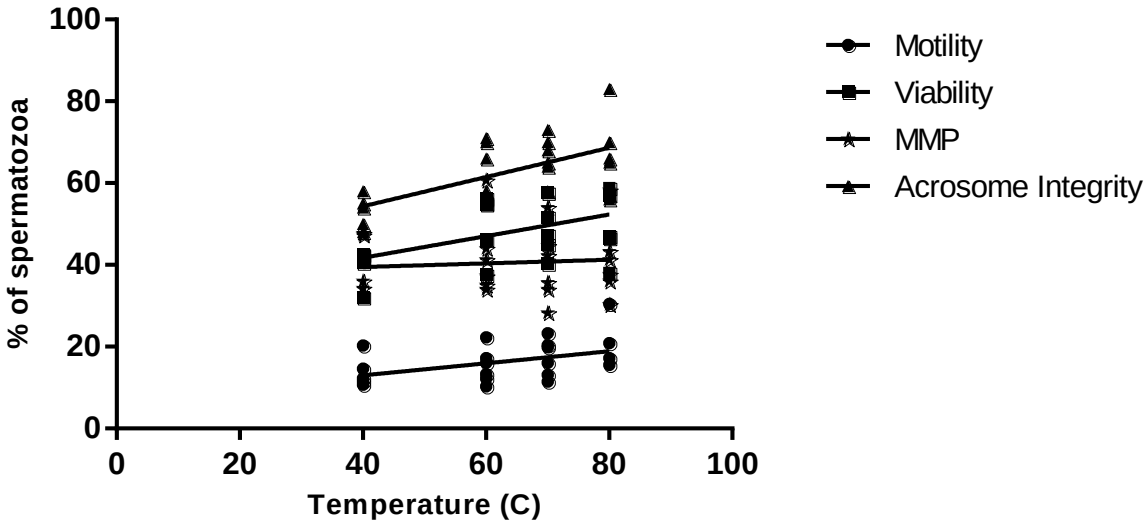

Fig. 4.2 Relationship between thawing temperature and post-thaw quality of frozen boar spermatozoa thawed by using rapid thawing method.

Table 4.3 Post-thaw quality parameters of boar spermatozoa thawed rapidly and transiently at different temperatures and then maintained at 39°C

Thawing temperature (°C)	Thawing duration (sec)	Maintained temperature (°C)	Maintained duration (sec)	Percentage of spermatozoa (mean + SEM)			
				Motility	Viability	HMMP	Acrosome Integrity
39	60	39	0	19.2 ± 1.3^{a}	34.8 ± 3.3^{a}	30.3 ± 4.1^{a}	52.2 ± 4.1^{a}
60	10	39	50	34.0 ± 2.6^{ab}	49.3 ± 2.0^{b}	42.4 ± 1.7^{ab}	71.5 ± 4.4^{b}
70	8	39	52	38.4 ± 7.8^{b}	51.6 ± 0.9^{b}	47.3 ± 4.3^{b}	74.2 ± 3.9^{b}
80	6	39	54	33.3 ± 6.0^{ab}	51.3 ± 2.4^{b}	47.5 ± 4.8^{b}	67.7 ± 5.1^{ab}

P ^{a,b} < 0.05, HMMP: High Mitochondria Membrane Potential, n= 6

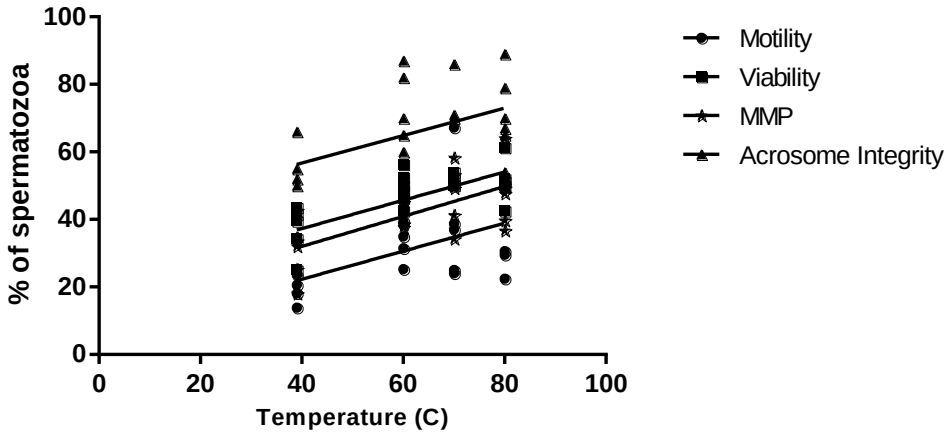

Fig. 4.3 Relationship between thawing temperature and post-thaw quality of frozen boar spermatozoa thawed by using rapid transient thawing method with maintaining procedure at 39^{o}C.

Table 4.4 Effect of thawing temperature on penetrability boar spermatozoa frozen in glycerol-free trehalose extender

Thawing temperature (°C)	No. of mature oocytes	Percentage (No.) of oocytes		
		Penetrated[1]	Monospermy[2]	Formed pronuclei[2]
39	107	46.7 ± 11.4 (52)[a]	63.3 ± 11.3 (29)	97.5 ± 2.5 (51)
70/39	121	70.7 ± 9.5 (87)[b]	58.5 ± 8.8 (47)	95.0 ± 2.9 (82)

[1]Percentage to the number of oocytes examined.

[2]Percentage to the number of oocytes penetrated.

[a,b]$P < 0.05$, n = 4.

4.0 Discussion

Results of the present study clearly indicated that rapid thawing improve post-thaw survival of boar spermatozoa and critical damage zone was travesed faster when thawed rapidly. Frozen boar spermatozoa have been thawed mostly between 37 to 40^{o}C for 20-40sec(Juarez et al., 2011; Gomez-Fernandez et al., 2012; Tomas et al., 2013). It has already been proposed repeatedly that there is a dangerous temperature zone, somewhere between -60^{o}C and the freezing point, as the critical range of temperature where harmful ice formation or the recrystalization easily occurs during cooling or warming process, respectively (Mazur, 1985; Bwanga, 1991; Gao et al., 2000). In the current study, we observed that spermatozoa passed faster through the critically dangerous zone when the straws were exposed to water at 70 or 80^{o}C than 40^{o}C ($P < 0.01$). Furthermore, boar spermatozoa are also more susceptible to cold shock damage when exposed to the temperature below 15^{o}C during cooling (Pursel et al., 1973). This phenomenan could be expected to reappear during thawing as so called "warm shock" (Bamba and Crang 1988). Our current results demonstrated that spermatozoa passed through the warm shock zone (from -196 to 15^{o}C) much faster when the straws were exposed to water at 70 or 80^{o}C than 40^{o}C (control). The calculated time at 70 and 80^{o}C was 66.9 and 50.2% of the time at 40^{o}C (13.81 sec), respectively. Therefore, thawing at 70 and 80^{o}C for a transient period should have an advantage to pass faster not only through the critical dangerous zone between -60 to 0^{o}C but also the warm shock temparature zone (less than 15^{o}C), consequently to reduce recrystallization of intracellular ice and warm shock during thawing.

In the current study, in fact, we observed significantly higher viability, motility and acrosome integrity of spermatozoa when frozen straws were thawed at 70 or 80^{o}C for 11 and 9 sec, respectively, having a positive liner correlation with the thawing temperature. Our current results were supported by previous studies having improved motility and acrosome integrity of boar spermatozoa with increased thawing rates (Pursel and Park, 1987; Fiser et al., 1993; Pursel and Johnson, 1975;Dhami et al., 1992; Hernandez et al., 2007a). However, a number of studies have been still reported that thawing rate had no or little improvement in plasma membrane integrity or normal acrosome ridge in spermatozoa (Eriksson and Rodriguez-Martinez, 2000; Cordova et al., 2006; Pugliesi et al., 2014). Bamba and Cran (1985;1988) reported that fast warming of boar semen in the temperature between 5 to 37^{o}C damaged acrosome membrane. However, according to our results, thawing at 60, 70 or 80^{o}C

for 13, 11 or 9 sec, respectively, considerably improved the incidence of spermatozoa with intact acrosome. It suggests that fast enough warming between 5 to 37^{o}C may have an advantage on the sperm acrosome membrane. Recently, Tomas et al., (2014) also reported greater sperm viability, motility and kinetic variables after thawing at 70^{o}C for 8 sec and Hernandez et al., (2007a) reported a greater survival rate after thawing at a rate of 1800^{o}C/min. Therefore, these rapid thawing protocols at high temperature guaranteed to improve not only the viability and motility, but also acrosome integrity of boar frozen spermatozoa.

We also found that maintaining straws at 39^{o}C for 52 sec after thawing at 70^{o}C for 8 significantly improved all sperm parameters (the viability, motility, acrosome integrity, and mitochondria membrane potential) as compared with controls (thawed and warmed at 39^{o}C for 60 sec). O'Connell et al., (2002) observed swelling, coiling, vacuolization and structural disorganization of mitochondria after sperm cryopreservation. In the present study, thawing at high temperature itself did not improved MMP of boar spermatozoa, but improved after maintaining straws at 39^{o}C after thawing at 70 or 80^{o}C. Post-thaw boar spermatozoa were maintained at 39^{o}C after washing with thawing solution in both thawing methods, However, maintaining procedure of boar spermatozoa within the straw allow spermatozoa to keep intact with egg yolk and trehalose further after rapid thawing and before washing. Rapid thawing results a swelling of spermatozoa due to influx of water and later reach the equilibrium. Presence of trehalose further at 39^{o}C probably induce efflux of water even after thawing and help reach the equilibrium faster and it may encourage the recovery of the MMP of boar spermatozoa. Moreover, as the temperature change between two seconds is very high, particularly at 60,70 and 80^{o}C during rapid thawing, a safe margin for transition near 39^{o}C may also attributed to better MMP after rapid transient thawing followed by maintain procedure at 39^{o}C. Following a processing procedure after thawing has been reported better membrane integrity supporting the findings of the current study (Correa et al., 1996), while, detrimental effects have also been reported (Senger et al., 1976; de Abreu et al., 1979) for boar spermatozoa.

In the present study, we also demonstrated a significantly higher penetrability after thawing frozen straws of boar spermatozoa at 70^{o}C for 8 sec and then maintaining at 39^{o}C for 52 s. Comparable with our study, improved IVF parameters have been reported for boar spermatozoa, however with rapid thawing at 50^{o}C (Selles et al., 2003; Pursel and Johnson,

1975). According to Shi et al., (2014), there are roughly 72–80 mitochondria are present in the mid piece and the tail of the mature mammalian spermatozoon, to provide immense energy for sperm motility. Therefore, a significant thawing protocol to maintain the MMP could have promoted the motility and penetrability of boar spermatozoa.

In the present study, we used trehalose, a non-permeating cryoprotectant, which is known to decreases the intracellular freezable water due to its osmotic effect and to be a biological antifreeze having an ice recrystalization inhibiting activity (Tam et al., 2007). This kind of biological antifreezes bind to the surface of ice and facilitate a localized freezing point depression and induces a change in the ice crystal habit (Capicciotti et al., 2013). Very rapid thawing in a glycerol-based extender has also known to result in some damages in spermatozoa due to unbalanced rates of efflux of glycerol and influx of water (Seidel, 1986). Very slow thawing rate results in recrystallization of micro crystals of intracellular ice and subsequent damage to subcellular organelles (Hammerstedt, 1990). The current results clearly demonstrated that rapid thawing of boar spermatozoa frozen in a glycerol-free extender containing trehalose could overcome the cryo-damage, consequently improved viability, motility, acrosome integrity, MMP and the penetrability in vitro.

Although use of high temperatures is a limiting factor in the protocol as it is far from being a practical method of thawing the straws, especially in the field conditions, it has showed a lower degree of cellular damage. Moreover, keeping spermatozoa under high temperatures for a longer period than recommended may risk the post-thaw survivability causing sever damages to membrane and intracellular proteins. As the warming rate of a thawing protocol is depend on the surface to volume ratio of the packaging, future studies are required on the potential of applying the current thawing procedure on other packaging systems.

4.5 Conclusion

Rapid thawing at 70^{o}C for 8 sec and maintaining at 39^{o}C for 52 sec enhanced motility, viability, acrosome integrity, mitochondria membrane potential and in vitro penetrability of boar spermatozoa frozen in a glycerol-free extender containing tehalose, and will be proposed as the optimum thawing protocol.

Chapter 5

Effect of milk supplements in glycerol-free trehalose freezing extenders on cryosurvival of spermatozoa

5.1 Introduction

Sperm cryopreservation is the most efficient method for storing boar spermatozoa for a long period, even though their fertilizing ability is lower than that of fresh or refrigerated semen (Rath, et al., 2009). Not all the boar ejaculates hold the capacity to withstand freeze thawing to the same level. Differences in sperm freezability have been reported to exist between breeds, within and between boars, between fractions coming from the same ejaculate and even between the seasons (Holt et al., 2005; Hernández et al., 2006; Pena et al., 2006; Barranco et al., 2013). Therefore, boars and their ejaculates have been rated as 'good' or 'bad' freezers and as 'good freezability ejaculates' or 'poor freezability ejaculates' (Watson, 1995).

Various extenders and supplement in glycerol-based extenders have been described for cryopreservation of spermatozoa to improve the post-thaw survival. Researchers constantly experiment to develop more practical and less-expensive methods to improve cryosurvival and freezability of spermatozoa of low freezability boars. Various milk based extenders have been proven to be effective for bovine (Singh et al., 2013; Foote et al, 2002), goat (Melo and Nunes, 1997), dog (Baran et al., 2012; Roca et al., 2001), ram (Kulaksiz et al., 2012), buck (Sule et al., 2007) and equine (Filho et al., 2009) spermatozoa. Although these additives have been using for a long time for sperm storage in the liquid or frozen states, effect of milk supplementation on boar spermatozoa cryopreservation is rarely studied, particularly in glycerol-free freezing extenders.

In our previous study we have demonstrated that non-permeating sugar trehalose can maintain motility, viability, acrosome integrity, mitochondria membrane potential and in vitro penetrability when boar spermatozoa were frozen in a glycerol-free trehalose freezing extender and thawed for 8 and 52 sec at 70^{o}C and 39^{o}C respectively. However, some of the boars demonstrated a lower level of frezability even though the fresh semen showed greater motility and viability. Therefore, current study was commenced to improve the cryosurvival of spermatozoa using milk supplements in the glycerol-free trehalose freezing medium.

5.2 Experimental design

5.2.1 Effect of milk supplementation on post-thaw cryosurvival

Sperm samples were cooled in 20% egg yolk extender and frozen with 100 mM trehalose + 0.25% (v/v) Equex STM™ freezing extender supplemented with 2% or 5% coconut milk or/and skim milk (5% CM- TC5, 5% SM- TS5, 5% CM + 5% SM -TCS5, 2% CM -TC2, 2% SM-TS2, 2% CM + 2% SM-TCS2 and Trehalose only control- T). Sperm cells were packed into 0.5-ml straws after cooling and cryopreserved in LN_2. Samples were thawed in two steps at 70°C for 8 sec and 39°C for 52 sec after storage of 2-3 days. Thawed samples were evaluated for motility, viability, MMP and acrosome damage by using fluorescence multiple staining procedure.

5.2.2 Statistical analyses

Statistical analyses were carried out by one-way ANOVA followed by Tukey's multiple range test using GraphPad Prism 6 Statistical Software (GraphPad Software Inc., California, USA). All the percentage data were subjected to arcsine transformation before statistical analysis if the percentage data contained values less than 10% and/or more than 90%. All data were expressed as mean ± SEM. Differences were considered significant at $P < 0.05$.

5.3 Results

The effect of milk supplements added to the glycerol-free trehalose freezing extender, on motility, viability, MMP and acrosome damage of boar spermatozoa after freezing–thawing was evaluated. Motility was significantly higher (60.2 ± 3.0) in the extender supplemented with 2% SM (TS2) as compared with control (38.3 ± 4.7) and other extenders ($P < 0.05$, Fig.5.1). Post-thaw viability of spermatozoa extended in TCS2 (57.0 ± 2.8) was considerably higher than TS5 (40.8 ± 2.4) and control (40.4 ± 1.6, $P < 0.05$). It was also significantly higher in TS2 (55.6 ± 3.4) as compared with controls ($P < 0.05$, Fig.5.2). Spermatozoa extended in TS2 exhibited a significantly higher percentage of MMP (59.0 ± 2.7), having orange-yellow multimeric JC-1 aggregates in their midpiece, than in trehalose only control (41.1 ± 0.7, $P < 0.05$, Fig. 5.3). When the acrosome status was evaluated by FITC-PNA, spermatozoa in the trehalose extenders containing 2% coconut milk (TC2) exhibited significantly lower acrosomal damage (9.5 ± 0.7) compared to other treated extenders and control (17.2 ± 2.0, $P < 0.05$, Fig.5.4). Higher concentration (5%) of either CM or SM was not effective in maintaining any of the post-thaw parameters ($P > 0.05$).

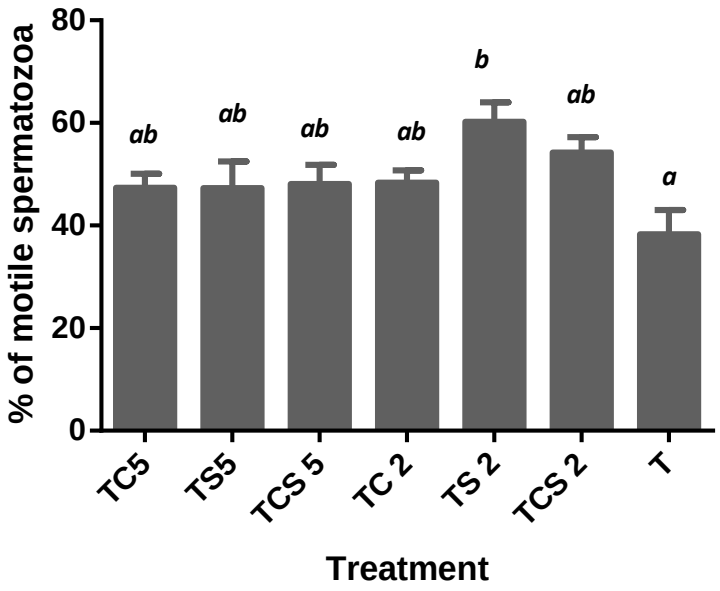

Fig.5.1 Post-thaw motility in boar spermatozoa frozen in glycerol-free trehalose extenders supplemented with coconut and/or skim milk. Error bars represent SEM. $^{a,b,}P < 0.05$, n = 5. TC5- 5% CM, TS5- 5% SM, TCS5- 5% CM + 5% SM, TC2- 2% CM, TS2- 2% SM, TCS2- 2% CM + 2% SM and T- Trehalose only.

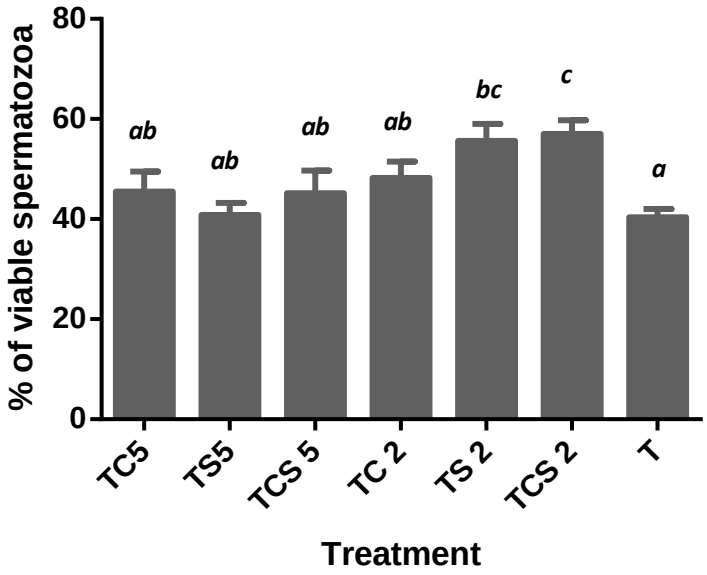

Fig.5.2 Post-thaw viability in boar spermatozoa frozen in glycerol-free trehalose extenders supplemented with coconut and/or skim milk. Error bars represent SEM. $^{a,b,c}P < 0.05$, n = 5. TC5- 5% CM, TS5- 5% SM, TCS5- 5% CM + 5% SM, TC2- 2% CM, TS2- 2% SM, TCS2- 2% CM + 2% SM and T- Trehalose only.

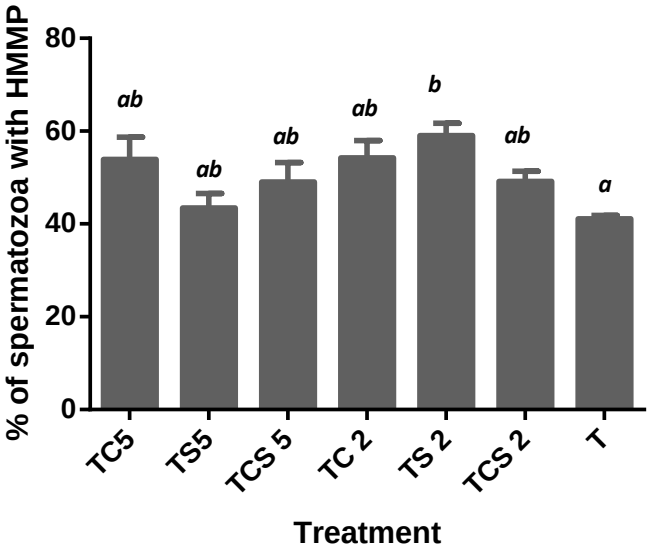

Fig.5.3 Percentage of spermatozoa with high mitochondria membrane potential (HMMP) in boar spermatozoa frozen in glycerol-free trehalose extenders supplemented with coconut and/or skim milk. Error bars represent SEM. [a,b] $P < 0.05$, n = 5. TC5- 5% CM, TS5- 5% SM, TCS5- 5% CM + 5% SM, TC2- 2% CM, TS2- 2% SM, TCS2- 2% CM + 2% SM and T- Trehalose only.

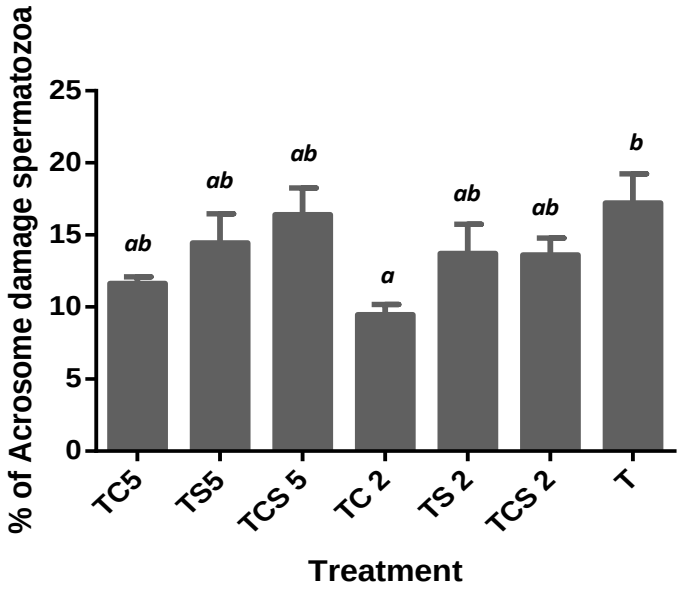

Fig.5.4 Percentage of spermatozoa with damaged acrosome in boar spermatozoa frozen in glycerol-free trehalose extenders supplemented with coconut and/or skim milk. Error bars represent SEM. [a,b] $P < 0.05$, n = 5. TC5- 5% CM, TS5- 5% SM, TCS5- 5% CM + 5% SM, TC2- 2% CM, TS2- 2% SM, TCS2- 2% CM + 2% SM and T- Trehalose only.

5.4 Discussion

Results shown in this study indicate that addition of 2% skim milk into glycerol-free trehalose freezing extender improve post-thaw motility, viability and MMP while adding 2% coconut milk reduce acrosome damage. These superior results could be due to the synergetic effect of milk and yolk in the freezing extender.

Bergeron et al., (2004) reported that proteins secreted by the seminal vesicles (BSP proteins) are added to sperm, bind to sperm membrane choline phospholipids, and cause a continuous loss of cholesterol and phospholipids from the sperm membrane upon ejaculation, which is detrimental to sperm storage. They further reported that, upon dilution of semen with extender that contains egg yolk, the LDLs present in egg yolk sequestered the BSP proteins and prevented their binding to the sperm membrane, thereby preventing the stimulation of loss of cholesterol and phospholipids in bovine spermatozoa. As the same way, skim milk prevented the binding of these proteins to sperm and reduced sperm lipid loss while maintaining sperm motility and viability (Bergeron et al., 2007). The foremost protective constituent of milk is most likely to be micelles of caseins, which are the major proteins of milk. However, seminal plasma was removed prior to cryopreservation in the current experiment. Results of the current study suggest that casein micelles probably able to bind and remove BSP proteins that are already bound to sperm membrane and prevent further lipid loss. Thereby, maintain good levels of motility, viability and MMP. In agreement with results obtained in the present study, significantly higher motility was recorded for ram spermatozoa extended in skim milk-egg yolk extender as compared with Tris, Bioxcell® and Sodium citrate extenders (Kulaksiz et al., 2012). Moreover, canine sperm viability was significantly greater when an Tris-glucose-citric acid buffer in an extender was completely replaced with 100% skim milk after 120 min of thawing, although the motility was not significantly different (Roca et al., 2001).

Moreover, whey protein fraction of the milk (protein fraction other than casein), may also have an effect on sperm membrane stabilization. Jay et al., (2004) revealed that cow milk composed of 80% casein protein while whey proteins accounts only for 20%. In a study done on effect of milk fractions, including whey proteins, on equine spermatozoa, some milk fractions (ultrafiltrate, microfiltrate, and α-lactalbumin fraction) decreased spermatozoa1 survival while others (O-lactoglobulin and native phosphocaseinate) were protective after 48 and 96 h storage at 4 or 15°C (Batellier et al., 1997). However, any protective effects of whey

proteins against cryodamage are yet be elucidated.

It is well known that sperm from species with low levels of cholesterol in their sperm membranes have decreased tolerances to cold shock, as compared to sperm from species with high levels of cholesterol (Darin-Bennett and White, 1977). In addition, the incorporation of cholesterol into boar, bull or stallion sperm membranes increases sperm tolerance to cryopreservation (Blanch et al., 2012; Purdy and Graham, 2004b; Moore et al., 2005), while the removal of lipids from the sperm during storage is deleterious for sperm functions (Bergeron et al., 2004). Thus, another plausible explanation for the reduced damage to the plasma membranes during cooling and freezing could be due to the effect of cholesterol in milk supplementation and yolk in a synergetic manner, in order to increase in the fluidity of boar spermatozoa membrane. At the same time, cholesterol in milk and yolk probably incorporated in to the phospholipids bilayer and prevented further removal cholesterol from the sperm membrane during storage and freezing.

Another possibility for having greater cryosurvival of boar spermatozoa is the effect of sugars present in milk. Lactose is predominant in skim milk while sucrose, glucose and fructose are present in coconut milk. These sugars in egg yolk based extenders has been tested in freezing boar (Malo et al., 2010; Chanapiwat et al, 2011; Corcuera et al, 2007; Yi et al., 2002), goat (Naing et al, 2010), ram (Molinia et al, 1994), mouse (Sztein et al., 2001) and stallion (Heitland et al., 1996; Loomis et al., 1983) spermatozoa. In combination with trehalose in the medium, these sugars probably acted as a cryoprotectant and minimized osmotic stress during freezing and thawing. Dehydration effect of sugars in turn prevents intracellular ice formation and membrane injuries due to ice. When milk was added at 5%, lower cryosurvival was resulted probably because high concentrations of sugars are detrimental for freezing spermatozoa. However, milk filtrate that contains only lactose and minerals was not sufficient to protect stallion sperm during storage (Batellier et al., 1997).

Inclusion of 2% coconut milk maintained viability and acrosome intactness to a large extent. Generally, milk proteins (Sodium Caseinate) are added to commercially produced coconut milk powders, although in minute amounts. Moreover, based on the solubility characteristics, at least 80% of the proteins in the coconut endosperm are classified as albumin and globulins (Balachandran and Arumughan, 1997). Freezability of semen is positively correlated with amount of these proteins in the seminal plasma and improving sperm motility resided in the content of albumin (Barrios et al., 2000; Georgiev et al., 1983).

However, coconut milk did not affect the motility in the present study. In contrast, 20% coconut milk and 80% citrate buffer significantly improved sperm motility (52.6%) at 2 hours post-extension in extended buck semen (Sule et al., 2007). Norman (1962) reported that the fertilizing capacity of bovine spermatozoa stored at 5° C in an extender, which contained 15% coconut milk and 5% egg yolk, compared favourably with the fertilizing capacity reported for sperm stored at 5° C in other more widely used diluents. He further reported that bovine spermatozoa aged for 6–7 days at room temperatures (18–30° C) in a coconut milk extender retained their fertilizing capacity.

Major protective effect of coconut milk could be due to the enormous amounts of antioxidants. Coconuts are rich in phenols and polyphenols, which are stronger antioxidants than the vitamins (Vinson et al., 2001; Dande and Manchala, 2011). Antioxidant content, measured using DPPH (2,2'-Diphenyl-1-picryl hydrazyl) radical scavenging activity and FRAP (Ferric reducing antioxidant power) assay, was reported as 129 and 920 mg/100g respectively and phenolic content was recorded as 31mg/100g (Dande and Manchala, 2011). In addition, coconut milk is rich in amino acids, sugars, sugar alcohol, vitamins, nitrogenous compounds, organic acids, growth substances and electrolytes (George et al., 2008). Skim milk also possesses antioxidant properties and Filho et al., (2009) reported that dilution of semen with skim milk-egg yolk based extender compensates for the non-enzymatic antioxidant protection lost with seminal plasma removal. Sperm membranes are rich in polyunsaturated fatty acids and can easily undergo lipid peroxidation in the presence of reactive oxygen species (ROS), leading to changes in membrane fluidity causing oxidative stress (Alvarez and Storey, 1982; Sikka et al., 1995). Involvement of ROS in capacitation and acrosome reaction has been proposed as a physiological role rather than pathological function (Kothari, 2010). Antioxidants in the milk supplementation probably control the balance between production and neutralization of ROS and protect spermatozoa against crocapacitation, hence the acrosome damage.

5.5 Conclusion

Supplementing 2% skim milk in a glycerol-free trehalose extender maintained good levels of motility, viability, and MMP while 2 % coconut milk supplementation reduced the acrosome damage. Viability was also preserved when the extender was supplemented with both skim milk and coconut milk at 2%.

Chapter 6

General Discussion

Cryopreservation of spermatozoa is a key factor in safe handling and transporting genetic materials and it plays an important role in the artificial insemination industry. Freezing and thawing of spermatozoa has been shown to result in a loss of motility and membrane integrity and even cell death to a larger portion of the original sperm sample. Although, advances in cryopreservation techniques have been taken place over last few decades, a considerable percentage of the boar spermatozoa are still not survive or lose their fertilization ability. The relatively low fertility of frozen thawed boar semen is associated with many factors including cytotoxicity of the cryoprotectant, highly sensitivity of plasma membrane, osmotic stresses, injuries due to ice formation during freezing and thawing, cold shock damages and even inter and intra variations present among boars.

Results of the first experiment revealed that glycerol in the freezing medium can be replaced with trehalose and 100 mM concentration was optimum for boar spermatozoa. Cryosurvival of boar spermatozoa is still considering as suboptimal and one of the main reason is the toxicity of the commonly used cyroprotectant, glycerol. Boar spermatozoa react variably to glycerol and generally, < 3% is considered as an optimal concentration. Therefore, first experiment was carried out to evaluate the effect of trehalose as a substitute for glycerol in boar spermatozoa cryopreservation. Several investigations have been carried out on adding sugars to the freezing medium but, all if not most of them use glycerol as well. In the first experiment we found that, glycerol can be successfully replaced with trehalose, a non-permeating cryoprotectant. To the best of our knowledge, this was the first attempt to replace glycerol completely with a sugar. Trehalose is a disaccharide and it exerts an osmotic effect in the spermatozoa before freezing. Our findings clearly demonstrated that the beneficial effect of trehalose is effective even in a glycerol-free condition. Trehalose is also known to stabilize the membrane by interacting with the polar head groups of phospholipids and increasing its fluidity. It also induces a decline in the membrane phase transition temperature due to its glass forming ability. Even though the post-thaw parameters show positive linear relationship with trehalose concentration, high concentrations were not good for maintaining motility due to its high viscosity and concentration-depend effect which hinders the movement of spermatozoa through the medium.

Rates of temperature change during the cooling and freezing phases of cyropreservation, as well as the thawing rate have a major impact on controlling damage to the sperm cell. During the cooling process, exposure to the non-frozen, hyperosmotic solute fraction causes water to withdraw from cells and results in cell shrinkage as well as an influx of ions. Upon re-warming, these dehydration effects are reversed and the subsequent water influx occurs and it may disrupt the plasma membrane. Straws are the most widely used packages (0.25 mL- 5 mL), even though the high surface-to-volume ratio makes it more vulnerable to temperature changes. Temperature gradients within the straw can also induce sperm functional and biochemical damage. Thermodynamics inside the straw is of paramount importance to study and it will give new insights to the evaluation of cryopreservation protocols. Thus, second experiment was carried out to understand the effect of different thawing methods (one-step and two-step) and rates using high temperatures on post-thaw survival of spermatozoa. At the time of thawing, sperm cells are rapidly re-hydrated and exposed to the majority of the osmotic stressors endured during the freezing and cooling phases in reverse order. Therefore, sperm cells are subject to severe changes in osmotic pressure and the formation of ice crystals.

Findings of the second experiment shows that thawing at 70^{o}C (8sec) followed by warming at 39^{o}C (52sec) resulted a better post-thaw survival and in vitro fertility. In conventional methods, thawing of straws is achieved at 37^{o}C to 40^{o}C for 20-40 sec. However, results of this study revealed that spermatozoa travese -196 ^{o}C to 15^{o}C region very fast when thawed at high temperatures than 40^{o}C. Holding at 15^{o}C has been suggested to prevents cold shock damages during freezing. Traversing this region fater could prevent the reversed effects of cold shock during thawing. The lethality of the intermediate temperature zone where extensive damage occurs is a challenge to cryopreservation. Therefore, passing of spermatozoa through the critical intermediate zone (-60^{o}C - 15 ^{o}C) at a faster rate and reaching 39^{o}C quickly collectively attributed to reduced recrystallization of intracellular ice. Therefore, reduced cell damages result significantly high post-thaw survival when thawed at temperatures of 80^{o}C and 70^{o}C. Temperature transition point at 39^{o}C could be critical in isothermic conditions as temperature difference between two seconds was high. At the same time, rapid thawing itself did not improve MMP. All the post-thaw parametes were improved when a second rapid transient thawing method with maintaining procedure at 39^{o}C, providing

a safe margin to the temperature transition point was adapted. This suggested that keeping the spermatozoa intact with cryopreservation solution further at 39^{o}C within the straw before washing, helped recovering of MMP. Moreover, Trehalose, itself is biological antifreeze having an ice recrystalization inhibiting activity. Presumably, rapid thawing together with trehalose prevented the recrystalization of ice during thawing and prevented the membrane damages.

Results of the third study indicated that 2% coconut milk or/and 2% skim milk can be used to improve survivability of boar spermatozoa. The discovery of the protective effects of egg yolk and cryoprotectants enabled sperm to survive the cryopreservation process and to preserve their fertility. However, some boars show low cryosurvival as compared with others in the same species even using better combination of above factors. This variation could be attributed to genetic variation, greographic distribution, season, and individual history. Therefore, third experiment was carried out examine the effect of milk supplementation to improve cryosurvival of boar spermatozoa. Reason for these results presumably due to the synergetic effect of egg yolk and milk supplements. LDLs in the egg yolk and casein micelles in the milk probably remove binding proteins, originated in the seminal plasma, already bound to plasma membrane. Thereby, prevent the stimulation of loss of cholesterol and phospholipids in the membrane. Moreover, sugar compounds in the milk act as cryoprotectants in combination with trehalose. Therefore, higher concentrations of milk supplements were not effective in providing protection during freezing. In addition phenolic and polyphenolic compounds together with other antioxidants present in the coconut milk probably protect spermatozoa from cryodameges. However, future studies are required regarding the in vitro penetrability of spermatozoa cryopreserved with milk supplements.

Conclusions

Better cryosurvival and post-thaw parameters were achieved when of boar spermatozoa were cyopreserved in a glycerol-free trehalose extender and thawed rapidly using two- steps method. Cryosurvival boar spermatozoa can be improved by supplementing trehalose medium with 2 % skim milk and/or coconut milk.

Chapter 7

Summary

Frozen thawed boar spermatozoa are still considered suboptimal due to the low conception rates and smaller litters after artificial insemination. The relatively low fertility of frozen thawed boar semen is associated with many factors including cytotoxicity of the cryoprotectant, osmotic stress, injuries due to ice formation during freezing and thawing, cold shock damages and even inter and intra variations present among boars. These areas were addressed in the current study using Berkshire boar semen. Results of the first study revealed that trehalose, a non-permeable sugar can be used to replace glycerol completely. It was capable of maintaining good levels of motility, viability, acrosome integrity, mitochondrial membrane potential (MMP) and in-vitro penetrability of boar spermatozoa after cryopreservation in a glycerol-free freezing extender. Optimum concentration was found to be 100 mM trehalose. According to the findings of the second study, rapid thawing and warming in water at 60, 70 and 80^{o}C enhanced motility, viability, acrosome integrity, MMP and in vitro penetrability of boar spermatozoa frozen in glycerol free tehalose extender. Rapid transient thawing at 70^{o}C for 8 sec followed by maintaining procedure for 52 sec at 39^{o}C is recommended as the optimum thawing conditions. Results of the third experiment revealed that supplementing 2% skim milk in a glycerol-free trehalose extender improved motility, viability, and MMP while 2 % coconut milk reduced the acrosome damage. Viability was also preserved when the extender was supplemented with both skim milk and coconut milk at 2%.

In conclusion, better cryosurvival and post-thaw parameters were achieved when of boar spermatozoa were cyopreserved in a glycerol-free trehalose extender and thawed rapidly. by Supplementing trehalose medium with 2 % skim milk and/or coconut milk improved cryosurvival of boar spermatozoa.

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APPENDIX 1

modified Modena Solution

		Mol Wt	mM	g/l
Glucose*	Sigma G8270	180.2	152.61	27.50 g
Sodium citrate $2H_2O$	Ishizu 894193	294.1	23.46	6.90 g
$NaHCO_3$	Ishizu 014-4083	84.0	11.90	1.00 g
EDTA-2Na $2H_2O$*	Sigma E4884	372.2	6.99	2.60 g
TRIS	Sigma T1410	121.1	46.66	5.65 g
Citric acid	Sigma C2404	192.1	15.10	2.90 g
Gentamicin**	Gibco 15710-064		(10mg/ml)	2.5 ml
Water				1000 ml

* Original: Glucose monohydrate (mol.wt. 198.2, 27.5 g/l, 138.7 mM); EDTA-2Na (mol.wt. 336.2, 2.35 g/l, 6.99 mM)

**Hiro original

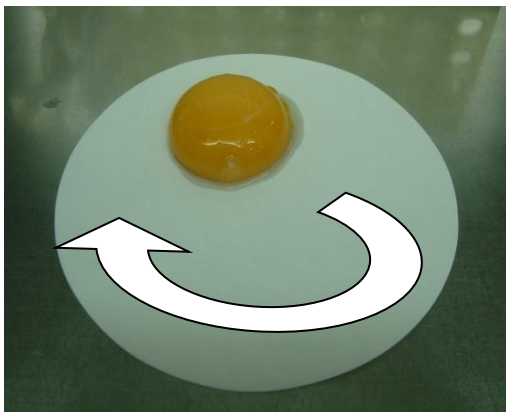
APPENDIX 2

Preparation of 20% egg yolk extender

a. Clean the egg shell with 70% alcohol and paper towel.

b. Break egg manually and separate egg white from egg yolk (an egg white separator can be used if needed); strain egg white into a beaker.

c. Place egg yolk on a filter paper (Whatman circle filters) and carefully roll the yolk on the filter paper to remove the remaining albumin (egg white) and the chalazas.



d. Fold a separate filter in half two times to form a pointy tip; puncture the vitelline (yolk) membrane with the filter tip; allow yolk to drain into a beaker while holding the membrane in the filter papere. Add 10 mL of yolk to 40 mL of mMS in a falcon tube.

f. Centrifuge at 3000 rpm for 15 min at room temperature.

g. Filter the supernatant and discard the precipitate.

APPENDIX 3

Modified PVA-TL-HEPES Medium

Component	Cat #	Mol. wt	Conc (mM)	g/1000 ml	g/2000 ml
NaCl	Fisher S271	58.45	114.00	6.6633	13.3266
KCl	Sigma S5405	74.55	3.20	0.2386	0.4772
$NaHCO_3$	Sigma S5761	84.00	2.00	0.1680	0.3360
NaH_2PO_4	Sigma S5011	120.00	0.34	0.0408	0.0816
Na-lactate*	Sigma L4263	112.10	10.00	1.868 ml	3.736 ml
$MgCl_2 \cdot 6H_2O$	Sigma M2393	203.30	0.50	0.1017	0.2034
$CaCl_2 \cdot 2H_2O$	Sigma C7902	147.00	2.00	0.2940	0.5880
Sorbitol	(Sigma S1876)	182.20	12.00	2.1864	4.3728
HEPES	Sigma H9136	238.30	10.00	2.3830	4.7660
Na pyruvate	Sigma P2256	110.00	0.20	0.0220	0.0440
Gentamicin	Sigma G1264			0.0250	0.0500
Penicillin G	Sigma PEN-K			0.0650	0.1300
Polyvinylalcohol	Sigma P8136	0.1%		1.0000**	

Adjust pH to 7.4.
Filter (0.22 µm) with vaccum filter system (Corning #25942-500).
Store at 4ºC and use within 2 weeks.
*60% syrup
**Dissolve in 100 ml of hot water first.

APPENDIX 4

Porcine Oocyte Medium (POM)

Component	Concentration	
NaCl	108.00 mM	6.3115 g/L
KCl	10.00 mM	0.7456 g/L
KH_2PO_4	0.35 mM	0.0476 g/L
$MgSO_4 \cdot 7H_2O$	0.40 mM	0.0984 g/L
$NaHCO_3$	25.00 mM	2.1003 g/L
Na-pyruvate	0.20 mM	0.0220 g/L
Ca-$(lactate)_2 \cdot 5H_2O$	2.00 mM	0.6166 g/L
Glucose	4.00 mM	0.7200 g/L
L-Glutamine	2.00 mM	0.2922 g/L
Hypotaurine	5.00 mM	0.5455 g/L
L-Cysteine	0.60 mM	0.0727 g/L
BME Amino Acids Solution	total 1.52 mM	2% v/v
MEM Non-Essential Amino Acids Solution	total 0.7 mM	1% v/v
Gentamicin	10 µg/ml	0.0100 g/L
PVA	3 mg/ml	3.0000 g/L

For Hepes-bufferred mPOM, the concentration of NaHCO3 may be reduced to 2 mM and 10 mM HEPES may be added.

APPENDIX 5

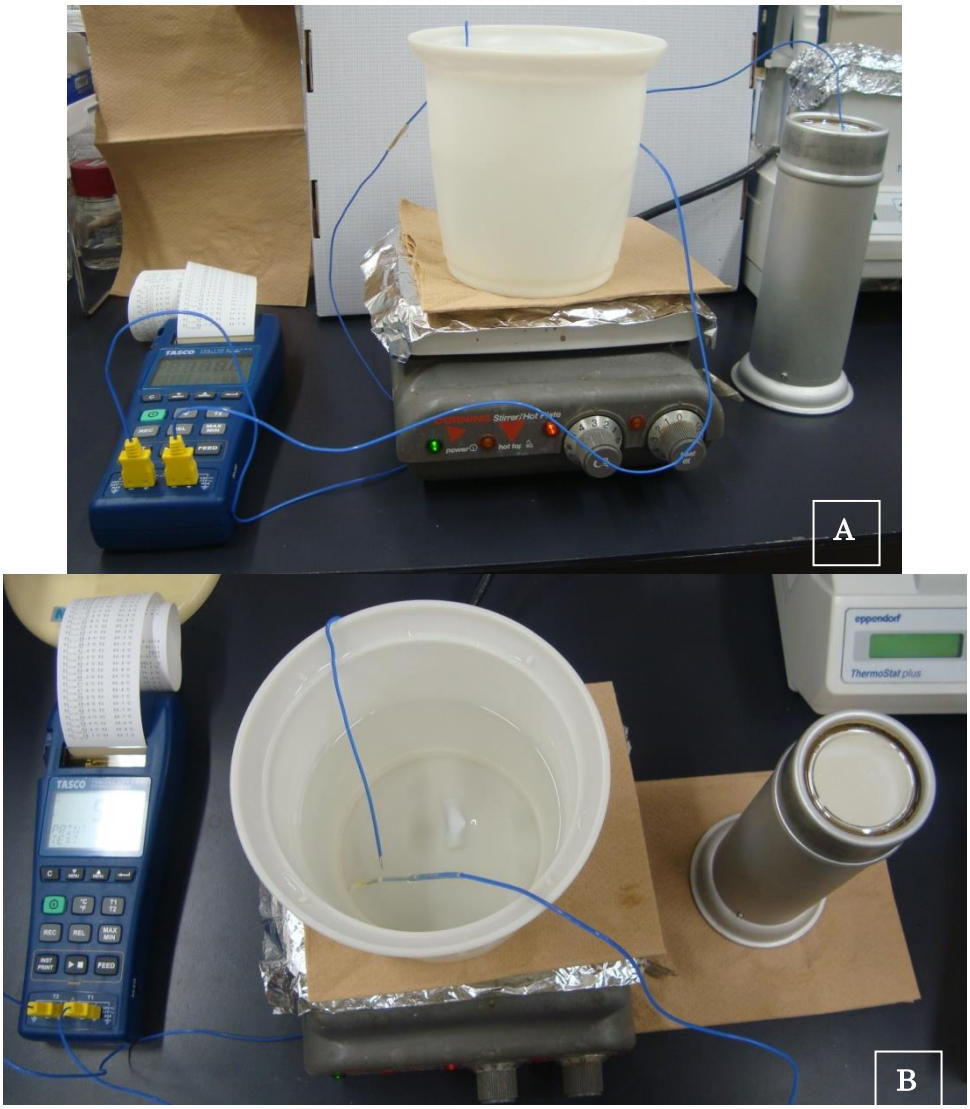
mM199 (Modified Medium199)

Medium 199 with Earle's salts supplemeted with;

Component	Concentration	
D-glucose	3.05 mM	0.5496 g/l
Hemi-Ca lactate	2.92 mM	0.3186 g/l
Sodium pyruvate	0.91 mM	0.1001 g/l
Sorbitol	12.00 mM	2.1864 g/l
Penicillin G potasium		0.0750 g/l
Gentamicin		0.0250 g/l
pH	7.4	

Filter (0.22 µm) with vaccum filter system (Corning #25942-500).
Store at 4ºC.

APPENDIX 6



Experimental setup used to measure the temperature inside the straw using two channel digital record thermometer (TASCO TNA- 140, Japan). (A) One sensor wire (T1) is inside the thawing water bath and the other sensor wire (T2) is inside the straw (filled with extender) immersed in LN_2. (B) After transferring the straw (T2) to water bath. Temperature readings were recorded at every 2 sec in the digital recorder.