CHAPTER II

LITERATURE REVIEW

Male reproductive system are structurally composed of testes, reproductive ducts (epididymis, vas deferens, ejaculatory duct, urethra), accessory glands (seminal vesicles, prostate gland, bulbourethral glands) and other supporting structures (scrotum, penis, spermatic cords). The testes have two main functions that are the productions of male gametes by spermatogenesis, and production of male sex hormones. In horse, spermatogenesis begins at puberty (18-24 months) and continues throughout the adult life (Clay and Clay, 1992). At the onset of puberty, the testes secrete large amounts of testosterone, which stimulates growth of the testes and initiates maturation of the seminiferous tubules.

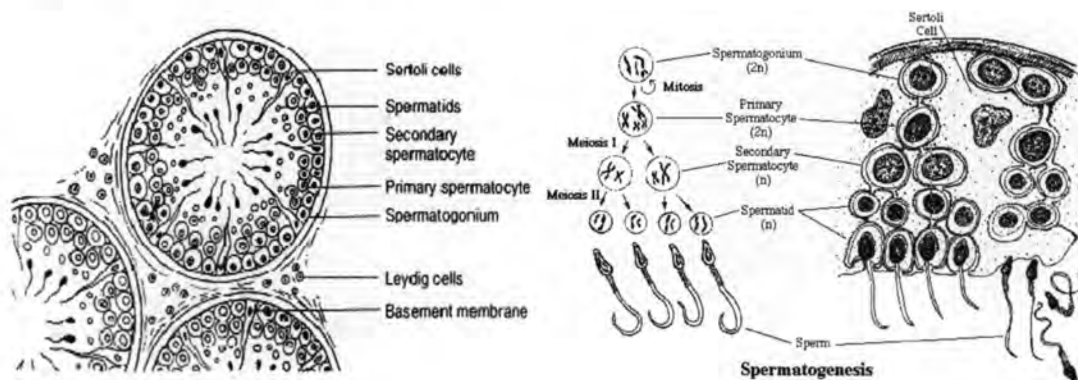

Figure 1 A cross-section view through a seminiferous tubule within the stallion's testis, illustrating the gradual division of spermatogonia to spermatozoa (Davies Morel, 2003)

The development of male gametes from spermatogonia is composed of two serial steps of sperm maturation: spermatocytogenesis and spermiogenesis. Spermatocytogenesis is a process where spermatogonia located at the basement membrane of seminiferous tubule undergo a marked modification to become spermatid. Each spermatogonium is attached to an adjacent sertoli cells by specialized membrane tight junctions. Spermatogonia undergo mitosis to form primary spermatocytes. Each primary spermatocyte posses the first meiosis to form two secondary spermatocytes. Each secondary spermatocyte undergoes a second meiosis to form two spermatids (Figure 1). As each division takes place, daughter cell migrates closer to the lumen of

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the seminiferous tubule, so that spermatids are immediately adjacent to the lumen. The process that converts spermatids into mature sperm (spermatozoa) is called spermiogenesis. The duration of spermatogenic cycle in horse can be divided into 3 phases: spermatocytogenesis (19.4 days), meiosis (19.0 days) and spermiogenesis (18.6 days). The whole cycle of development takes 57 days and occurs in a wave-like fashion. The mean daily sperm production of mature stallion is in the order of 7-8 x 10^9 sperm, and it takes 8-10 days for sperm to pass from the testis to the exterior (Johnson et al., 1997; Davies Morel, 1999; 2003).

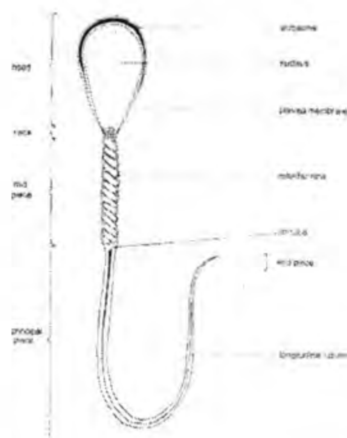


Figure 2 Structural details of stallion spermatozoon (Davies Morel, 2003)

The head of a spermatozoon contains the condensed nucleus and is capped by an apical vesicle (acrosome) filled with hydrolytic enzymes (Figure 2). The acrosome plays an important role in fertilization. The mid-piece contains large helical mitochondria that generate the energy. The tail contains microtubules. Sperm plasma membrane, composes of lipids and proteins arranged as a bilayer with the hydrophilic ends of the lipids externally and the hydrophobic fatty chains internally. Proteins are intermingled with the lipids. The predominant lipids of stallion spermatozoa are phospholipids and cholesterol at ratio 0.64:0.36. Following spermatogenesis, spermatozoa are then released into the lumen of the seminiferous tubule and transported to the epididymis where they are stored and final maturation of sperm takes place. During ejaculation, the spermatozoa are propelled through the vas deferens and urethra and are mixed with secretions from the seminal vesicles, prostate and bulbourethral glands. Composition of stallion semen was described by Mann (1975) as shown in Table 1.

Table 1. Composition of stallion semen (adapted from Mann, 1975)

Components	concentration (mg/ml)	Components	concentration (mg/ml)
Protein	1.2-12	Sodium	2.57
Fructose	0.02-0.08	Potassium	1.03
Glucose	0.82	Phosphorus	0.02-0.07
Sorbitol	0.2-0.6	Calcium	0.26
Citric acid	0.08-0.53	Magnesium	0.09
Inositol	0.19-0.47	Chloride	4.48
Glycerylphos-		Ergothioneine	0.03-1.1
Phorylcholine	0.4-3.8		

Artificial insemination (AI) technique was first established in horse in 1322, but overall success of AI remains poor especially when frozen-thawed semen is used. The first success in artificial insemination of cryopreserved equine semen was reported in 1957 (Barker and Gandier, 1957). Stallions have shown a particularly high degree of individual variation with respect to the cryopreservability. It has been estimated that about 20% of stallions produce semen that freeze well, 60% freeze acceptably and 20% freeze poorly (Vidament et al., 1997). To date, the mechanisms by which the differences in cryopreservability between different individuals stallion have not yet to be clear. It has been postulated to involve genetic in origin, genetic selection of stallions and also non-genetic reasons such as feeding and season.

Pregnancy rates of mares bred with frozen–thawed semen are highly variable (range 0–100% per-cycle; Samper and Morris, 1998). Loomis (2001) reported that per-cycle and per-season pregnancy rates were 53.5 and 71.9%, respectively. Similar results were reported by Vidament (2005) based on post-thawed motility of frozen–thawed semen, per-cycle pregnancy rates ranged from 43 to 52%. These studies are the results of many inseminations performed by several individuals, thereby lacking in intensive quality control. It is possible in highly managed mare herds to achieve

higher per-cycle pregnancy rates. Per-cycle pregnancy rates have been variable in principle by several factors such as mare management, the number and concentration of progressively motile spermatozoa, the timing of insemination, and also site of semen deposition. In addition, protocols from different laboratories, methods for freezing stallion semen and instructions for inseminations of mares with frozen–thawed semen have been dissimilar as a result of highly variable sperm quality after freezing and thawing (Metcalf, 1995; Samper and Morris, 1998). Different procedures and freezing techniques for stallion semen are summarized in Table 2.

Table 2. Processing for cryopreservation stallion semen

Protocols	*Primary extender Centrifugation*	*Freezing extender (%) Glycerol*	*Freezing and Thawing techniques*
Loomis et al., (1983)	Glucose-EDTA 650 g, 15 min	Lactose-EY-EDTA 5%	10 min, LN_2 vapour 38 °C, 30 sec.
Vidament, (2005)	UHT skim milk 600 g, 10 min	INRA-82-EY 2.5%	Controlled-rate (40-60°C /min) 37 °C, 30 sec.
Clulow et al., (2006)	Kenney extender 400 g, 10 min	L-EDTA-Duck 4 %	7 min, N_2 vapor 37 °C, 30 sec.
Janett et al., (2003)	INRA82+20%EY 1000 g, 2 min	Lactose-EY 5%	Controlled-rate (30-60°C /min) 37 °C, 30 sec.
Häard and Häard, (1991)	Citrate-EDTA 400 g, 15 min	Lactose-EDTA 5%	Controlled(10°C/min) 75°C, 7sec, then 35 °C 10-30 sec

Current freezing protocols for stallion semen involve a two-step dilution procedure in which semen is first diluted with a primary extender, centrifuged and then diluted a second time prior to freezing in an extender containing a cryoprotectant. The first dilution contains saline/sugar extenders or skim milk extenders used to dilute fresh semen. The dilution rate is either 1:1 or 1:3. The success of centrifugation depends on duration (10–15 min) and centrifugation force (350-700x g). Moore et al. (2005) reported

the deleterious effect of seminal plasma on stallion spermatozoa during cryopreservation, while retention of 5–20% seminal plasma in the suspension after centrifugation improved cryosurvival of stallion spermatozoa.

Principle of semen cryopreservation

During the cryopreservation, equine spermatozoa are exposed to a rapid change in osmotic pressure. Equine spermatozoa are highly susceptible to cryo-damage in particular plasma membrane, organelles and also sperm functions (Aurich, 2005). During cryopreservation, plasma membrane of spermatozoa is prone to damage and often results in the irreversible loss of motility and/or fertilizing capability (Tischner, 1979; Lagares et al., 2000). The primary causes of cryodamage induced plasma membrane disruption are thermal, mechanical, chemical and osmotic stress imposed upon the membrane (Watson, 1995). This damage can cause cellular dehydration or intracellular ice formation within the cell (Figure 3). During freezing process (-15 to -60°C), ice typically forms in the extracellular compartment, and thus the plasma membrane acts as a barrier to preventing intracellular ice crystal growth by changes from a liquid crystalline stage to a gel stage. The increase in osmotic pressure in environment can cause intracellular water to diffuse out of the cell, dehydration in sperm cell. If cooling rates are too fast, intracellular ice crystal increasingly forms and leads to the cellular injury and death (Amann and Pickett, 1987; Neild et al., 2003). By contrast, too slow freezing rate induces severe dehydration of sperm cells and also results in cell death. Cryopreservation of stallion sperm therefore requires an optimal procedure during freezing and thawing.

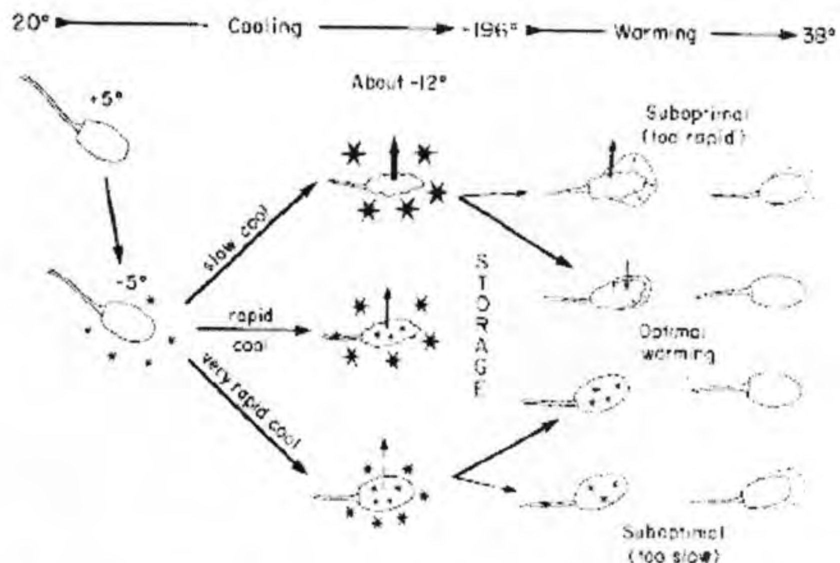


Figure 3 Diagram representing the interaction among physical changes in equine spermatozoa and sperm viability during freezing (Amann and Pickett, 1987)

To minimize cryoinjury during freezing and thawing, cryoprotectants are usually added into freezing medium. The cryoprotectants often used for freezing semen can be broadly classified in 2 major groups that are penetrating (glycerol, ethylene glycol, etc.) and non-penetrating cryoprotectants (skim milk, egg yolk, sugars, etc). They potentially protect spermatozoa against rapid cooling rates or cold shock (Noiles et al., 1992; Squires et al., 2004). In addition, reduction of storage temperature from body temperature (~37°C) to 5°C substantially reduce cell metabolism to approximately 10% of the original level, while their metabolism essentially ceases when the sperm cells are frozen at sufficiently low temperatures (Cochran et al., 1984; Bedford et al., 1995).

Factors affecting the cryopreservability of stallion semen

Cryopreservation of stallion spermatozoa causes physical and chemical stress that result in poor cryosurvival rate of spermatozoa (Jasko et al., 1992; Loomis and Squires, 2005). Several factors have been postulated to impair the membrane integrity, structure and function of the spermatozoa such as lipid phase transition, intracellular ice formation and osmotic-induced excessive water influxes (Amann and Pickett, 1987; Samper et al., 1991). Differences in fatty acid composition and sterol are associated with

its tolerance to cold shock and freezing-thawing process. This can be varied among species and individual stallion (Loomis and Graham, 2008).

Because of the importance of breeding soundness examination, semen quality before and after freezing is fairly important. Indeed, techniques for semen collection such as frequency and intervals also play an essential role for obtaining good quality semen prior to cryopreservation (Samper, 1991). Siemer et al. (2004) reported that a double semen collection at 1 h apart and with a 48 h interval gave as good or better results than single daily collection. However, Pickett et al. (1985) contradictorily reported that double collection schedules did not affect on sperm motility and total number of sperm. In practice, it is advisable to collect the stallion semen twice daily and every other day as the most cost-effective procedure. However, this procedure may affect the semen quality in some stallions. The optimal semen collection frequencies and intervals for individual stud farms are often determined at the basis of a compromise between different factors.

In equine industry, stallions are not selected by ability of sperm to withstand the standard cryopreservation protocols as in bulls and rams (Loomis and Graham, 2008) because stallion's semen quality is largely variable among individual stallion. In 1987, Amann and Pickett reported that 38% of 40 stallions produced spermatozoa with average post-thaw motility of 80–100% compared to initial pre-freeze values. Similar results, Muller, (1987) was evaluated from 341 stallions and classified 35% as good freezers base on >60% initial motility, >70% normal morphology and ≥30% post-thaw motility; 25% were considered "average" and 40% were "poor" freezers. Amann and Picket (1987) estimated that around 25% of stallions could achieve "acceptable" pregnancy rates with frozen semen, while 30% would yield extremely low pregnancy rates. Even when ejaculates were selected for post-thaw progressive motility ≥35%, poor pregnancy rates after insemination of mares can be ranged from 8 to 61% per estrous cycle. Many reasons have been suggested such as inherent differences, sperm biochemistry and metabolism between stallions (Amann and Pickett, 1987; Loomis and Graham, 2008). The membrane cholesterol to phospholipid ratios differ markedly between stallions and ejaculations. The membranes containing higher cholesterol:phospholipid molar ratios (0.9, 0.3, 0.45, 0.36, 0.26 in human, rooster, bull,

stallion and boar sperm, respectively) exhibits lower transition temperatures. Thus, overall lipid composition of a cell appears to affect its ability to survive cooling damage.

Seminal plasma is generally removed from stallion spermatozoa before cryopreservation process to prevent a deleterious effect on post thawed motility (Amann and Pickett, 1987; Aurich et al., 1996). However, seminal plasma has been shown to positive effect for cryopreserved stallion spermatozoa in several studies (Pickett and Amann, 1987; Katila et al., 2002). Aurich et al. (1996) reported that post-thawed semen quality of stallion having poor semen freezability (post-thaw motility <20%) was improved when the seminal plasma was removed and then replaced with seminal plasma from a good freezer stallion (>30% motile sperm after thawing). In addition, Katila et al. (2002) also reported a beneficial effect on sperm cryosurvival when 20% seminal plasma was added back to sperm after centrifugation, and then incubated at 5 °C for 2 h prior to freezing. Moore et al. (2005) reported that stallion spermatozoa incubated with 5% seminal plasma had higher motile sperm after thawing, than sperm incubated with 20% seminal plasma for minimum of 2 h at 5°C before freezing. These results indicate that seminal plasma is either detrimental to sperm cryosurvival or beneficial to sperm cryosurvival.

Centrifugation/glycerol-adding is one of the steps that affect post-thaw sperm quality (Vidament et al., 2000). This step is also necessary for addition of freezing extender and adjust sperm concentration prior cryopreservation (Amann and Pickett, 1987). Changing temperature and time of centrifugation/glycerol-addition has significantly related with post-thaw motility and fertility. Vidament et al. (2000) reported that the post-thaw motility was improved when centrifugation/glycerol-addition was performed at 22°C instead of 4°C. And also the post-thaw motility was higher when cooling from 37°C to 4°C was done within 1 h compared to 4 h. It has been proposed that, during centrifugation, spermatozoa were damaged when they are pelleted. Membranes are still in liquid phase at 22°C and could therefore undergo less damage during centrifugation at 22°C than at 4°C (Cochran et al., 1984).

In addition to the physical changes occurred during cryopreservation process, cryoprotectant such as glycerol, ethylene glycol, dimethyl sulfoxide, methyl formamide or dimethyl formamide also play a critical role in determining the sperm quality after

freezing and thawing (Squires et al., 2004). Glycerol is a commonly used as permeable cryoprotectant in stallion semen cryopreservation (Loomis et al., 1983; Vidament, 2005; Clulow et al., 2007). The optimum concentration of glycerol is varying from 2.5-5% (Table 2.). The composition of the freezing extender may influence the rate of the cooling phase required before freezing. Essentially the semen should cool slowly from room temperature to 4 ^{o}C before storage at 4 ^{o}C for equilibration at least 60 minutes, the semen are then loaded into 0.5 straws at 4 ^{o}C (Vidament et al., 2000).

As previously stated, it becomes clear that many factors are contributed to the success of semen cryopreservation. Although several strategies have been tested to improve the freezability of stallion semen, the overall success of semen cryopreservation, to date, remains relatively poor when compared to other domestic animals such as bulls. Thus, it is essential to further investigate the factors involving the cryopreservability of spermatozoa and also to generate optimal procedures for freezing stallion semen.

Stress occurred during cryopreservation

Cold shock is detrimental for movement pattern of sperm, premature loss of motility, reduction of energy production, increase of membrane permeability and loss of intracellular molecules and ions. Stallion spermatozoa are most susceptible to cold shock between 19°C and 8°C (Amann and Pickett, 1987; Crockett et al., 2001). It was occurs when cells exposed to low temperatures without freezing. The degree of damage of cold shock is dependent upon rate of cooling and final temperature to which spermatozoa are cooled. In contrast to rapid cooling, rapid warming to 37°C is rarely detrimental. The susceptibility of the spermatozoa against cold shock is associated with the lipid composition of the membrane (Watson, 2000). Bull spermatozoa can be cryopreserved successfully because it contains high levels of DHA (Kampschmidt et al., 1993). In contrast, DHA levels of stallion spermatozoa are lower than bull spermatozoa. Cholesterol / phospholipids ratio in the stallion spermatozoa is low (Aurich, 2005). Compared to other species such as boar, the sperm plasma membrane of stallions has a relatively high cholesterol content of about 37%. However, the content of cholesterol does not only differ between species, but also between individual males within a species

and between individual ejaculates of a single male (Gadella et al., 2001). In addition to cold shock, intracellular oxidative stress also plays a key role in sperm viability because this condition induced by oxygen and oxygen-derived oxidants, commonly known as Reactive Oxygen Species (ROS) cause an increasing rate of cellular damage (Sikka et al., 1995). ROS are highly reactive oxidizing agents belonging to the class of free-radicals. The mechanism of losing sperm function due to oxidative stress involves excessive generation of ROS. The main resources of ROS production in semen are leukocytes and defective or damaged spermatozoa. This result in decrease sperm motility, decrease sperm viability and increase midpiece defects (Neild et al., 2005). Finally, osmotic stress occurs when cell volume changes during adding and removing the cryoprotectants. Although cryoprotectant removal appears to be more damaging than addition, the extent of damage incurred (due to cell swelling) depends upon both the size and shape of the sperm and the permeability of the membrane to the cryoprotectant. Equine spermatozoa appear to have a limited osmotic tolerance similar to boar spermatozoa (Gilmore et al., 1998). Total motility of equine spermatozoa reduced to less than 50% at iso-osmolar ±100 mOsm and declined to less than 10% at 100 and 500 mOsm. Although motility declined rapidly under hyperosmotic conditions, there was no change in plasma membrane integrity through 600 mOsm. The reduction in sperm viability appeared linear in the range of 100–325 mOsm, which is well above the critical osmolality of 47 mOsm estimated previously for equine spermatozoa (Noiles et al, 1992). In a recent study, the volume of equine spermatozoa increased linearly on the basis of spermatocrit measurements between 300 to 100 mOsm, and there was a rapid loss of viable spermatozoa between 100 and 50 mOsm (Lagares et al., 2000). The critical osmolality of equine spermatozoa is approximately 50 mOsm.

Polyol pathway in mammalian epididymis

Mammalian spermatozoa are produced in seminiferous tubule, and they later on have to transit along the epididymis for their maturity. Frenette and Sullivan (2001) reported that the epididymal epithelium secretes in a complex patterns of proteins which are associated with an apocrine mode of small membranous vesicles, called epididymosomes. Within the epididymal compartment, specified proteins of epididymosomes are transferred to define sperm surface domains (Frenette et al.,

2002). In bovine semen, aldose reductase is one of the major proteins associated with epididymosomes. Aldose reductase (AR) uses nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor to reduce aldose and ketoses (Frenette et al., 2004). This enzyme is involved in the reduction of glucose to sorbitol in the polyol pathway (Figure 4). The next step of this sugar pathway involves sorbitol dehydrogenase (SORD), which oxidizes sorbitol by using nicotinamide adenine dinucleotide (NAD+) as an electron acceptor to produce fructose (Oates, 2002; Pruneda et al., 2006). It well known that glucose and fructose can be energy sources for spermatozoa from a wide range of species, including the stallion (Mann, 1975; Leese et al., 1981) and sorbitol has been shown to be oxidized by ovine spermatozoa *in vitro* (King and Mann, 1959). Both glucose and sorbitol are present in stallion semen, although the mechanism or pathway for utilization of sorbitol by stallion sperm remains unclear.

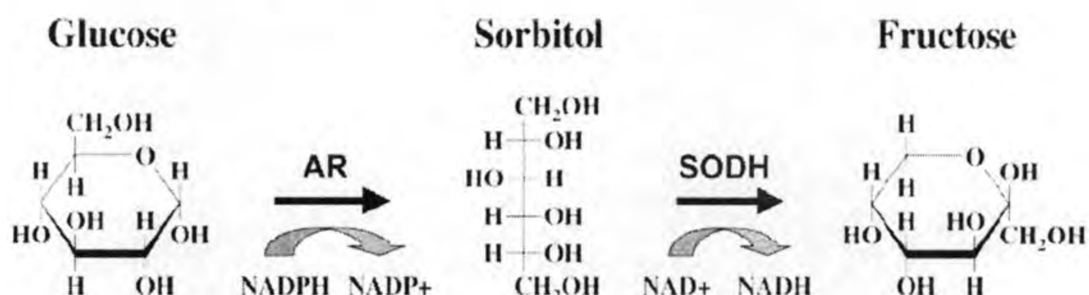
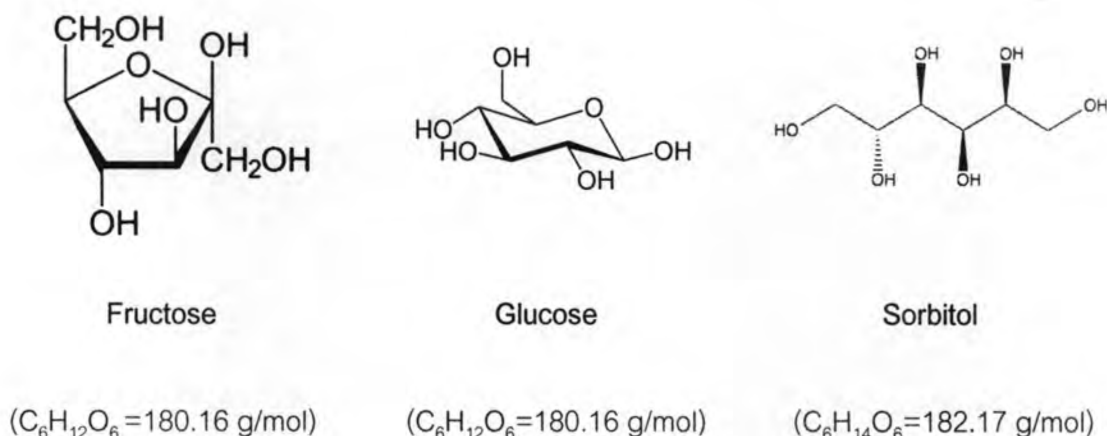


Figure 4 Polyol pathways. Aldose reductase (AR); Sorbitol dehydrogenase (SODH); Nicotinamide adenine dinucleotide (NAD+); and Nicotinamide adenine dinucleotide phosphate (NADPH). (Frenette et al., 2006)

Importance of glucose, fructose and sorbitol in stallion semen

Mammalian spermatozoa can obtain energy through glycolysis and the Krebs cycle by consumption of monosaccharides, such as glucose, fructose and mannose (Mann, 1975), or other compound such as glycerol (Jones et al., 1992). Although all species can use most monosaccharides, the specific manner of metabolism varies within species (Rikmenspoel and Caputo, 1966). Accordingly, stallion spermatozoa are able to use glucose and some fructose, although the formation of metabolic intermediaries such as glucose 6-phosphate or ATP is very different between the two

hexoses. This implies that the effects of other monosaccharides (fructose, glucose and sorbitol) on the stallion spermatozoa qualities may be different.



($C_6H_{12}O_6$ =180.16 g/mol) ($C_6H_{12}O_6$=180.16 g/mol) ($C_6H_{14}O_6$=182.17 g/mol)

Figure 5 Structure and molecular weight of each monosaccharide sugar (Palmer, 2008)

Sugars are often used as non-penetrating cryoprotectants in combination with penetrating cryoprotectants. Differences in the cryoprotectant abilities of different sugars have been demonstrated for bulls and rams (Arns et al., 1987). Sugars are naturally present in seminal plasma in varying quantities (see Table 1 for stallion semen compositions). Fructose and sorbitol are among the most widespread carbohydrates in the seminal plasma of rams and bulls. In this species, the glucose and sorbitol are produced in the seminal vesicles and is expressed by the blood glucose in the seminal plasma. The effect of these compounds, especially sorbitol, on sperm survival before and after freezing has not been reported in this species.

Glucose is the major glycolysable sugar found in stallion semen (concentration 0.82 mg/ml; Mann, 1975) and is a major energy source for stallion spermatozoa, unlikely the bull in which fructose is the major energy source (Garcia and Graham, 1989). The presence of significant concentrations of sorbitol and lactic acid indicates that some of enzymes necessary for the conversion of glucose to fructose are present within the stallion reproductive tract. However, stallion spermatozoa show limited ability to use fructose as an energy source under anaerobic conditions.

Sorbitol is a monosaccharide sugar alcohol that is also presented in equine semen (Table 1). It is a linear molecule, which makes it difficult to penetrate eukaryotic cell plasma membranes (Burg, 1995). Within the semen, sperm plasma membrane interacts with SORD and will be able to convert sorbitol into fructose. As a consequence, the transporter (glucose-transporter 5) could move sorbitol-derived fructose into sperm and could then be used as an energy source for glycolysis and oxidative phosphorylation (Glander and Dettmer, 1978). Sorbitol is formulated by reduction of glucose changing the aldehyde group to an additional hydroxyl group. It is a normal intermediary product of the conversion of glucose to fructose. Its function is to ensure an equilibrium in osmotic pressure between seminal plasma and spermatozoa (Mann, 1975) and it was used as organic stabilizer of the cells (Yu and Chang, 1987). Garcia and Graham, 1989 reported the benefits of sorbitol in freezing extender for sperm cell motility of frozen semen in bull. Interestingly, many organisms such as bacteria, yeast, and fungal, sorbitol acts as an osmostabilizing agent to protect the plasma membrane from oxidative stress conditions (Katrina et al., 1999). These findings suggested that sorbitol would have beneficial effect for maintenance of plasma membrane integrity of stallion spermatozoa under stress conditions.

To date, comparative study regarding the effect of freezing technique and sugar types (glucose, fructose and sorbitol) has not been previously examined on maintaining sperm quality after cryopreservation. This study aimed to compare the effect of freezing techniques (Conventional versus Controlled-rate freezer: *experiment 1*) and to study the effect of freezing extenders supplemented with different monosaccharide sugar (glucose, fructose, sorbitol) (*experiment 2*) on the post-thawed semen quality.

Objectives

1. To compare the effect of freezing techniques (conventional versus controlled-rate freezer) on post-thawed sperm motility and viability
2. To study the influence of freezing extenders supplemented with different sugars on post-thawed stallion semen quality

Expected output

1. Gain more knowledge regarding responses of stallion sperm during cryopreservation process

2. Optimization of freezing technique and freezing extender for stallion semen

3. Obtain more basic knowledge about stallion semen cryopreservation technique for further development of stallion semen cryopreservation

4. One international publication