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Effect of Cryopreservation on Sperm Quality and Fertility

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1. Introduction

Geographical barriers to breeding animals have long been reduced because of possibilities of semen transportation. In modern cattle breeding, where artificial insemination (AI) is the most widely applied tool facilitating extensive utilization of frozen semen from genetically superior sires, cryopreservation has been an invaluable technique. In order to extend the time span of the viability of spermatozoa, their metabolic rate has to be slowed down thereby reducing the rate at which substrates are used and toxins are produced. As a general rule cooling of spermatozoa is the simplest method that can successfully depress spermatozoal metabolic rate and therefore, prolong sperm survival. The use of carbon dioxide and other metabolic inhibitors like proteinase inhibitors are also known to produce a similar but less successful effect (Colenbrander *et al,* 2003; Cremades *et al,* 2005; Curry *et al,* 2000).

Semen stored after cooling to 5-8°C will survive for 24-48 h without a significant decline in motility and even up to 96 h without a significant drop in fertilization rates. Though chilling semen provides an efficient and successful means of short-term storage, it has yet some adverse effects on the spermatozoa manifested as a depression in viability rate, structural integrity, depressed motility and conception rates (Batellier *et al,* 2001; Medeiros *et al,* 2002; Watson, 2000). Preferably, the spermatozoa of many species can now be stored indefinitely at -196°C in liquid nitrogen for future use, while still retaining acceptable fertilization rates postthaw. The techniques for successful cryopreservation of spermatozoa have also slowly progressed over the past several decades (Hammerstedt *et al,* 1990) and are now fairly standardized. However, cryopreservation is also known to be detrimental to sperm function and fertility even with the most up to date techniques. Generally, sperm viability is decreased by 50%, whereas fertilizing capacity is affected by a factor of sevenfold after cryopreservation (Lessard *et al,* 2000).

The effects of cryopreservation on sperm function and fertility have been widely studied, particularly in bovine. Various sperm organelles have been known to be affected due to the detrimental effects of cryopreservation. Induction of premature acrosomal reaction, altered mitochondrial function, reduction of motility and failure of chromatin decondensation, all of which influence the viability and fertility of the sperm cells have been reported by different authors (Chaveiro *et al.* 2006; Cooter *et al,* 2005; Watson, 2000; Wongtawan *et al,* 2006). Cooling is a major stressor, as a result of which membrane bound phospholipids reorient themselves into a different configuration that disrupt membrane function and permeability

(Amman and Graham, 1993; Lessard *et al,* 2000). The stress response shown by spermatozoa as a reaction to a drop in temperature is referred as cold shock. Generally, cold shock damage manifests itself as a decline in cell metabolism, altered membrane permeability, loss of intracellular components, irreversible loss of spermatozoan motility and an increase in the number of dead spermatozoa. The damage to the cellular membranes is of most significance because it has a carry-over effect on other cellular structures and functions.

The severity of the cold shock depends upon the final temperature and the rate of temperature drop. The cellular damage resulting from cooling or freezing affecting both the structure and function of the cells can be categorized as direct or indirect (Amann and Pickett, 1987; Watson, 1990). Direct damage is more definable and is the type usually associated with cold shock evident shortly after the drop in temperature and is affected by the rate of cooling. Indirect or latent damage is more difficult to quantify and may not be initially apparent; it tends not to be dependent upon the rate of cooling. Addition of cryoprotectant agent (CPA) such as glycerol, or of other components such as egg yolk, milk, bovine serum albumin, polyvinyl alcohol and liposomes in extenders have been used in an attempt to providing some protection to spermatozoa and minimizing the adverse effects of cryopreservation (Katila, 1997). Direct cellular damage is irreversible and is usually apparent at later stage during postthaw analysis particularly in samples that are to rapidly frozen (Bildeau *et al,* 2000; Pommer *et al,* 2002; Samper, 2001). Hence, the ability to predict postthaw sperm quality and fertility from a routine sperm function assay would be greatly beneficial to the success of cryopreservation.

2. Cryopreservation of semen

In order to realize many of the potential advantages of AI, long-term storage of semen is necessary. This is only possible by freezing, a system which halts the metabolic processes of the spermatozoa, allowing indefinite storage without a significant loss of fertility. The discovery of the cryoprotectant properties of glycerol has made cryopreservation possible. Among the many benefits resulting from the process of cryopreservation are the genetic improvement of important farm species and control of diseases affecting them both of which have a highly significant impact on the sustainability of the agri-food industry (Bailey *et al,* 2000). Cryopreservation has been widely used in the modern cattle industry and AI has been the most widely applied tool in facilitating the extensive utilization of frozen semen. Today, cryopreservation has enabled producers to have the ability to access superior genetics for a fraction of the price of buying a bull. Increased breeding efficiency and exploitation of sires through AI programs has become widespread in both the dairy and equine industry. However, the development of a reliable method to cryopreserve sperm is extremely important for preservation of superior genes from valuable animals. The maximum time allowed from semen collection to insemination varies considerably, ranging from immediate insemination requirements to an indefinite in vitro semen storage period. As a result of the growing popularity of these artificial breeding programs, the need to maintain fertility of sperm after varying periods of storage has become increasingly important. In addition to this cryopreservation has enabled the storage of animal genetics to keep allele variation and keep hope for endangered species. Genome resource banking to preserve the biodiversity of rare and endangered species or valuable transgenic lines would also benefit from sperm cryopreservation. Moreover, reproductive research with non-domestic animals particularly those involving cryopreservation of sperm, oocytes, and

embryos can also provide insight and direction into establishing more effective genetic and conservation management programs.

The success of cryopreservation depends upon many other factors, including interactions between cryoprotectant, type of extender, cooling rate, thawing rate and packaging, as well as the individual animal variation (Andrabi, 2007; Clulow *et al*, 2008; Cooter *et al*, 2005). Some loss in spermatozoan viability is inevitable due to the processing procedures prior to freezing as well as during the actual freezing process. Research reports of the success of cryopreserved semen vary significantly often affected by the method of experimentation and recording, which is unstandardized in many reproductive researches. Information about pregnancy rate to a single insemination, timing of insemination, the number of spermatozoa inseminated, volume of inseminate used or type of extender used are still incomplete for some farm animals. Moreover, motility of spermatozoa has proven to be an even poorer indicator of fertility in frozen–thawed samples (Samper *et al*, 1991). Regardless of all these considerations, for cryopreservation to be considered a success the process should enable a spematozoon to retain its fertilizing capacity at postthaw. To achieve this it must retain its ability to produce energy via metabolism; to maintain normal plasma membrane configuration and integrity; retain its motility; and enzymes, such as acrosin, within the acrosome to allow penetration of the ova. Disruption of any of these functions or abilities will significantly affect the spermatozoon's ability to achieve fertilization. The greatest risk to the maintenance of these attributes is presented by the formation of ice crystals and the resultant movement of water up osmotic gradients during the process of cryopreservation.

During the process of freezing, several biophysical changes are evident within the semen sample. As the temperature drops to below freezing the sample undergoes supercooling. As the temperature drops further beyond supercooling, extracellular ice crystals begin to form from the water within the surrounding medium. This ice formation increases the concentration of solutes, such as sugars, salts and proteins. In response to this newly developed osmotic pressure gradient and the fact that water within the spermatozoon is slower to form ice crystals than the water in the surrounding medium, water passes out of the spermatozoa, particularly from the spermatozoon head, across the semi-permeable plasma membrane. Consequently, the spermatozoon becomes increasingly dehydrated (Andrabi, 2007; Watson, 2000; Woelders, 1997). The rate of efflux of water from the spermatozoa also depends upon the speed of temperature drop: the slower the drop, the greater would be the time needed for the efflux of water, and hence a much greater dehydration. This does reduce the chance of ice crystal formation within the spermatozoon, which could cause considerable physical damage (Amann and Pickett, 1987; Hammerstedt *et al*, 1990), but an even greater damage occur due to increased intracellular dehydration and solute concentration. On the other hand, if the cooling rate is rapid, water has little time to move out of the spermatozoon and hence large intracellular ice crystals form, causing physical damage to cell membranes and other intracellular components. However, the problems of dehydration and solute concentration are less evident with rapid cooling. A successful cryopreservation should, therefore, aim at arriving at an optimum cooling rate that will provide a compromise between all these factors.

There are two main temperature ranges of concern regarding damage to spermatozoa during freezing: the period of supercooling (0°C to -5°C) and the formation of ice crystals (-6°C to -15°C) (Woelders, 1997). Excessive supercooling results in a rapid ice formation, with

the possibility of physical damage. In samples other than semen, this problem can be overcome by a technique termed seeding, which is designed to induce ice formation more gradually over a greater temperature range. However, there is little or no evidence that seeding a semen sample during the freezing process has any advantageous effects. The second area of concern is known to have a significant effect on spermatozoan function post thaw. In an attempt to overcome some of these problems, the use of CPA has been investigated. Cryoprotectants may be divided into either penetrating or non-penetrating depending on their action. Penetrating cryoprotectants are able to penetrate the plasma membrane of the spermatozoa and, therefore, act intracellularly as well as extracellularly. The second type of CPA is non-penetrating and can only act extracellularly.

Glycerol remains to be one of the most favored CPA, especially with bovine semen. It is a penetrating cryoprotectant, acting as a solvent and readily taken up by spermatozoa, entering the cell within one minute of addition to the surrounding medium (Pickett and Amann, 1993). Its presence, both intra- and extracellularly, acts to lower the freezing point of the medium to a temperature much lower than that of water. This in turn reduces the proportion of the medium which is frozen at any one time, reducing the effect of low temperature on solute concentrations and hence on osmotic pressure differences (Amann and Pickett, 1987; Medeiros *et al*, 2002; Watson and Duncan, 1988). It also provides channels of unfrozen medium, between ice crystals, in which spermatozoa may exist while at low temperatures. A further effect of glycerol may be a salt buffering action. Other penetrating CPAs include dimethyl sulphoxide (DMSO) and propylene glycol. Non-penetrating CPAs include sugars like lactose, mannose, raffinose, trehalose and proteins, such as egg yolk lipoprotein. These CPAs are believed to act by increasing the osmotic pressure of the extracellular fluid and hence drawing water out of the spermatozoa, thereby decreasing the risk of formation of ice crystals and hence physical damage. However, they do not alleviate, and may even exacerbate the problem of dehydration and increases in solute concentration (Steinmann, 1996).

Other alternatives have been used as CPA, including Orvus ES paste, a mix of anionic detergents, and the synthetic detergent OEP, an amino-sodium lauryl sulphate. OEP apparently alters the composition of egg yolk, improving its cryprotectant properties. Its inclusion was initially tried in extenders for use with boar semen with some success. It was used in an extender containing 5% egg yolk, 2.5% glycerol at 0.4% proportion of OEP, along with lactose, fructose, glucose, ethylenediamine tetraacetic acid (EDTA), sodium citrate and sodium bicarbonate (Christanelli *et al*, 1984).

Both penetrating and non-penetrating CPAs themselves were later on known to cause some form of damage to the spermatozoa (Fiser *et al*, 1991). This was believed to be either due to physical damage as a result of the changes in osmotic pressure gradients or to biochemical disruption of subcellular components. The addition of cryoprotectant such as glycerol has a more adverse effect on motility than on mortality or fertility (Blach *et al*, 1989). A study was carried out in stallion semen sample (n=41) to compare two freezing techniques (cooling at +4°C for 2.5 hrs, suspending the straws on a rack in a Styrofoam box 3cm above liquid nitrogen for 7 minutes followed by plunging the straws in to the liquid nitrogen; and programmable freezer; IceCube 14S, Vers. 1.30, SY-LAB Gerate GmbH, Austria) using an extender containing egg yolk and glycerol (Gent extender, Minitube Int., Germany). The mean (±SD) total motility for samples frozen in liquid nitrogen was 32.8 ±13% (range=5-55%) while the mean (±SD) live percent was 43.3 ±13% (range=14-63%). Total motility and live sperm percent were also highly correlated (Fig.-1) in the samples frozen by liquid

nitrogen. Though greater numbers of sperms were live, not all were found to be motile in both methods of freezing. Conversely, other studies indicate that the use of motility as an indication of viability is not a very accurate assessment in post–thaw samples. This is believed to be because of a greater detrimental effect of cryopreservation agent on the mitochondria than on the acrosome region of the spermatozoan head. The detrimental effect of glycerol on such function is apparently more evident in stallions than in other species such as bull.
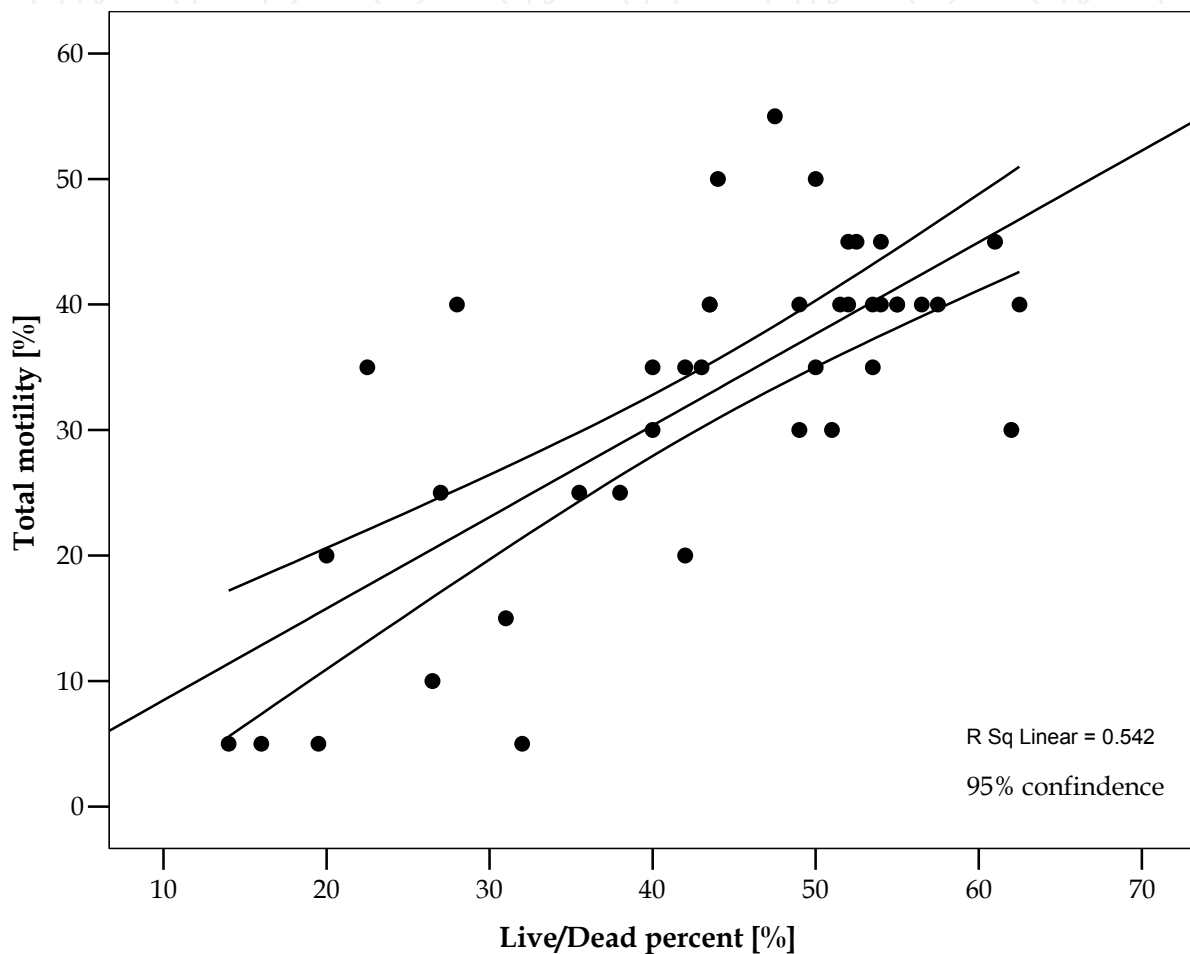

Fig. 1. Correlation between total motility and live/dead percent of stallion semen preserved in Gent extender containing egg yolk and glycerol preserved using liquid nitrogen

A further detrimental effect of the use of glycerol is the rapid efflux of glycerol from the spermatozoa into the glycerol-free secretions of the female's tract at insemination. This rapid exit across the plasma membrane is also believed to cause damage to the spermatozoa and, hence, loss of sperm function (Pickett and Amann, 1993). This accounts in part for the apparent poor positive correlation between motility and fertilization rates in post thaw equine spermatozoa. Computer-automated sperm head morphometry has been used to determine the effects of cryopreservation on bovine sperm head. The protocol for the use of cryoprotectants is ultimately a compromise between the advantages and detrimental effects of their incorporation. Consequently, the exact protocol may vary with individual breeding males in order to obtain optimal results. However, such individual tailoring is not practical in a commercial situation and hence further compromise is normally required.

3. Effect of cryopreservation on spermatozoa

Spermatozoa continuously change and develop from their origins as somatic cells until their destination as highly specialized cells capable of fertilization. They have basically three functional regions comprising a head that contain the condensed nuclear material, a mid piece serving as a powerhouse and a tail which is the propulsive region. Subsequent maturation occur within the epididymis, followed by further development induced first by contact with seminal plasma and then by the secretions of the female tract (Varner and Johnson, 2007). The final stages of spermatozoon development are induced by the immediate environment of the oocyte and its zona. In the process, most of the organelles are lost together with the cytoplasm, and the spermatozoal chromatin is remodeled. This specialization, though, is achieved at a cost, reducing the spermatozoon's ability to repair itself leading to a greater susceptibility to environmental change. Hence, even under ideal conditions, it is inevitable that some damage will occur to spermatozoa during the freezing process (Andrabi, 2007).

3.1 Effect on spermatozoal metabolism

The structural changes produced in the postthaw sperm cells membrane are primarily linked to altered abilities for energy sourcing. This would later on influence both cellular metabolism and other sperm functions such as motility (Cerolini *et al*, 2001; Dziekonska *et al*, 2009; Gillan *et al*, 2004). A spermatozoon is one of the smallest cells in the body specifically designed for propagation of genetic material achieved through fertilization. Like many other cells in the body, it requires a constant supply of energy for maintenance of cellular order and functions needed for survival and accomplishment of its task. This energy requirement increases significantly with the onset of activated motility, and becomes even more pronounced when hyperactivated motility is initiated (Granish and Suarez, 2002; Varner and Johnson, 2007). Plenty of exogenously derived nutrients are required by the spermatozoa to gain the strength needed for the long journey from the epididymis to the ovum in the female reproductive tract. These nutrients are metabolized intracellularly, resulting in the release of useable energy available for cellular processes primarily in the form of ATP. Like many metabolically active body cells, spermatozoa possess the metabolic machinery required for glycolysis, the citric acid cycle, and oxidative physphorylation. ATP for spermatozoa is mainly derived either by glycolysis in the cytoplasm or through oxidative phosphorylation in the mitochondria (Dziekonska *et al*, 2009; Januskauskas, and Zillinskas, 2002). The relative contributions of the two processes to ATP generation are as yet unclear. A gradual reduction in the metabolic activity of spermatozoa during storage at cold shock temperature could limit the production of detrimental by-products, which might compromise sperm function but metabolic activity altered in this way does also influence essential sperm functions such as motility. Among the different alterations of activity of the intracellular enzymes, glucose-6-phosphate-dehydrogenase is the first enzyme which leaves the cell when the cellular membrane is damaged during cold shock. Generally, the intracellular concentration of ATP is decreased or lost and the AMP/ADP-rate is increased by the cryopreservation.

Spermatozoal motility, considered to be one of the most frequently used characteristics for evaluating the fertility potential of ejaculated spermatozoa, is known to be dependent on mitochondrial function. The ATP generated by oxidative phosphorylation in the inner mitochondrial membrane is transferred to the microtubules to drive motility. Hence reduced

sperm motility induced by cryopreservation is believed to be mainly associated with mitochondrial damage (Januskauskas, and Zillinskas, 2002; Ruiz-Pesini *et al*, 2001). In human spermatozoa, mitochondrial enzymatic activities were shown to be correlated with spermatozoal motility (Ruiz-Pesini *et al*, 2001). Male infertility can result from a significant decrease in the number of motile forms and/or from movement quality disorders. Some studies reported a significant correlation between computer assessed spermatozoal motility and field fertility (Januskauskas *et al*, 2003) however, in a more recent study (Garcia-Macias *et al*, 2007), spermatozoal total and progressive motilities and velocity parameters were known to have no correlation with fertility though the authors noticed that velocity parameters were highest in the high-fertility group in their study. The decrease in correlation between motility and fertility has been suggested to be due to a difference in the change of permeability of the acrosome and mitochondrial membranes to calcium ions. The acrosome membrane suffers most from cold shock which accounts for most of the fertility failure (Deneke N., Lemma A., and Yilma T., 2010, Unpublished information). Vesiculation of the acrosomal and plasma membranes occurs during sperm cell death which is termed as false acrosomal reaction. A true acrosome reaction, which precedes fertilization, occurs only in live, intact spermatozoa. Thus, it is important not only to analyze semen for sperm viability but also to determine the alteration of acrosomal integrity simultaneously. HOST and acrosomal integrity tests were employed to evaluate sperm membrane integrity in Holstein Friesian AI bulls belonging to the National Artificial Insemination Centre (Ethiopia). The proportion of sperm cells that reacted to HOST (n=36) was 60.6±9.2% and the proportion of sperm with altered acrosome in the same sample was 50.6%. HOST reaction was highly correlated ($r = 0.82$, $p<0.001$) to higher percent live sperm in frozen sample indicating that live sperms whose membrane is relatively intact will react to the HOST solution. When the two tests are performed simultaneously, acrosomal alteration seem to be directly correlated with membrane integrity as evidenced in the correlation study (Fig.-2). Similar correlation result between the incidence of swollen tails after incubation for 20-30 min, and of altered acrosomes and total spermatozoan motility has also been previously reported for horses (Cueva *et al*, 1997).

Storage of spermatozoa outside the body cavity can impact availability of oxygen and metabolic processes. Cryopreservation of spermatozoa is associated with both oxidative stress and physical stress (Chatterjee *et al*, 2001; Mazur *et al*, 2000). If raw or extended semen is left undisturbed in a laboratory setting, use of dissolved O_2 by aerobic respiration leads to depletion of O_2 and the need to resort to glycolysis for meeting energy demands. Spermatozoa such as that of a stallion are highly susceptible to extreme oxidative stress in view of their absolute reliance on the aerobic metabolism to meet their ATP requirements. Moreover, their inability to synthesize antioxidants, their exuberant content of polyunsaturated fatty acid, their unique capacity to generate superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) through mitochondrial respiration, and their restricted endogenous antioxidant defense mechanism which is lost with the cytoplasm during the final stages of spermatogenesis all contribute to the extreme oxidative stress also observed during cryopreservation (Aitken and Baker, 2004; Parks and Lynch, 1992;). Compared to the raw fresh semen, cryoprserved spermatozoa experience a shorter lifespan and a lower fertility (Andrabi, 2007; Samper, 2001). This was partially attributed to the great difference between fresh and frozen sperm in the generation rate of O_2^- and H_2O_2 or in the intracellular concentration of free calcium ions (Ca^{2+}) (Ball and Vo, 1999; Pommer et al 2002; Samper, 2001). Protection against the effects of reactive oxygene species like O_2^- and H_2O_2 in
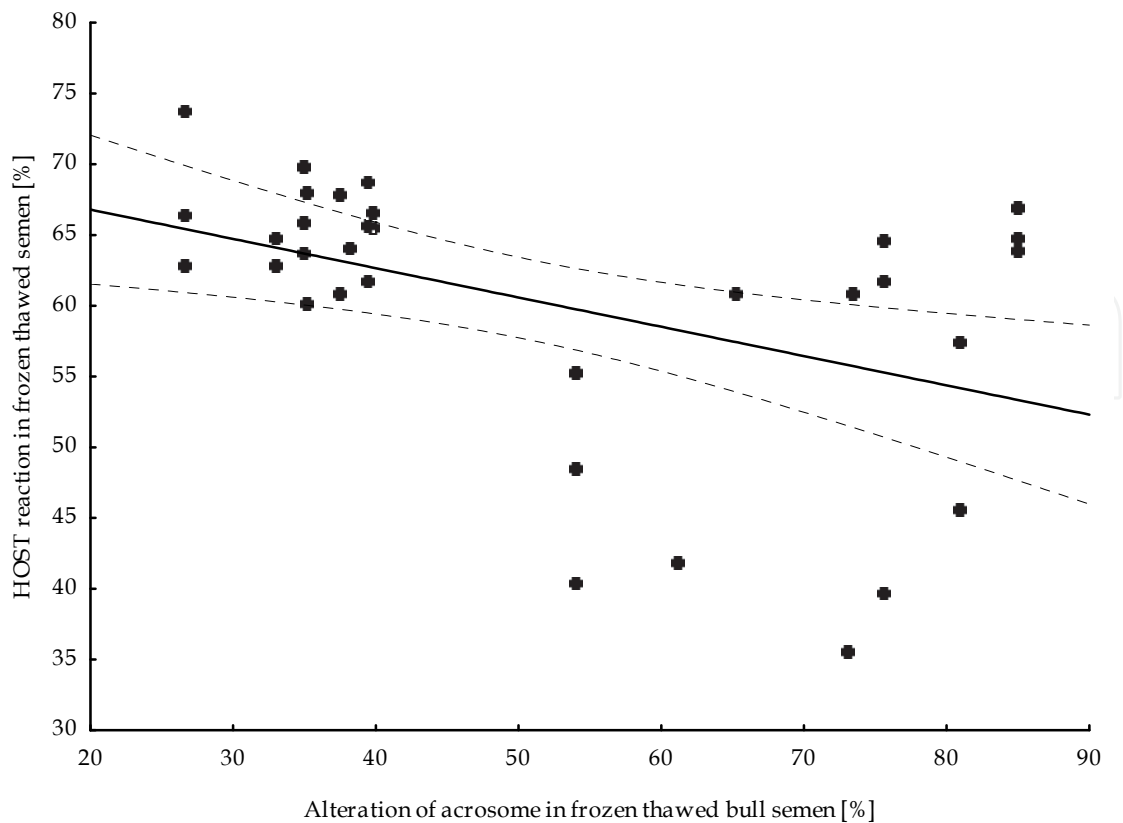
Fig. 2. Correlation between HOST reaction and alteration of acrosomal integrity in frozen-thawed bull semen (n = 36; r= -0.45; p<0.001)

spermatozoa is afforded by a variety of scavenging molecules. Pyruvate, for instance, is a potent scavenger of H_2O_2 and its supplementation to a chilled stored stallion semen or to a frozen thawed bull semen resulted in a significant augmentation of sperm motility and ATP levels. Oxidation of thiols in sperm proteins by O_2^- and H_2O_2 was found to be associated with inhibition of sperm motility and fertilizing ability (Mamoto *et al*, 1996). Cryopreservation of bull sperm in egg yolk based extenders significantly reduced the intracellular level of thiols and post-thaw treatment of frozen semen with thiols containing antioxidants prevented H_2O_2 -mediated loss of sperm motility (Bilodaeu *et al*, 2001).

3.2 Effect on spermatozoal ultra-structure
Mammalian sperm are very sensitive to lower temperature past body temp down to the freezing point of water mainly due to the sensitivity of the plasma membrane which is the primary site of injury in cryopreserved spermatozoa. The freezing induces membrane alteration, which when thawed, causes changes in protein activity and subsequently altered permeability to water and solutes. This results in a substantial loss of viable spermatozoa (Bailey *et al*, 2003). The characteristics of membranes that affect their sensitivity include cholesterol/phospholipid ratio, content of non-bilayer-preferring lipids, degree of hydrocarbon chain saturation and protein/phospholipid ratio (Medeiros *et al*, 2002). Each species of animal contains a different membrane composition. This causes different effects from cooling and subsequently different cryosensitivity of sperm across various species.
Membranes are naturally fluid, a prerequisite for their efficient function. The two main factors known to affect fluidity are the relative concentrations of phospholipids and

cholesterol in which higher concentration of phospholipids results in a more fluid membrane, and the temperature of the membrane. As membranes are cooled, lipids undergo transition from their normal fluid state to a liquid crystalline state, in which the fatty acyl chains become disordered (Medeiros *et al*, 2002; Parks and Lynch, 1992; Watson, 2000). During freezing this liquid crystalline state is transformed to a gel state where the fatty acyl chains become re-ordered in a parallel fashion producing a rigid structure. The phase transition temperature for these changes varies with different lipids and depends upon their structures. In general, the longer the fatty acyl chains, the higher is the phase transition temperature. As each lipid class within a membrane reaches its phase transition temperature it conforms to the gel configuration and tends to aggregate together with other similarly conformed lipids within the membrane (Parks and Graham, 1992). The remainder of the lipids within the membrane may still be fluid, so areas of gel membrane can be identified within a mainly fluid structure. In addition, the junction areas between the gel and the other lipid and protein fractions become areas of weakness, subject to fusion and rupture as well as being permeable to ions (Hammerstedt *et al*, 1990).

The peak phase transition temperature for phospholipids within the membrane of boar's, bull's and stallion's spermatozoa are 24.0°C, 25.4°C and 20.70°C, respectively. Similarly, the peak transition temperature for glycolipids in stallion spermatozoon membranes is 33.4°C, compared with 36.2°C and 42.8°C for boars and bulls, respectively (Parks and Lynch, 1992). These differences in peak transition temperatures account for the variable tolerance to cold shock exhibited by spermatozoa from these species of domestic animals. The membrane configuration has a roughly even distribution of phospholipids in both the outer and inner layers for reasons of stability. The major phospholipids within the spermatozoon plasma membrane namely phosphatildylcholine, sphingomyelin and phosphatidylethanolamine have differing positions within the membrane bilayer. Phosphatidylcholine and sphingomyelin are associated with the outer layer of the bilayer, whereas phosphatidylethanolamine has an affinity for the inner, cytosolic layer. These affinities are not normally evident except when the membrane is under stress. Hence, cold shock causes changes to the distribution of the phospholipids across the bilayer which results in altered membrane function (Amann and Pickett, 1987; Hammerstedt *et al*, 1990). Generally, changes in plasma membrane integrity and motility are both indicators of sperm viability and metabolic intactness. In this regard boar spermatozoa are known to suffer extensive membrane and tail damage during freezing and thawing, and those spermatozoa that survive suffer from a shortened lifespan, requiring AI to be carried out with large numbers of spermatozoa closely timed to the moment of ovulation (Wongtawan *et al*, 2006).

Another major component of the spermatozoon membrane is protein. The protein–lipid interactions are critical for the efficient functioning of the membrane. It is important to ensure even distribution and molding of proteins into the bilayer, thus eliminating pores and membrane faults. These interactions may also be required for the efficient functioning of these proteins as enzymes, receptors or channels for the movement of ions like calcium ions. These configurations of the membrane and interactions between its components function ideally at a normal body temperature. Hence, freezing beyond the transition phase temperature results in a change to the gel state and a gradual aggregation of specific lipids within the membrane. Consequently, these protein-lipid interactions are disrupted and therefore, the proteins no longer act efficiently as enzymes, receptors or ionic channels (Medeiros *et al*, 2002). The membrane as a whole loses some of its structural and functional integrity. Disruption of the membrane configuration also interferes with the function of the

glycocalyx components, peripheral proteins known to confer stability to the spermatozoa in their passage through the female system. This will influence peripheral protein attachment, causing them to aggregate in the areas of membrane still in the fluid state once gel formation has begun (Andrabi, 2007; Housley and Stanley, 1982). Many of these changes to membrane configuration involving lipids are known to be irreversible and subsequent warming of the spermatozoa does not restore the original membrane configuration.

Another sperm alteration linked to freezing is related to the transfer of proteins through the cell, which is modulated by the distribution of lipids along the membrane, altering the response to induction of capacitation and the acrosome reaction of frozen/thawed spermatozoa during fertilization (Guthrie and Welch, 2005). Dislocation of proteins in the plasma membrane, such as those belonging to the glucose transporter (GLUT) family have also been reported as major problem related to cryopreservation. These GLUT proteins are mainly responsible for the transport of hexose across mammalian sperm membranes (Kokk et al, 2005). GLUT proteins have been detected in the spermatozoa membrane of dog (Rigau et al, 2002), human (Kokk et al, 2005) and boar (Medrano et al., 2006), highlighting their important role in the regulation of sperm glucose and fructose metabolism.

Ultrastructural studies have shown the presence of detrimental effects of cryopreservation on various sperm organelles such as altered spermatozoal mitochondria (Nishizono et al, 2004). Microscopic examination of stallion spermatozoa indicates that the function of the mitochondrial cristae is also affected by cold shock. This damage to mitochondrial structure and hence its function within the spermatozoa is likely to account for the decrease in motility observed after freezing (Ruiz-Pesini et al, 2001). Moreover, swelling of the acrosomal area was observed to be a consequence of cold shock, which indicates a loss of integrity as membranes are normally unable to stretch. Freezing is also known to change motion characteristics of spermatozoa due to the irreversible changes to the mid-piece and coiling of the tail (Watson, 1990). Particularly in donkeys, spermatozoa show an increased incidence of backward motion because of an over-bending of the tail area (Ayalew E. and Lemma A., 2010; Tsega A. and Lemma A., 2009; Unpublished observation).

Cryopreservation has also been shown to induce the acrosome reaction in spermatozoa. DNA integrity of sperm is essential for accurate transmission of paternal genetic information. Normal condensation and stabilization of sperm chromatin in the nucleus followed by decondensation after sperm penetration and injection into the cytoplasm of the oocyte are pre-requisites for fertilization. However, sperm chromatin structure and DNA are known to be altered or damaged during cryopreservation (Donnelly et al, 2001; Fraser and Strzezek 2004; Hammadeh et al, 2001; Peris et al, 2004). It is reported that the cryopreservation process of freezing and thawing can increase abnormal chromatin condensation in human (Donnelly et al, 2001; Hammadeh et al, 2001; Royere et al, 1991), boar (Fraser and Strzezek, 2004), and ram (Peris et al, 2004) sperm. Normal chromatin packaging is also known to significantly decrease after the freeze-thawing procedure in human sperm. The chromatin structure of a mature spermatozoon is normally highly condensed, making about 5-10% in volume of that of a somatic cell. This packing is a result of a marked alteration in the composition of nucleoproteins that occurs during epididymal transit. The spermatid genome encodes for protamines, a unique type of spermatozoal protein, which predominate as nucleoproteins during spermatozoal maturation in the epididymis. The cysteine residues of this protein establish intramolecular and intermolecular disulfide linkages that result in compaction and stabilization of the associated DNA. This design is believed to provide protection to the chromosomes during their transport within the female

reproductive tract (Varner and Johnson, 2007). It has been postulated that a reduction of sperm surface area due to alteration of sperm chromatin may ultimately be manifested in abnormal morphology of the sperm head. A decrease in the percentage of normal sperm heads in the ejaculate has been correlated with lowered fertility in bulls and over-condensation of chromatin appears to be associated with reduced fertility in men (Royere *et al*, 1991). Moreover, cryopreservation appears to reduce the ability of sperm chromatin to decondense. The adverse effects of cryopreservation on sperm chromatin and head morphology may be responsible for lowered fertility of spermatozoa observed after cryopreservation.

3.3 Cpacitaion-like effect of cryopreservation

Once spermatozoa reach the site of fertilization, there appear to be highly coordinated cellular and molecular events that should happen before the actual fertilization. The sperm cells first attach temporarily to oviductal epithelial cells, a process that requires specific cell to cell attachment, possibly mediated through spermatozoal surface carbohydrate-binding proteins, termed lectins (Varner and Johnson, 2007). They then undergo capacitation and hyperactivation, bind to the oocyte zona pellucida, undergo the acrosome reaction, penetrate the zona, and finally fuse with and penetrate the oolemma. Intracellular calcium levels increase in sperm during capacitation, hyperactivation and the zona pellucida-induced acrosome reaction. Increased concentrations of calcium ions are believed to trigger an intracellular signalling way associated with capacitation. Capacitaion and cryopreservation induce several similar changes to the sperm including calcium influx in to the cells (Bailey *et al*, 2000). However, during cryopreservation sperm cells fail to properly moderate normal internal calcium levels. Restructured membranes and distorted lipid-protein associations are believed to favour further calcium ion influx during cryopreservation. Disruption of the normal capacitation and/or the acrosome reaction due to abnormal concentrations of calcium ion would severely compromise the fertilizing potential of spermatozoa post-thaw. On the other hand, cryopreserved sperm cells exhibit a capacitaion-like behaviour and appear to be in a partially capacitated state due to the cryopreservation-induced membrane changes that makes the cells to be more active to their environment after thawing. As demonstrated by different authors, capacitaion normally creates a state of destabilization with which the sperm cell acquires the fertilizing capacity while remaining susceptible to membrane degeneration and spontaneous acrosomal reaction when fertilization fails (Bailey *et al*, 2000). Cryopreservation creates a subpopulation of killed and partially or fully capaciatated sperm thereby reducing the heterogeneity of the sperm population. This will produce a sperm subpopulation with a shortened lifespan in vivo and whose fertilization potential has been severely compromised reducing the fertility of the semen sample as a whole.

Capacitaion like effect or 'Cryo-capacitation' is one of the major factors associated with reduced longevity and poor survivability of cryopreserved spermatozoa in female reproductive tract (Bailey *et al*, 2000; Watson, 2000), resulting in reduced fertility of frozen-thawed semen. At present, it is generally accepted that poor survival of spermatozoa in the female reproductive tract is among the most important consequences of sperm cryoinjury caused by cryopreservation. This concept of premature capacitation and reduced longevity of sperm cells in the female reproductive tract has led to the routine use of oviductal insemination by laparscopy rather than vaginal or even transcervical insemination in different animals (Bailey *et al*, 2000). The capacitation-like changes have been demonstrated

by greater proportion of chlortetracycline fluorescent pattern "B" due to freezing thawing in bull (Cormier *et al*, 1997), boar (Maxwell, and Johnson, 1997), equine spermatozoa (Thomas, *et al*, 2006), and in buffalo bull semen (Kadirvel *et al*, 2009). Impaired sperm membrane function due to cryopreservation inevitably diminishes the successful union of the oocyte and spermatozoa during in vivo fertilization. The structural reorganization of the sperm head plasma membranes after cryopreservation appears to disrupt the ability of the sperm to interact normally with cells of the female genital tract (Lessard *et al*, 2000; Medeiros *et al*, 2002; Watson, 2000). Poorly motile spermatozoa are also less likely to arrive at the site of fertilization in vivo or to penetrate the zona. Moreover, the proportion of motile sperm population itself is adversely affected by cryopreservation (Cerolini *et al*, 2001; Gillan *et al*, 2004). Reduced sperm binding is likely a result of membrane injury, possibly by structural damage to the sperm receptors or by incomplete receptor aggregation.

4. Evaluation of post-thaw semen quality

The semen quality and its relationship with fertility have great importance in animal production. Hence, in vitro tests are frequently applied to determine the quality of semen for its approval and use in both AI and other biotechnology procedures. Conventional laboratory tests for assessment of semen quality include light microscopic study of spermatozoal morphology, and estimation of spermatozoal motility which in turn encompass percentages of motile and progressively motile sperm; velocity of spermatozoal movement; and longevity following in vitro storage. Other features of semen quality include concentration, volume, detection of the presence of urine, blood, or potentially pathogenic bacteria and functional integrity tests. The choice of adequate parameters by reproducible, fast and sensitive methods is of increasing concern. This is because the predictive value of the standard seminal parameters is limited or insufficient for the identification of subfertile individuals (Clement, 2001; Love *et al*, 2000).

The nucleus, acrosome, the flagellum, mitochondria, and the plasma membrane are the most important regions of the spermatozoa that need to be assessed during postthaw semen evaluation. A series of laboratory tests devised to evaluate these various compartments will aid in the improved localization of spermatozoal dysfunction thereby improving the predictive values of laboratory-based semen evaluation to a relatively more accurate level. More specific techniques such as testing the mitochondrial function, flagellar substructure, and plasma membrane integrity are already available (Graham and Moce, 2005; Gravance *et al*, 2001; Thomas *et al*, 1998). A variety of laboratory procedures used today in the assessment of the integrity of the plasma membrane are important components of the postthaw semen evaluation. Among them is the evaluation of the ability of spermatozoa to exclude extracellular dyes, such as eosin, which are non permeable when the membrane is intact. Another approach is the hypo-osmotic swelling test (HOST) in which the spermatozoa are exposed to a hypotonic media (50 to 100-mOsm range) to test their osmoregulatory function (Davies-Morel, 1999; Neild *et al*, 1999). Dyes that can traverse the membrane and those that are membrane-impermeable can be combined in a solution before spermatozoal exposure to provide a more accurate reflection of membrane integrity. For instance, fluorescent plasma-membrane dyes can be combined with mitochondrial dyes or acrosomal dyes to provide more thorough coverage of the functional regions in the assay (Garner *et al*, 1994; Kavak *et al*, 2003; Love *et al*, 2003). More recently, a computer assisted semen analysis (CASA) is in use and gives extensive information about the kinetic property

of the ejaculate based on measurements of the individual sperm cells. Using CASA, motility and movement characteristics of spermatozoa have been correlated to in vivo fertility. Still, CASA-assessed motility is done on a rather limited number of spermatozoa and is predisposed to a certain degree of human bias.

An understanding of how molecular and ultra-structural basis of spermatozoal function, spermatozoa-oviductal interactions, and gamete engagement are influenced by cryopreservation will undoubtedly lead to many practical applications in semen evaluation. From this may arise possibilities for detailed laboratory tests to assess spermatozoal function, to introduce improved methods of semen preservation, options for applications of assisted reproductive technologies and even treatment options for subfertile animals. The chromatin structure assay (SCSA) tests, for instance, is a flow cytometric procedure that uses the metachromatic fluorochrome to test the denaturability of spermatozoal chromatin that is not normally monitored by conventional methods in various species (Evenson *et al*, 1995; Love 2005; Makhlouf and Niederberger, 2006). Chromatin susceptibility to denaturation is correlated with the level of actual DNA strand breaks and might be indicative of genetically defective spermatozoa (Evenson *et al*, 1995). Several types of defective sperm organelles and DNA can be detected in large number of sperm by immunochemical assays and flow cytometry. Microarray profiling of sperm mRNA has been shown to indicate gene expression associated with both fertile and infertile males (Evenson *et al*, 2002; Thomas *et al*, 1998). More importantly, spermatozoa affected by such damage might show no apparently detectable alteration in motility or membrane integrity, but may induce embryonic failure after fertilization (Fatehi *et al*, 2006). Further DNA damages are not evident until the time of fertilization making the chromatin defect clinically significant as it represents a potential non repairable defect. This becomes quite important clinically, because affected spermatozoa in an ejaculate may not be impaired from fertilization, and hence performing repeated insemination may not increase pregnancy rate.

The search for the identification of biochemical markers of spermatozoal function is still ongoing. Such finding will improve the efficiency of laboratory-based detection of infertility induced by the process of freezing/thawing by targeting specific cellular components. However, incorporation of detailed tests such as SCSA for semen evaluation should not replace or reduce the value of the conventional methods of spermatozoal motility or morphology tests. More recently, much attention has been given to the test of capacitation process, as an immediate precursor to fertilization. However, on the path to fertilization there are many preliminary steps prior to capacitation leading to the need for sperm evaluation involving tests of spermatozoal response to particular environmental conditions related to the overall fertilization process (Petrunkina *et al*, 2007).

5. Fertility of cryopreserved spermatozoa

The process of cryopreservation represents an artificial interruption of the progress of the spermatozoon towards post-ejaculation maturation and fertilization. Even with the best preservation techniques to date, cryopreservation process still causes harmful damage to the spermatozoa. As it has been discussed earlier in this chapter, cryopreservation affects fertility by virtue of its effect on sperm membranes, cytoskeleton, motile apparatus and nucleus, and cell metabolism. Moreover, freezing and subsequent thawing procedures render the remaining surviving spermatozoa physiologically different from spermatozoa before cryopreservation. Sprmatozoa become very sensitive to any form of stress in their

environment in vivo as well as in vitro. As a result, fertility from frozen thawed semen is poorer than that obtained from fresh semen. For this reason, proper evaluation of the post-thaw quality of spermatozoa is of utmost interest for AI industry to obtain information on the fertilizing capacity of the cryopreserved semen.

Many tests of sperm motility, morphology, acrosomal status, defective sperm organelles and DNA, and metabolism have been correlated with fertility (Evenson *et al*, 2002; Larsson and Rodriquez-Martinez, 2000; Muller, 2000; Saacke *et al*, 2000; Thomas *et al*, 1998). All of these spermatozoal attributes have been shown to be either directly or indirectly affected by cryopreservation or the thawing process. The correlation between fertility and percentage of motile sperm in a semen sample has already been demonstrated. In one study, after insemination of 55 cows with frozen semen a 30.9% (17 cows) pregnancy rate with an average number of services per conception of 2.7 was found. Conception rate to first service was only 7.2%. The mean ($\pm$SD) alteration of acrosome and positive reaction to HOST for successful (pregnant) and failed insemination (non pregnant) were 47.6 ± 9.9% and 64.7 ± 3.0%, and 62.7 ± 7.3% and 42.1 ± 3.9%, respectively with a highly significant (p<0.001) difference in both tests between the successful and failed inseminations. Semen that did not impregnate contained the highest proportion of sperm with altered acrosome that did not react well to HOST. This shows the significance of the use of combination of semen evaluation methods to avoid the use of poor quality semen and hence reduce the male factor from fertility assessments (Deneke N., Lemma A., and Yilma T., 2010, Unpublished information). Significant variation in the success rates for frozen semen has been reported with an apparently much wider variation in the performance of equine semen post freezing and thawing than is with bovine semen. For instance, one-cycle pregnancy rates of 32-51% have been reported in mares (Muller, 1987; Pickett and Amann, 1993).

In general, pregnancy rates for frozen semen at best reach values approaching those of natural service but may also result in complete failure, despite the protocol for freezing apparently being unchanged (Pickett and Amann, 1993). It is very difficult, therefore, to predict likely conception rates with any certainty and it is also very difficult to compare, with accuracy, the pregnancy rates obtained in different research work using frozen semen. In most of the research carried out there are many variables, the details of which are often not specified. The results reported for pregnancy rates depend, among other things, upon: the individual animal and the quality of semen produced; the minimum standard set for semen quality prior to acceptance for freezing; the number of spermatozoa per straw and straws per insemination; the freezing protocol; the thawing protocol; post-thaw semen quality control; the numbers and reproductive ability of the females used for insemination; the timing of the insemination; the number of inseminations per cycle; and the number of cycles and inseminations per pregnancy. Hence, no absolute figure has been arrived at for pregnancy rates using frozen semen (Pickett and Amann 1993). Fertility of an ejaculate from a male is also highly dependent on the fertility of the females. Conversely, a more recent review indicates that male fertility should not be evaluated independently of female fertility (Amann, 2005). The same author reported that a stallion with 95% fertility can have an observed fertility as high as 90% if bred to mares with 95% fertility, and as low as 24% if bred to mares with 25% fertility. Subsequently lower fertility is seen if the stallion's fertility is lower. Because of this female factor in fertility using a single male on a group of females to ascertain fertility is not sufficient to extrapolate the results to a population.

6. Extenders used in freezing semen

The addition of a cryoprotectant in to the semen sample is needed in order to protect spermatozoa from cold shock. A large variety of extenders combining various components (sugars, electrolytes, buffers, egg yolk, milk and milk products), have been proposed and used for extending sperm. Milk and milk-based extenders are known to be practical and efficient in protecting spermatozoa of various species (Batellier *et al*, 2001; Varner *et al*, 1989). Based on the composition and dynamics of the spermatic membranes, some substances such as lipids, fatty acids and proteins have been incorporated to the semen with the goal of decreasing sperm damages related to cryopreservation. Glycerol and egg yolk extenders are amongst the first to be used for freezing semen (Curry, 2000; Garner *et al*, 1999; Holt, 2000; Medeiros *et al*., 2002), and today many extenders use glycerol as the major cryoprotectant. Glycerol is used at a relatively high concentration which can be detrimental to spermatozoan viability at higher temperatures hence it is added after the semen has been cooled (Fahy, 1986). The deleterious effects are due to osmotic stress, changes in membrane organization, fluidity and permeability, as well changes in the lipid composition. Thus, a compromise has to be reached with regards to the concentration of glycerol and the length of time that the glycerol is in contact with the spermatozoa prior to freezing, in order to maximize the beneficial effects of glycerol as a cryoprotectant but minimize its toxic effects. In one study, the inclusion rate of 4% glycerol in an extender containing 20% egg yolk was found to be superior to 2% or 6% glycerol as regards to progressive motility (Cochran *et al*, 1984). In addition, the efficiency of glycerol may be affected by the diluents to which it is added, as well as the method of storage.

The cryoprotectant nature of many other substances, including sugars and liposomes has also been demonstrated. In equines, it is a common practice today that the preparation of semen for cryopreservation involves the use of two extenders: a primary extender for initial dilution, which is aspirated off after centrifugation, prior to the addition of a secondary extender for freezing. Numerous extenders have been used as primary or secondary extenders. Examples of extenders for freezing include egg yolk and those based on skimmed milk with egg yolk (Pickett and Amann, 1993). Good success has been reported with the use of trehalose as a cryoprotectant within a skimmed milk–egg yolk extender. It is suggested that trehalose has a stabilizing effect on the spermatozoon plasma membrane (Steinmann, 1996).

Unlike the bull and the ram in which fructose is the major energy source, most extenders use glucose as the major source of energy for metabolic activity and movement of spermatozoa in equines (Katila, et al, 2001). In this regard, three extenders were evaluated for their efficiency of sustaining the viability of jack sperm as measured by motility characteristics (Ayalew E. and Lemma A., 2010; Unpublished observation). The first extender was a heated skimmed milk (95°C for 10 minutes and cooled to 37°C before use). The second extender was prepared from glucose (4gm), glycerol (4%), and crystalline penicillin (150,000 IU) diluted in heated skimmed milk to make up 100ml extender (represented as SMGLU). The third extender contained 4% glycerol in skimmed milk (represented as SMGLY). SM was used for storage in Equitainer (Agtech Inc, Manhattan, USA) while SMGLU and SMGLY were used for liquid nitrogen storage after a 2.5hr optimization at +4°C, suspension over liquid nitrogen vapour for 7 minutes and plunging the straws immediately into the liquid nitrogen. SMGLU preserved sperm showed superior results (p<0.05) for both total and forward progressive motility than either SM alone or SMGLY (Table 1).

Semen parameter	SM [Equitainer] n=15	SMGLY [Liquid nitrogen] n=32	SMGLU [Liquid nitrogen] n=13
Live sperm after 24hr [%]	36.2±24.3	10.2±11.7	34.3±7.5
Total Motility after 24hr [%]	24 ±17.4	8.8 ±9.6	30±10.6
Progressive motility at 24hr [%]	19.3 ±14.5	7.2±7	24.6±10.5

Table 1. Mean (±SD) of semen parameters for three different extenders used to dilute jack semen

In another study, insemination of 34 animals (17 mares and 17 jennys) with jack semen extended in heated skimmed milk and stored in Equitainer (Agtech Inc, Manhattan, USA) for 24 hrs resulted in 38.2% pregnancy rate (Tsega A. and Lemma A., 2009; Unpublished observation). Addition of trehalose to bull semen extenders is known to provide a modest improvement in fertility when used in combination with glycerol, which remains the cryoprotective agent of choice. Egg yolk phospholipids can also lessen chilling injury on bull sperm by binding to low density lipoproteins of the membrane and by increasing the permeability of the membrane, although they do not alter intrinsic membrane composition and/or physical properties (Holt, 2000). The addition of concanavalin-A to the freezing diluent, a substance that has the ability to coat and thus protect spermatozoa membrane, has been suggested to provide additional protection for acrosome membranes and help to preserve motility post freezing and during thawing (Koskinen *et al*, 1989). The inclusion of liposomes, which have proved successful in bulls, has been tried with some success in equine semen (Heitland *et al*, 1995).

7. Freezing and thawing rates

Storage of semen at ambient temperature does not in itself significantly reduce the spermatozoan metabolic rate, thus limiting the potential length of storage and demanding cryopreservation at -196°C in liquid nitrogen for long term storage. The cooling/freezing rate in the critical temperature range is of considerable importance during cryopreservation process because this determines whether the spermatozoa will remain in equilibrium with their extracellular environment or become progressively supercooled with the increasing possibility of intracellular ice formation (Kumar *et al*, 2003). During slow cooling, the dehydration of the spermatozoa can proceed to the point of osmotic equilibrium between intracellular and extracellular space with maximal, often detrimental, cellular dehydration. However, raising the cooling rate too much will not prevent the formation of intracellular ice because of the slow dehydration. Therefore, the survivability of the spermatozoa depends upon the optimum cooling rate. Optimal cooling rate will reduce the excessive concentration of intracellular solutes and intracellular dehydration thereby reducing excessive shrinkage of the sperm cells. However, even at optimum cooling rates, spermatozoa remain vulnerable to the unfavorable conditions for a shorter period of time (Woelders, 1997).

The type of extender used and the speed of temperature drop are known to have an effect on susceptibility of spermatozoa to cold shock and the success rate of freezing semen. Moreover, the freezing rate depends on the method of processing and of storage. The cooling of straws can be conveniently done by either initial suspension in racks over a tank of liquid nitrogen or a computer-controlled programmable freezer followed by plunging

into liquid nitrogen for long-term storage (Clulow *et al*, 2008). The extent of damage to a spermatozoon as a result of cold shock depends not only on the drop in temperature but also the speed with which this drop is attained. The rate of temperature drop was found to be most critical over the specific temperature range of 0-5°C when motility was evaluated later. In general, the faster the rate of cooling, the more severe is the damage (Kayser, 1990). There is further evidence which suggests that the rate of temperature drop also determines the subsequent active life of the spermatozoa (Andrabi, 2007).

Frozen spermatozoa are further injured during the thawing process, which has been regarded as being due to re-crystallization of ultra-microscopic ice crystals to form comparatively large ice crystals (Watson, 2000; Woelders, 1997). The warming damage occurs when the spermatozoa pass through the critical temperature zone of -5°C to -15°C (Kumar *et al*, 2003). Water bath temperatures between 4°C and 75°C can be used to successfully thaw semen however, the temperature chosen for the water bath depends on the desired rate of thawing. During fast thawing (optimum; at 37°C for at least 45 sec) the time for re-crystallization to occur is limited and this increases the survivability of spermatozoa. However, when the duration of thawing is insufficient for the out-flow of excess cryoprotectant from the cell it suffers osmotic stress and the spermatozoa swells and lyses as the medium becomes abruptly diluted by the melting of extracellular ice (Pegg, 2002). The thawing rate can be influenced by factors such as the temperature and nature of the environment (air or water bath) and the thermal conductivity of the packaging as related to the diameter of the lumen of the packing. Some semen thawing protocols involve the addition of warmed extender to aid the process of thawing which will also increase the volume of the inseminate and aid preservation of spermatozoan viability. Thawing extenders may be used for semen stored in pellets, vials or straws, and are added as part of the thawing process.

8. Packaging for frozen semen

To maximally utilize the genetics of desired sires on a commercial basis, attempts are made to package a minimal number of spermatozoa per insemination unit without sacrificing fertility (Foote and Parks, 1993; Shannon and Vishwanath, 1995). Ultimately, the number of motile spermatozoa per insemination is determined by the postthaw motility evaluations and non return to estrus rates from a large number of inseminations. The ability to predict postthaw sperm quality and fertility from a routine sperm function assay is beneficial when one considers the extended period of progeny testing.

Several methods are available for the packaging of spermatozoa for freezing in different species. They include glass ampoules or vials, polypropylene, polyvinyl or plastic round or flat straws (usually 0.5-1.0 ml in volume), flat aluminium packets (10-15 ml); pellets (0.1-0.2 ml), and macrotubes (Heitland *et al*, 1996; Kneissl, 1993; Park *et al*, 1995). Both ampoules and straws are traditionally frozen by suspension over liquid nitrogen, followed by plunging into liquid nitrogen at -196°C. Subsequent work investigating the effect of the rate of freezing led to the current application of the use of computer-controlled programmable freezers at different packaging sizes (Clulow *et al*, 2008). Although pellets have the advantage of allowing a rapid drop in temperature to be achieved, they are not suited for easy identification after freezing. In addition, the re-use of the carbon dioxide block or metal plate carries the potential risk of cross contamination with spermatozoa from the previous freezing batch. On the other hand, the use of vials or straws readily allows the accurate

identification of samples and considerably reduces the risk of cross-contamination during cryopreservation.

As different methods of storage have been used, the question of whether the means of storage has any effect on the success rate of cryopreservation has been raised. In this regard, different authors have compared spermatozoa stored in different packages (Heitland *et al*, 1996; Kneissl 1993; Park *et al*, 1995). Their results showed an effect on spermatozoa quality manifested through reduced motility and conception rate. The reports further stressed the roles of different extenders used, the interaction between extender, and means of packaging. However, the reasons for these discrepancies was not fully explained, and it was also not clear in all work how the dimensions of the straws change with volume, in addition to which different extenders and concentration of spermatozoa were used. On the other hand, a more recent work in stallion demonstrated that stallion spermatozoa can be frozen at a concentration as low as 40×10^6 mL^{-1} in 0.25mL straws without a negative effect on sperm motility, morphology or acrosome integrity (Clulow *et al*, 2008).

9. Recent advances in cryopreservation

The application of frozen-thawed semen technology is currently increasing worldwide. Several studies have focused on identifying damages during freezing and thawing, tests to screen sperm quality post-thaw, evaluation of alternative cryoprotectants and other additives, and freezing procedures to improve sperm viability and fertility (Clulow *et al*, 2008; Goolsby *et al*, 2004; Medeiros *et al*, 2002; Squires *et al*, 2004;). Most of the progress in improving survival of frozen-thawed spermatozoa centers on minimizing the oxidative damage and decreasing the osmotic stress on spermatozoa. Equine sperm are particularly known to be susceptible to oxidative stress, relative to other species, because of their high content of unsaturated fatty acids. In addition to membrane effects, lipid peroxidation can also damage DNA. The addition of antioxidants to extenders has been used as a method to decrease lipid peroxidation and oxidative stress associated with cryopreservation (Bilodaeu *et al*, 2001; Pe~na *et al*, 2003; Roca *et al*, 2004). Different amides, compounds with lower molecular weight than glycerol and penetrate the sperm plasma membrane more readily, have been evaluated as alternative cryoprotectants to glycerol in different animals (Bianchi *et al*, 2008; Medeiros *et al*, 2002; Squires *et al*, 2004;). These compounds include methyl formamide (MF), dimethyl formamide (DMF) or ethylene glycol (EG) and dimethyl acetamide. They were known to provide greater post-thaw motility when used at different concentrations. Particularly, MF and DMF or EG have been used as alternative cryoprotectants for individual males whose sperm has lesser post-thaw motility when frozen in glycerol (Bianchi *et al*, 2008; Squires *et al*, 2004). The use of low-density lipoproteins (LDLs), most often isolated from egg-yolk from different species, as additive has proven beneficial for sperm function post-thaw, particularly for DNA-integrity(Rodriguez-Martinez and Wallgren, 2011). Attempts to minimize osmotic stress during cryopreservation have included step-wise dilution of cryoprotectants, by incorporating cholesterol-loaded cyclodextrins (CLC) in freezing diluents (Wessel and Ball, 2004). As an alternative to adding CLC to extenders provision of polyunsaturated fatty acids in the feed as a means of altering the sperm-lipid membrane profile has been tried with some success in boars and stallions (Brinsko *et al*, 2005; Purdy and Graham, 2004).

Different kinds of freezing procedures have also been reported in the last several years in an attempt to controlling the rates of cooling. Recent results indicate that the cryopreservation

of bull, stallion and boar semen could be improved by using a programmable freezer (Bianchi *et al*, 2008; Clulow *et al*, 2008; Woelders and Chaveiro, 2004). An interaction between glycerol concentration and cooling rate has been described for boar semen. Current cryopreservation methods based on optimal combinations of glycerol and cooling rate has allowed consistent sperm survival in the frozen semen, with acceptable variation among individuals. Another method, termed multi-thermal gradient (MTG), that aims to overcome the problems of conventional freezing protocol has also been reported (Arav *et al*, 2002). This freezing technology is based on directional freezing in which the spermatozoa are moved through a linear temperature gradient so that, theoretically, the cooling rate and ice front propagation are precisely controlled. Thus, the spermatozoa are preserved gently between horizontal columns of ice thereby avoiding the damaging effects of the random ice crystal formation observed in conventional freezing. The technique is also known to allow the incorporation of controlled seeding into the freezing process and prevent the dehydration of sperm commonly seen in conventional freezing while halving the level of glycerol required (Arav *et al*, 2002). A slightly different technique, termed unique freezing technology (UFT) which was originally designed to freeze foodstuffs, has been recently tested for semen cryopreservation (Goolsby *et al*, 2004). The UFT involves placing extended samples in a bath that contains an organic fluid with a heat capacity similar to water with a freezing rate of -6.1°C /min. Similar results of post-thaw motilities with sperm frozen in traditional liquid nitrogen procedures have been reported for four UFT treatments (Goolsby *et al*, 2004).

Another front of investigation in the last decade has been the development of methods of examining sperm ultrastructural characteristics and alterations. Amongst these are sperm kinematics assessed by computer-assisted motility analysis, osmotic resistance tests, plasma membrane integrity evaluation with fluorescent membrane-impermeable dyes, evaluation of acrosomal status with fluorescein isothiocyanate-conjugated lectins, investigation of DNA integrity using the SCSA, or assessment of membrane architectural status (Gillan *et al*, 2004; 2005). Most methods require the application of fluorescence microscopy and/or flow cytometric techniques. Because of their highly quantitative, repeatable, and sensitive nature, the techniques are already getting their place in many modern semen laboratories.

10. Conclusion

Cryopreservation continues to be one the most frequently employed technique for use in modern animal production. Commercial AI will inevitably use this technique to preserve and transport semen over a wider area around the world. However, even with the most up to date procedure cryopreservation still causes detrimental effect on sperm compartments and their function. To ensure that semen used for AI are of a relatively uniform, and of high quality, artificial breeding organizations should discard ejaculates based on seminal quality tests immediately after ejaculation and after freezing and thawing. There are evidences resulting from different investigation that different sperm compartments are interrelated, the defect in one will invariably affect the other compartment. The success of sperm cell in its ability to fertilize is also affected at different level during its course from its origin until it reaches the ovum. Cryopreservation would be an additional artificial interruption in this journey. Therefore, the knowledge of the biochemical basis of the detrimental effects of cryopreservation and the means to detect these changes easily, cheaply and accurately during semen evaluation would be of great significance. The use of combination of tests, rather than the employment of a single test, would give superior and more complete

information about the status of the spermatozoa. There are already successes in improving the methods of detection of high quality sperm with good fertility using combination of tests addressing different functions and compartments of the sperm. Tests addressing integrity of sperm chromatine structure are able to identify sperm defects not normally detected during conventional semen analysis. They are highly useful in that their application can avoid carryover problems reflected in the embryo after fertilization has taken place. While most of the investigations were carried out in bovine and equine, the lack of complete information for other species has led to extrapolation of knowledge which in some instances has not given acceptable results. For this reason, the evaluation of different extender combinations, freezing techniques, and developing new methods of semen evaluation should be undertaken for various species of animals worldwide.

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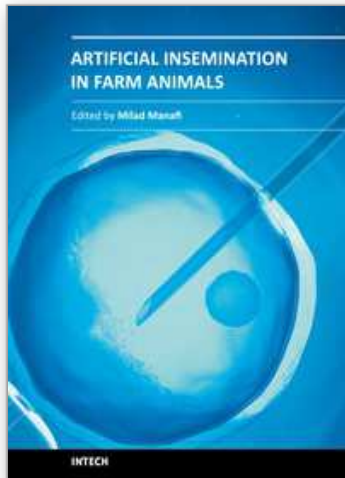
Artificial Insemination in Farm Animals

Edited by Dr. Milad Manafi

Artificial insemination is used instead of natural mating for reproduction purposes and its chief priority is that the desirable characteristics of a bull or other male livestock animal can be passed on more quickly and to more progeny than if that animal is mated with females in a natural fashion. This book contains under one cover 16 chapters of concise, up-to-date information on artificial insemination in buffalos, ewes, pigs, swine, sheep, goats, pigs and dogs. Cryopreservation effect on sperm quality and fertility, new method and diagnostic test in semen analysis, management factors affecting fertility after cervical insemination, factors of non-infectious nature affecting the fertility, fatty acids effects on reproductive performance of ruminants, particularities of bovine artificial insemination, sperm preparation techniques and reproductive endocrinology diseases are described. This book will explain the advantages and disadvantages of using AI, the various methodologies used in different species, and how AI can be used to improve reproductive efficiency in farm animals.

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