# CHAPTER V

# DISCUSSION

The present study demonstrated clearly that cryopreservation potentially induced cell injury, in particular cell membrane integrity as measured by aniline blue-eosin or calcein AM/EthD-1 staining and cell function by means of sperm motility. Although percentage of post-thaw progressive motility has been variable between laboratories, it appeared that there is an effect of individual semen property on cryopreservability of stallion semen. The technical terms of "good-freezer" and "bad-freezer" are therefore, generally applied (Samper, 2001; Loomis and Graham, 2008). To date, a number of strategies have been tested, for example, types of cryoprotectant and also freezing techniques aimed specifically to improve post-thaw semen quality and also sperm longevity. Cold shock has been postulated as a potential cause of cell injuries, and this has been believed to involve the sperm cell's plasma membrane composition especially phospholipids and cholesterol in the plasma membrane (Parks and Graham, 1992; Kampschmidt et al., 1993; Neild et al., 2003).

As previously described, stallion spermatozoa are very susceptible to cold shock (Watson, 1981; Borg et al., 1997; Crockett et al., 2001), and membrane phase transition from liquid to gel state at a certain temperature (ranged from 20 to 0°C) alter the property and functions of sperm plasma membrane as reported in human (Crowe et al., 1989), ram (Holt and North, 1986), boar (Drobnis et al., 1989) and also in stallion (Moran et al., 1992). A number of strategies have been tested in equine semen (Loomis et al., 1983; Janett et al., 2003; Vidament, 2005; Clulow et al., 2007), in order to improve post-thawed sperm quality and pregnancy rate after artificial insemination. This is of importance because the quality of frozen-thawed sperm in this species has been marked variable among the laboratory and individual stallion. Indeed, the rate of temperature reduction during sperm cryopreservation plays a key role in determining the post-thawed sperm quality, principally by the fact that too rapid or too slow freezing curve induces cell damage by intracellular ice formation and high concentration of solutes that increase tremendously within the spermatozoa (Amann and Pickett, 1987).

The controlled rate freezing technique has been used to minimize the cryoinjuries during freezing and thawing (Moran et al., 1992; Borg et al., 1997). In the present study, total motility of the spermatozoa cryopreserved in controlled-rate freezer was higher at 2 hrs and 4 hrs post-thawing compared to spermatozoa frozen in liquid-nitrogen vapors (in a Styrofoam box). Several previous reports suggested that freezing curve of the conventional freezing technique with Styrofoam box is not exactly known and also more variable than controlled rate freezing technique (Palmer and Magistrini, 1992; Clulow et al., 2007). Moreover, the evaporation of the nitrogen vapors in the Styrofoam box can be varied in each time of freezing (Muller, 1982). Moore et al. (2005) reported that stallion spermatozoa can withstand with a wide range of freezing rates ranged between 5°C and 45 °C/min without any significant difference in sperm motility and viability, although it is still essential to further analysis for other sperm parameters such as acrosomal membrane and also the fertility of these frozen-thawed spermatozoa. However, the main advantage of conventional freezing technique is the easy to use in practice, less expensive and is logistically acceptable.

This study also demonstrated the importance of freezing extender, in particular the role of sugar supplementation on the cryopreservability of stallion spermatozoa. Several attempts have been made to improve the quality of frozen-thawed semen thereby increasing pregnancy rates of frozen semen. To date, various extenders have been shown to improve the quality of frozen semen (Loomis et al., 1983; Squires et al., 2004; Vidament, 2005). Stallion spermatozoa are most susceptible to cold shock between 19°C and 8°C (Amann and Pickett, 1987; Crockett et al., 2001) where phase transition of liquid to a gel stage takes place. Spermatozoa undergo rapid changes in response to thermal and osmotic stresses during cooling and cryopreservation that render spermatozoa becoming injured at levels of plasma membrane, mitochondria and also DNA (Gao et al., 1997; Aurich, 2005; Neild et al., 2005). Several types of sugars such as glucose, fructose and trehalose are often added to freezing medium for stallion spermatozoa (Squires et al., 2004; Roasa et al., 2007). These sugars act as non-penetrating cryoprotectant and protect spermatozoa against cryoinjury occurred during

freezing and thawing, principally by reducing the intracellular ice formation (Amann and Pickett, 1987). However, the effects of sugar in protecting sperm viability depend also on other factors such as storage temperature (Arns et al.,1987), molecular weight of sugar (Glander and Dettmer, 1978) and the type of buffer used (Garcia and Graham, 1989). Sugars are also energy substrates during sperm incubation (Yildiz et al., 2000; William and Ford, 2001) and have also been shown to interact with phospholipids and proteins, thereby altering their phase transition and hydration state (Aboagla and Terada, 2006) and stabilizing the plasma membrane (Hincha et al., 2006; Aisen et al., 2000). Among monosaccharide sugars used for stallion semen, glucose is most supplemented in the freezing extender, while the knowledge on interaction of sugar types and sperm quality during freezing and thawing is obscure. Owing that aniosmotic condition plays a central role in determining the sperm quality and the osmotic tolerance of stallion spermatozoa appears to be limited (Wessel and Ball, 2004), we therefore only examined the effects of the three types of monosaccharide sugars (glucose, fructose and sorbitol) as they have similar molecular weights rather than other types of sugar in order to minimize the effect of different osmotic stress.

In the present study, sorbitol was substituted to glucose and fructose in the semen freezing extender. The study clearly demonstrated that sorbitol improved equine sperm quality post-thaw, in terms of motility, viability, plasma membrane functionality (HOS test), acrosome integrity and longevity compared to glucose and fructose as determined at 10 min, 2 hrs and 4 hrs post-thawing. Many reports have shown that sugars protect sperm against the damage during cryopreservation (Arns et al., 1987; Alvarez and Storey, 1993; Storey et al., 1998; Aboagla and Terada, 2006; Fernández-Santos et al., 2007). Sorbitol has been demonstrated to improve cryotolerant in microorganisms (Hubalek, 2003; Suree Nanasombat and Niracha Sriwong, 2007) and also sperm of bull (Garcia and Graham, 1989), man (Alvarez and Storey, 1993) and ram (Molinia et al., 1994). Although it remains unclear regarding the protective effects of

sorbitol against cryoinjury during cryopreservation, a wide range of mechanisms has been postulated. Sorbitol is a sugar alcohol that has unique molecules, in which the aldehyde group is replaced by an alcohol group (Garcia and Graham, 1989) and this is structurally similar to the glycerol (Nagase and Tomizuka, 1968). Furthermore, sorbitol also has ability to osmostabilize the sperm plasma membrane from oxidative stress conditions (Katrina et al., 1999) and acts as an alterative energy source for sperm motility and signal transduction in proposed metabolic pathway (King and Mann, 1959; Storey, 2008). Jain and Roy (2009) indicated that during cryopreservation, proteins structure are interaction with sugars molecule to stabilize the cell plasma membrane, that has a hydrogen bonding together and can induce protein unfolding, while cells loss water, thereby preventing cell dehydration. Sorbitol, unlike glucose and fructose, is a linear molecule similar to glycerol (Burg, 1995) and should be converted into fructose by sorbitol dehydrogenase enzyme (SORD) before it can be penetrate cell's plasma membrane (Frenette et al., 2006) and is used as an energy source for sperm glycolysis and oxidative phosphorylation (King and Mann, 1959; Storey, 2008). In addition, it has been known that sorbitol is naturally presented in seminal plasma (Mann, 1975) and female reproductive tract fluid (Frenette et al., 2004 and 2006). When semen is exposed to this fluid, sperm will become hyperactivated, theoretically because sorbitol increases the protein tyrosine phosphorylation (Cao et al., 2009).

Previous reported by King and Mann (1959) indicated that in anaerobic condition, fructose, but not sorbitol, is broken down glycolytically to lactic acid that may impair the sperm function. These results suggested that mechanism and permeability of stallion spermatozoa to sorbitol, glucose, and fructose may be different. Riedel (1972) reported that sorbitol and trehalose had 18.7% and 16.7% unfrozen water of total water during deep freezing. These may be postulated that the medium which have high amount of unfrozen water may lethal to spermatozoa, occurred from ice crystal forming during freezing and thawing process. In addition, because the sorbitol has its molecular

form similar to glycerol, it is therefore of importance to investigate the interaction of concentration of glycerol and sorbitol, since some of stallion spermatozoa are sensitive to the toxicity of glycerol (Alvarenga et al., 2005). It might be possible to replace high concentration of glycerol with low-toxic sorbitol. For instance, trehalose helps to diminish the acrosomal damage and reduce toxicity of glycerol during cryopreservation of boar (Gutiérrez-Pérez et al., 2009) and ram (Aisen et al., 2002) spermatozoa, in terms of maintaining sperm motility and viability. We suggested that sorbitol could well influence osmotic properties, affects membrane permeability and protects equine spermatozoa against cold shock during cooling and thawing process in combination with glycerol (Devireddy et al., 2002; Wessel and Ball, 2004).

In this study, sorbitol preserved quality of frozen-thawed equine spermatozoa better than the other 2 sugars, in terms of motility viability acrosome integrity and plasma membrane integrity. Moreover, the results showed that sorbitol-based extender gave significantly higher in the percentage of plasma membrane functionality (positive sHOS test) than the others 2 sugars. The sHOS test has been proposed to relate with fertility (Neild et al., 1999 and 2000), as well as, the results from *in vitro* study that stallion sperm cryopreserved with sorbitol-based extender gave similar result of longevity test to glucose-based extender. Pregnancies (50% pregnancy rate) obtained in this study indicated convincingly that stallion spermatozoa cryopreserved with sorbitol-based semen extender attained their viability and fertilizability *in vivo*, although the number of inseminated mares could have been increased.

Although this study demonstrated that sorbitol can potentially be used in freezing extender, however, it is necessary to observe the optimal concentration of sorbitol added into freezing extender and also to study the interaction of sorbitol to sperm plasma membrane in order to decrease the toxicity and concentration of glycerol.

## Conclusions

*Experimental I* and *II* demonstrate that stallion spermatozoa can be successfully cryopreserved using either conventional or controlled-rate freezing techniques. Although sperm from individual stallion demonstrate different freezability, controlled rate freezing technique appears to improve sperm motility and viability post-thawing when compared to conventional freezing (using Styrofoam box). Cryopreservation is often associated with poor semen quality. Improving freezing extenders for stallion's semen is therefore essential for clinical aspects since overall pregnancy rate of mares inseminated with frozen-thawed semen remains variable. Sugar provides a microenvironment that protects sperm from cryoinjury. Sorbitol can be alternatively used as cryoprotective agent that has protective ability better than glucose and fructose, although the interaction of sorbitol and sperm plasma membrane remains to be elucidated. This is the first report that sorbitol can be used in freezing medium for stallion semen, while semen quality is improved beyond the glucose-based semen extender especially at 10 min post-thawing used worldwide.