Wayamba Journal of Animal Science – ISSN: 2012-578X; P753-P567, 2013
First Submitted September 23, 2013; Number 1379950661

CRYOPRESERVATION OF SPERM IN RUMINANTS-A REVIEW

Sonia Saraswat [*], SK Jindal[1] and SD Kharche
Physiology, Reproduction and Shelter Management Division, Central Institute for Research on Goats, Makhdoom, Uttar Pradesh- 281122, India

*****Corresponding author: sonia.saraswat@gmail.com

The ability to cryopreserve spermatozoa from all of the domestic species is challenging. Even though all the cells must endure similar physical stresses associated with the cryopreservation processes, sperm from the different species are very different in size, shape and lipid composition, all of which affect cryosurvival. Thus, when a cryopreservation protocol has been optimized for sperm of one species, it may not be ideal for sperm of other species. Bovine and caprine sperm-freezing diluents contain similar ingredients, but interactions between goat seminal plasma and egg yolk are deleterious to the sperm, a situation not observed with bovine seminal plasma and egg yolk. Therefore, a thorough understanding of the specifics of sperm freezing from a particular species will improve the cryosurvival of sperm from that species. This review updates information relating to the cryopreservation of buck semen, with emphasis on the peculiarities specific to the species. The topics discussed include the effects of cryopreservation, sperm dilution and concentration, freezing and thawing methodologies, the components of cryopreservation diluents, traditional and recently investigated cryoprotectants and additives. In addition, suggestions for creating a standardized freezing protocol for buck semen are also presented.

Keywords: Semen cryopreservation, cryoprotectants, antioxidants

The procedure that stabilizes the cells at cryogenic temperatures is called cryopreservation. Advances in the cryopreservation technology led to the development of methods that allow maintenance of a variety of cell types including male and female gametes, small multicellular organisms and even more complex organisms such as embryos. Semen cryopreservation is an extensively used technique for the expansion of reproductive techniques, such as artificial insemination (AI) and *in vitro* fertilization (Medeiros et al., 2002), it causes partial irreversible damage to the sperm cells (Amann and Pickett, 1987, Purdy, 2006) due to cold shock, oxidative stress, sperm membrane modification, cryoprotectant toxicity, intracellular ice crystal formation, and fluctuations in osmotic pressure (Watson, 1995, Isachenko, 2003). Such sperm injuries then ultimately lead to a reduction in sperm motility and poor fertility following AI (Matsuoka et al., 2006, Rodriguez-Martinez and Barth, 2007).

Intracellular ice formation in the sperm cell is one of the major detrimental factors that reduce the viability and membrane integrity of frozen-thawed sperm (De Leeuw et al., 1993). Goat sperm is an excellent example, require unique attention to maximize the post-thaw viability as the deleterious interaction between egg yolk and the bulbourethral gland secretions exists for goat semen that does not exist for other species such as the bull, boar or ram (Purdy, 2006)

General aspects of semen Handling

Sperm dilution and concentration: It is essential that a semen sample be diluted properly to have a sufficient number of sperms in order to accommodate the cells in an insemination straw. So that a high fertility rate can be achieved using the least number of inseminations and the lowest number of sperm per insemination. Studies

reveal that semen samples in farm animals diluted by either diluting semen with specific volumes of diluents or by diluting semen to a specific spermatozoa concentration. Dilution rates of 1:1–1:23 (semen to diluent) have been used successfully (Evans and Maxwell, 1987, Ritar et al., 1990a, b). Reports of sperm being successfully frozen and reasonable fertility have been obtained with samples ranging from 80 to 500×10^6 cells/ml (Ritar et al., 1990a,b, 12 Karatzas et al., 1997, Purdy, 2006).

Extenders: The purpose of a freeze extender is to supply the sperm cells with a source of energy, protect the cells from temperature-related damage and maintain a suitable environment for the spermatozoa to survive temporarily (Purdy, 2006). The extender to be use must have adequate pH and buffering capacity, suitable osmolality and should protect sperm cells from cryogenic injury (Salamon and Maxwell, 2000). Apart from the cryopreservation protocol used, the extender composition and the nature of the cryoprotectants are of great importance for sperm survival during cryopreservation (Hammerstedt et al., 1990, Curry et al., 1994, Salamon and Maxwell, 2000, Purdy, 2006). In buck and ram frozen semen extenders, dried skimmed milk or tris–glucose based hypertonic diluents are used frequently (Evans and Maxwell, 1987). Extender pH values range from 6.75 to 7, as mammalian semen has a pH of 7.2–7.8.

Till date several extenders have been used for preservation of mammalian sperm so can be grouped according to their chronological use or development (Salamon and Maxwell, 2000) such as (a) Citrate-sugar based diluents: The citrate–sugar-based diluents was used for freezing ram semen after the late 1960s. (b) Milk diluents: Skimmed milk protection is based in the protein fraction, namely caseins Medeiros et al., 2002). Milk has been adapted for freezing mammalian semen, mostly in reconstituted form combined with arabinose, fructose or egg yolk. However, addition of egg yolk to heated homogenised milk did not increase post-thaw sperm survival (Salamon and Maxwell, 2000). (c) Lactose-based diluents: Successful use of lactose, as the main component of diluents for freezing bull semen, stimulated its application for other mammalian semen. Lactose–yolk was used for both the non-glycerolised and glycerolated diluent's portions, or only for the non-glycerolated portion followed by glycerolated INRA medium (Salamon and Maxwell, 2000). (d) Saccharose based diluents: Saccharose has been used as the principal component of synthetic extenders because it protects the acrosome integrity of sperm better than glucose, fructose or lactose (Milovanov and Sokolovskaja, 1980, Barbas and Mascarenhas, 2009). Synthetic antioxidants have been used in saccharose extenders to inhibit peroxidation of sperm phospholipids particularly unsaturated fatty acids. Lipid peroxidation is also detected in thawed semen.

Synthetic tocopherol (Vitamin E) has been used as antioxidant in freezing extenders (Salamon and Maxwell, 2000). (e) Raffinose based diluents: In ram and bull freezing extenders some authors have detected better cryoprotective effect with trisaccharides than with mono or disaccharides in stabilizing protein-lipid complex of the sperm membrane (Salamon and Maxwel, 2000). (f) Tris based diluents: Tris extenders are frequently used for semen cryopreservation of bulls, rams and bucks (Purdy, 2006). Ram spermatozoa can tolerate Tris concentrations from 250 to 400 mM, and glucose is a better sugar component in Tris medium than fructose, lactose or raffinose (Salamon and Maxwell, 2000). Fisher et al.,(1987) reported Tris to be best in preserving acrosomal integrity and motility after thawing with an osmolality of 375 mOsm/kg containing 2% egg yolk. Semen frozen in Triladyl® (a Tris based diluent) have reasonable fertility after sheep transcervical insemination. This extender has given better *in vitro* results than lactose–yolk and saccharose–lactose–yolk, and the addition of 2% bovine serum albumin improved its protective effect on acrosome integrity. Tris based extenders used for semen freezing (buck and ram) normally contains fructose or lactose, lowering their normal concentrations compared to other extenders (Purdy, 2006).

However, currently the TRIS-egg yolk-glucose and non-fat dried skimmed milk extenders are most commonly used for cryopreserving goat sperm (Purdy, 2006). The dilution of goat semen into diluents containing egg yolk or milk can have a detrimental effect on the quality of the sperm cells during cooling and cryopreservation (Pellicer-Rubio and Combarnous, 1998 and Sias et al., 2005). The addition of egg yolk not only reduces sperm motility and sperm viability but also increases the incidence of acrosomal damage in several species (Aboagla et al., 2004, Ritar et al., 1991, Julian et al., 2006). Similarly, a protein from the goat bulbourethral gland (SBUIII), identified as 55–60 kDa glycoprotein lipase (BUSgp 60) is responsible for hydrolysis of plasma membrane triglycerides and triglycerides in the skimmed milk that results in fatty acid production which is toxic to sperm (Pellicer-Rubio and Combarnous, 1998, Pellicer-Rubio, 1997) *(g) Other diluents:* Zwitterion buffers which are synthetic, such as Tes, hepes, and pipes have been used with varying success as the basis of diluents for freezing ram semen. Fertility of semen extended in Test medium (Tes titrated with Tris) varied from poor to good results (13–67%). Fertility rate was lower in semen frozen in zwitterions buffers than in Tris–glucose–yolk (Salamon and Maxwell, 2000). *(h) soy-based extender*: the use of a soy based-extender rather than egg yolk to prevent large reduction in motility and result in better post-thaw motility(Roof *et al*., 2012). Soy lecithin and egg yolk differ in lipid composition and fatty acid content (Le Grandois et al., 2009) and might interact differently with the lipase enzymes in goat seminal fluid. Limiting negative interactions between the extender and lipases is useful in large scale goat AI programs (Nordstoga et al., 2011). Sariozkan *et al*. (2010) reported higher CASA motility values for goat sperm frozen in Bioxcell(Soy-based extender) compared to a Tris-based egg yolk extender.

Although iso-osmotic extenders have generally been used as semen diluents, previous reports have shown the freezing of mammalian sperm cells in hypertonic extenders to improve the rate of sperm viability and acrosome integrity (De Leeuw et al., 1993, Molinia et al., 1994a, Woelders et al., 1997, Yildiz et al., 2000, Farshad and Akhondzadeh, 2008, Khalili et al., 2009). According to these studies and the observations of Zuther et al.(2004) supplementation of extenders with sugars, such as sucrose, trehalose or raffinose protect the sperm cells against freeze-damage. These sugars, as non-permeating cryoprotectants are not able to diffuse across the plasma membrane, but create an osmotic pressure that induces cell dehydration before freezing, thus decreasing the extent of cell injury by intracellular ice formation (Molinia et al., 1994a, Liu et al., 1998, Jafaroghli et al., 2011). In addition, these sugars interact with the plasma membrane phospholipids, reorganizing the sperm membrane and increasing its fluidity. Thus inducing a depression in the membrane phase transition temperature of the dry lipids to form a glass (Molinia et al., 1994a, Aisen et al., 2002, Eiman and Terada, 2003, Fernandez-Santos et al., 2007).

Cryoprotectants

A cryoprotectant is a substance that is used to protect biological tissue from freezing damage (damage due to ice formation). It is included in a cryopreservation medium to minimize the physical and chemical stresses resulting from the cooling, freezing and thawing of sperm cells. In general cryoprotectants are broadly classified into two classes, penetrating and non penetrating cryoprotectants. Penetrating cryoprotectants pass through the sperm membrane and act both intracellularily and extracellularily while non–penetrating cryoprotectant acts only extracellularily, includes proteins such as in milk or egg yolk, sugars such as fructose, lactose, mannose, raffinose or trehalose, synthetic polymers such as polyvinylpyrrolidine, methyl cellulose and amides.

Glycerol is the most common penetrating cryopritectant although DMSO and propylene glycol had been used with some cells. Most penetrating cryoprotectants serve as both a solvent and sugars are

solutes or colloids and cannot serve as a solvent compound placed into a solvent, such as water which dissolve and from a true solution or ionized are termed as salutes. Many membrane–permeable cryoprotectants (glycerol, diethyl sulfoxide, ethylene glycol, and propylene glycol), and their combinations, have been tested with buck sperm (Ritar et al 1990a, Ritar et al 1990b, Singh at al, 1995, Leboeuf et al, 2000). Glycerol, dimethy1 sulfoxide (Me_2SO), and ethylene glycol are generally used in a range of 1-8 % but the greatest recovery of sperm post-thawing has been achieved with glycerol (Tuli and Holtz, 1994, Singh et al., 1995). Combinations of cryoprotectants, such as glycerol and Me_2SO, have also been used, and yielded positive results.

Antioxidants

Antioxidants are the compounds which dispose, scavenge, and suppress the formation of ROS, or oppose their actions. Spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma or in spermatozoa itself to prevent oxidative damage (Saraswat et al., 2012). An antioxidant that reduces oxidative stress and improves sperm motility could be useful in the management of male infertility (Bansal et al., 2008)]. Antioxidants are the agents, which break the oxidative chain reaction, thereby, reduce the oxidative stress. Vitamin E (antioxidant) may directly quench the free radicals such as peroxyl and alkoxyl generated during ferrous ascorbate-induced LPO, thus it is suggested as major chain breaking antioxidant (Saraswat *et al*., 2012). Thiol groups play an important role in detoxification and antioxidation of ROS, besides maintaining the intracellular redox status. These groups serve as defense mechanisms of sperm cells to fight against oxidative stress (Saraswat *et al*., 2012). A variety of biological and chemical antioxidants that attack ROS and LPO are presently under investigation (Sikka, 1996). Studies demonstrate that supplementation of cryopreservation extenders with antioxidants has been shown to provide a cryoprotective effect on bull, ram, goat, boar, canine, and human sperm quality, thus improving semen parameters, for example, sperm motility, membrane integrity after thawing (Bucak et al., 2010). Supplementation with these antioxidants prior to the cryopreservation process may be recommended to facilitate the enhancement of sperm cryopreservation technique for the goat breeding industry (Saraswat *et al*., 2012). Addition of antioxidants vitamin E, butylated hydroxytoluene (BHT), and Tempo to extended turkey semen improves sperm survival and membrane integrity and reduces the loss of motility after 48 h of storage (Uysal et al., 2007) similarly addition of vitamin C(3mM), glutathione reduced(5mM) in semen extender provided great benefit to buck semen during storage at refrigeration temperature (5^0C)(Saraswat et al., 2011 and 2012).

Cryopreservation principle and action

The cryopreservation of reproductive cells is the process of freezing, storage, and thawing of spermatozoa, to low sub-zero temperatures, such as (typically) 77 K or −196 °C (the boiling point of liquid nitrogen). At these low temperatures, any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. However, it involves an initial exposure to cryoprotectants, cooling to subzero temperature, storage, thawing and finally with a return to a physiological environment that will allow subsequent development. Proper management of the osmotic pressure to avoid damage due to intracellular ice formation is crucial for successful freezing and thawing procedure.

Main techniques for Cryopreservation

There are two main conventional freezing techniques: slow freezing and rapid freezing, currently vitrification is also used in sperm cryopreservation.

*Slow Freezing***:** The slow freezing technique proposed by Behrman and Sawada (Behrman and Sawada, 1966) consists of progressive sperm cooling over a period of 2–4 h in two or three steps, either manually or automatically using a semi-programmable freezer.

The manual method is performed by simultaneously decreasing the temperature

of the semen while adding a cryoprotectant (uses low concentrations of cryoprotectants which are associated with chemical toxicity and osmotic shock such as glycerol or dimethyl sulfoxide) in a stepwise manner. The sample is frozen from 5°C to −80°C at a rate of 1–10°C/min. The specimen is then plunged into liquid nitrogen at −196°C (Thachil and Jewett, 1981, Said et al., 2010).

In spite of reports showing successful sperm freezing with manual techniques, the reproducibility of this procedure could pose some problems. For this reason, programmable freezers have been investigated (Holt, 2000, Santo et al., 2012). The freezers use a plate to hold the straws; these are cooled by liquid nitrogen held in a storage tank under the plate. Liquid nitrogen is poured into the tank, and the machine, once programmed, uses the software data logging to obtain cooling from 20°C to −80°C at rate of 1.5°C/min and then at 6°C/min; at completion of the freezing the straws are removed and stored into liquid nitrogen at −196°C. This takes about 40 min (Holt, 2000). Programmes are simple to use and allow for a cooling combination which does not require continuous operator intervention and have been used to increase the reproducibility of the freezing operations (Holt, 2000).

Some authors argue that conventional slow freezing, either manual or automated, causes extensive chemical-physical damage to the sperm probably because of ice crystallization(Mazur et al., 1981, Santo et al., 2012).

*Rapid Freezing***:** Rapid freezing was first proposed by Sherman (Sherman, 1990). This technique requires direct contact between the straws and the nitrogen vapours for 8–10 min and immersion in liquid nitrogen at −196°C. Inside nitrogen vapours there is a thermal gradient, as a function of the distance and the volume of the liquid below. The sample is initially mixed in dropwise manner with equal volume of cold cryoprotectant; the mixture is loaded into the straws and left to incubate at 4°C for 10 minutes. The straws are then placed at a distance of 15–20 cm above the level of liquid nitrogen (−80°C) for 15 min; after this stage, the straws are immersed in liquid nitrogen. During cooling it is preferable to place the straws in horizontal position to minimize the heat difference between the two ends. This technique has some drawbacks for example, low reproducibility, and the temperature drop curve cannot be controlled, and the freezing temperatures may vary from −70, −80, and −99°C (Fabbri et al., 2004).

*Vitrification***:** Researchers have developed a new technique, vitrification, Vitrification does not need expensive freezing equipment and the method (vitrification/warming) only takes a few seconds (Isachenko, 2003). Classical vitrification requires a high proportion of permeable cryoprotectants in the medium (30–50% compared with 5–7% for slow freezing) and seems to be inadequate for sperm cells, due to lethal osmotic effects and possible chemical alterations. The cryoprotectants act like antifreeze, they lower the freezing temperature and increase the viscosity instead of crystallizing, the syrupy solution turns into an amorphous ice—i.e., it vitrifies. Rather than a phase change from liquid to solid by crystallization, the amorphous state is like a "solid liquid", and the transformation is over a small temperature range described as the glass transition temperature.

Two conditions usually required to allow vitrification, an increase in the viscosity and a depression of the freezing temperature. Many solutes do both, but larger molecules generally have larger effect, particularly on viscosity. Rapid cooling also promotes vitrification.

Semen processing and freezing rates

After dilution, semen is cooled to a temperature close to +4°C. During this period of refrigeration, there is an adaptation of sperm cells to a reduced metabolism. Equilibration means the time interval the sperm cell remains in contact with glycerol (before freezing), to allow cryoprotectant to penetrate the cells, enabling equilibrium between intra and extracellular concentrations of glycerol and other osmotically active extender elements (Evans and Maxwell, 1987).

Semen freezing is greatly influenced by the semen dilution rate. Semen is extended for protecting spermatozoa during cooling, freezing and thawing. Some authors use a dilution rate of 2–5 folds, with the extender composition adjusted to the dilution rate (Evans and Maxwell, 1987).

After dilution, semen is cooled to a temperature close to +4°C. During this period of refrigeration, there is an adaptation of sperm cells to a reduced metabolism. Equilibration means the time interval the sperm cell remains in contact with glycerol (before freezing), to allow cryoprotectant to penetrate the cells, enabling equilibrium between intra and extracellular concentrations of glycerol and other osmotically active extender elements (Evans and Maxwell 1987, Barbas and Mascarenhas, 2009). During equilibration, glycerol may participate in some harmful events, as alterations in the structure and biochemical integrity of spermatozoa and the increase of acrosome reaction that will reduce fertility.

Rapid cooling of extended semen from 30 to 15°C may not affect sperm survival (Lebouef et al., 2000). By the contrary fast cooling from 30 to 10, 5 or 0°C, decreases post thaw motility and sperm fertility (Fisher et al., 1987, Evans and Maxwell, 1987). Afterwards mini straws are horizontally placed in a rack inside a metallic box containing nitrogen (LN_2), 4–6 cm above the liquid nitrogen surface, undergoing freezing in LN_2 vapours during 20 min., freezing rate is regulated by the distance between the straws and the level of liquid nitrogen. Ram and buck spermatozoa packed in straws tolerate a variation in the freezing rates. Placing straws over liquid nitrogen vapours, at temperatures between −75 and −125°C has no effect on sperm quality. By the contrary, temperatures of −55°C reduce sperm survival (Evans and Maxwell, 1987). Freezing rates from 50 to 100°C/min are usually selected for freezing ram and buck semen. However, from −10 to −60°C, we must use cooling rates faster than 50°C/min. Afterwards we can slow freezing rate (20–30°C/min) until freezing is complete (Byrne et al., 2000, Anel et al., 2003). It is advised to freeze semen according to a parabola-shaped curve, which is possible putting the straws 4–6 cm above liquid nitrogen. The most adequate freezing rate is the fastest one that allows extracellular water freezing without intracellular ice formation. Optimal freezing rates for sperm vary according to species from 1 to 10°C/min for humans and 50 to 100°C/min for bulls (Woelders, 1997).

Thawing

Ram and buck semen is generally thawed at 38–42°C during 30 seconds, but thawing at higher temperatures (60–75°C) produces similar post-thaw motility, acrosome integrity and fertility of spermatozoa (Evans and Maxwell, 1987). Semen may be thawed in physiological serum or in a variable thawing solution, depending on the composition of the freezing extender (without glycerol). Some researchers found a relationship between the composition of the freezing extender and that of the thawing solution when Tris-based freezing media were used (Evans and Maxwell, 1987, Salamon and Maxwell, 2000).

Freeze- thawing and its effects

The sperm cryopreservation includes temperature reduction, cellular dehydration, freezing and thawing (Medeiros et al., 2002). Semen freezing induces the formation of intracellular ice crystals, the osmotic and chilling injury that gives rise to several sperm damages, namely cytoplasmic fracture, effects on the cytoskeleton and genome related structures (Isachenko, 2003).

Membrane permeability is increased after cooling and this may be a consequence of increased membrane leakiness and specific protein channels. Calcium regulation is affected by cooling and this has severe consequences in cell function, inclusively cell death. The uptake of calcium during cooling influences capacitation changes and fusion events between plasma membrane and acrosomal membrane. Sperm membrane is a structure that undergoes reorganization during capacitation. Cold shock reduces membrane permeability to water, solutes and injures acrosomal membranes (Purdy, 2006).

The main changes that occurs during freezing are mainly ultrastructural, biochemical and functional, which impairs sperm transport and survival in the female reproductive tract and reduces fertility in domestic species (Barbas and Mascarenhas, 2009). The ultra structural damage is greater in ram than bull spermatozoa. Greater damages have been detected in plasma and acrosome membranes, mitochondrial sheath and axoneme (Salamon and Maxwell, 2000).

In frozen-thawed semen, motility of sperm cells is better preserved than its morphological integrity. Plasma and outer acrosome membranes are the most cryo-sensitive. Biochemical changes have also been detected, including the release of glutamic-oxaloacetic transaminase (GOT), losses of lipoproteins and amino acids, decrease in phosphatase activity, decrease in loosely bound cholesterol protein, increase in sodium and decrease in potassium content, inactivation of hyaluronidase and acrosin enzyme, loss of prostaglandins, reduction of ATP and ADP synthesis and decrease in acrosomal proteolytic activity (Salamon and Maxwell, 1995b).

Progressive Motility

The evaluation of sperm cell motility and morphology is an essential parameter in the examination of sperm quality and the establishment of correlations between sperm quality and fertility (Voss et al., 1981, Salamon and Maxwell, 2000). Due to this, the biggest obstacle to the exploitation of frozen semen is that the freeze–thawing process of goat sperm generally leads to a decrease in the percentage of motile and viable sperm cells after thawing as a result of damage to membrane integrity and ultrastructure (Watson, 2000). The evaluation of sperm motility, morphology and acrosomal status is an essential criterion in the evaluation of the quality of a semen sample prior to its use for AI (Salamon and Maxwell, 2000). Also the number of motile cells in frozen-thawed semen is about 50% that in the corresponding fresh sample; however the number of motile sperm per insemination dose varies among species. A sperm cell may be motile but damaged what reduces its fertility (Medeiros et al., 2002). In this sense, it has been widely demonstrated that cryopreservation leads to a decrease in sperm motility measured objectively by computer-aided methods in the goat (Dorado et al., 2007, 2009) and other animal species (Thurston et al., 2001, Martinez-Pastor et al., 2005).

The most common methods to evaluate sperm motility is the microscopic observation of semen samples. This subjective estimation has a reduced fertility predictive value because of the high variability observed between individuals and laboratories (Holt et al., 1994, Verstegen et al., 2002). A more objective and precise assessment of sperm motility can be achieved with Computer-Assisted Sperm Analysis (CASA), in which each sperm head trajectory is reconstructed and its kinetics derived in different species (Verstegen et al., 2002).

Sperm Morphology

An important part of any breeding soundness is an evaluation of sperm morphology. In the most fundamental case, the size and shape of the head, mid piece and tail are examined. Additional information can be gained by evaluating integrity of the acrosome and sperm membranes. It is always the case that some sperm from an ejaculate are morphologically abnormal and a decrease in the number of morphologically normal sperm in ejaculates leads to reduced fertility (Chandler et al., 1988, Gravance et al., 1998). Therefore, the lower fertility of the cryopreserved semen samples may well be a result of a decrease in the number of normal sperm in these samples (Gravance et al., 1997). The presence of an acrosomal cap is an important parameter in the fertilization process and has been also highly related with fertility of frozen semen (Lindsay et al., 2005). Today there are techniques to evaluate sperm chromatin condensation and stability, namely the flow cytometric (FCM) sperm chromatin structure assay (SCSA) (Evenson and Jost 2000) that is a useful predictor of fertility, either *in vivo* or *in vitro* (Gandini et al., 2006). Also sperm chromatin may be

evaluated under a fluorescence microscope after Acridine Orange (AO) staining on slide (Gandini et al., 2006). These ultrastructural and biochemical cryogenic changes in spermatozoa causes reductions in their functional integrity, survival *in vivo* and fertilizing capacity (Evenson and Jost 2000). A lower packing quality (chromatin) in morphologically normal and motile spermatozoa is one of the limiting factors for fertilizing capacity (Gandini et al., 2006). The tertiary and quaternary chromatin structure is essential for protection of genetic information and in early post fertilization events. When DFI (DNA fragmentation index) in thawed semen is higher than 30%, it decreases *in vivo* and *in vitro* fertility (Gandini et al., 2006).

So, the semen quality evaluations are the most important factor in estimation of fertility in domestic animals. Acrosome reaction, Hypo osmotic swelling test and Live and dead test have been used for quality determination of semen.

Live and dead sperm count

Many different staining techniques have been devised for examining sperm morphology. A nigrosin-eosin stain is commonly used because it is effective, simple and in addition allow sperm to be readily visualized, it is so-called "live-dead" stain, allowing one to assess membrane integrity at the same time as morphology.

The assessment of sperm viability is one of the basic elements of semen analysis and is especially important in samples where many sperms are immotile, to distinguish between immotile dead sperm and immotile live sperm. The concept of using eosin to mark dead cell, which takes up eosin and nigiosin as a background stain to increase the contrast between faintly stained cells and an otherwise bright background is well known and widely used. Patil and Raja observed the parent live spermatozoa in Malabari bucks as 61.38 percent. Vital different staining technique are used for counting live and dead spermatozoa in semen smears, however, eosin-nigrosin stain is widely used for this purpose. In this staining technique eosin stains the dead spermatozoa as pink or red whereas live spermatozoa remain colorless, as they are impermeable to the Eosin stain. Nigrosin provides a blue-background. The total number of live spermatozoa is usually higher than total motile spermatozoa because all the live sperms are not necessarily motile. There exists a highly significant positive correlation between motility and percentage of living spermatozoa as well as normal live spermatozoa and fertility (Hafez et al., 1993).

Sperm Abnormalities

Sperm abnormalities is produced by-inheritance, adverse environmental effects, improper handling of semen, disease and extremes of temperature. There may be head, middle piece, neck or tail abnormality which interfere with the progressive motility and the fertility of the spermatozoa. There are several methods of classifying sperm abnormalities and the most convenient one is the classification of abnormality according to the region of spermatozoa. This includes primary and secondary abnormalities

Primary abnormalities: Primary abnormalities are of two types

1. *Head abnormalities*: Giant head (Macro cephalic), Small head (Micro cephalic), Double head, Pyriform heads, Long and narrow heads, Loose abnormal heads, Asymmetrical heads, Primordial cells, Diadem defect (invagination in nucleus).
2. *Middle piece abnormalities*: Double middle piece, Coiled middle piece, Swollen middle piece, Abaxial attached middle piece, Short or enlarged middle piece, Filiform middle piece, Vestigial middle piece. iii) Tail abnormalities: Tightly coiled tails, Double tails, Absent or short tails, Broken tails, Kinky tails, Truncated tails.

Secondary abnormalities: Loose or free normal heads, Detached galea capita, Proximal droplets, Distal droplets, Loose or free middle piece, Loose or free tail, Bent middle piece, Kinked middle piece, Coiled tails, Bent tails, Tufted tails, Dag defect (Split, shattered or fractured mid piece).

Acrosome Integrity

Acrosome is a golgi derived secretary granule that lies as a cap over the anterior portion of the sperm nucleus. It contains several hydrolytic enzymes (Mann and Lutwak–Mann,1981). The acrosomal status is considered as an essential prerequisite to ensure the fertilization (Kakar and Annand, 1984). The acrosome is a membrane enclosed structure covering the anterior part of the sperm nucleus. This structure contains powerful hydrolyzing enzymes and is a basic feature of the sperm head of all mammals (Yanagimanchi, 1994). Acrosome integrity is one of the earliest visual sigh of sperm damage since the spermatozoa with damaged acrosome do not possess fertilizing ability (Lightfoot and Salamon, 1970a, Lightfoot and Salamon, 1970b). As a prerequisite for normal fertilization the content of acrosome is released into the surrounding during the acrosome reaction (AR) (Brick et al., 2009) for the successful penetration of the oocyte. By fusion of the sperm plasma membrane with the outer acrosomal membrane, spermatozoa release their acrosomal enzymatic content by a modified form of exocytosis, enabling them to penetrate the zona pellucida. Purdy (2006) suggested that the decrease of intact acrosome percentage is induced by osmotic damage, but the extent of the damage varies according to the species.

Functional Membrane Integrity (Hypo – osmotic swelling test)

Hypo osmotic swelling test (HOST) developed by Jeyendran (1984) based on the observation of morphological alterations (size increase) when spermatozoa is exposed to hypo-osmotic condition. During the course of reaction (HOST), spermatozoa with intact and functionally active plasma membranes undergo swelling (due to influx of water) and subsequently increase in volume to establish equilibrium between the extra and intra cellular compartments. Thus this technique, being simple and inexpensive may prove useful in studies involving the functional of sperm membrane, possibly predict the spermatozoa ability to fertilize and be used as a means of evaluating male fertility. Functional membrane and acrosome integrities were also assessed using HOS-G test (Selvaraju et al., 2008). The cells expressing tail coiling patterns indicating membrane integrity were counted as HOS positive cells and number of spermatozoa with intact acrosome was also counted (Selvaraju et al., 2009).

Lipid- Peroxidation

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process in which free radicals steal electrons from the lipid in cell membranes, resulting in cell damage. This process proceeds by free radicals chain reaction mechanism. LPO composed of three major steps- Initiation, propagation and termination (Nogushi and Niki, 1999). The LPO reaction result in changes in sperm membrane Fluidity, Loss of membrane integrity as well as irreversible loss of sperm motility (Storey, 1997).

Current state of the art in sperm cryopreservation

Successful sperm cryopreservation requires maintaining the post thaw structural and functional integrity. However, to maintain functional integrity, the compartments of the sperm need to be fully protected so that frozen–thawed sperm can undergo normal fertilization under either *in vitro* or *in vivo* conditions. Differences in the various sperm compartments: we expect that each compartment (i.e. acrosome, flagella, midpiece) of sperm is affected by cryopreservation differently. While motility may be protected at a high level, acrosome integrity may be severely damaged under a similar physical alteration such as osmotic stress (Walters et al., 2005). Although the effects of an entire cryopreservation procedure on acrosome integrity have been investigated to some extent, the specific effects of anisosmotic stress on acrosome integrity have not been previously investigated. The methods of cryopreservation of bull sperm have not changed much in the past few years. There has been some work on the addition of cholesterol to the membrane of bull sperm prior to cryopreservation. Work by Purdy and Graham(2004) has shown that the addition of cholesterol to the membrane improves postthaw motility. In addition,

cholesterol added to the membrane did not inhibit fertility, the ability of the sperm to undergo the capacitation, or acrosome reaction. Work is also being done to improve the methodology for cryopreservation; however, it appears currently that the methods are equal to, but not improved over, current systems.

Currently, methods are being developed to freeze buck and boar sperm by alterations of the freezing medium composition such as the addition of antioxidants (Saraswat et al., 2012, Funahashi and Sano, 2005), various forms of packaging the semen for cryopreservation, and freeze-drying. In addition an effort has been made to investigate the effects of reactive oxygen species on cryopreservation of buck sperm by the addition of antioxidants to the extender prior to freezing and DNA stability (Saraswat et al., 2012). An alternative method of preserving sperm is freeze-drying, which has the advantage of room temperature storage, but freeze-dried sperm must be used in combination with intra cytoplasmic sperm injection (ICSI), as a high percentage of motility is lost. Currently, the biggest drawback to freeze-dried sperm is the low evidence of ICSI-derived offspring.

CONCLUSION

Cryopreservation has become an essential component of the artificial reproductive technologies provides a method to preserve spermatozoa from exotic or endangered species moreover allows import and export between countries and prevent vertical disease transmission. The vitrification method opened new perspectives in cryopreservation of semen. Authors believe that antioxidants addition in semen will gain importance as a major tool in mammalian gamete cryopreservation. Furthermore, cryopreservation of semen provides a means for reducing animal space requirements, and protects valuable animal lines from potential loss due to environmental disasters, genetic drift, reduction of polymorphism in breeding and in breeding depression, preservation of genetic diversity and infectious diseases.

REFERENCES

1. Aisen, E.G., V.H. Medina, A. Venturino. 2002. Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. Theriogenology 57: 1801–1808.
2. Amann, R. and B. Pickett 1987. Principles of cryopreservation and a review of stallion spermatozoa. Equine Ve. Sci. 7:145–173.
3. Anel, L., P. De Paz, M. Alvarez, C.A. Chamorro, J.C. Boixo and A. Manso. 2003. Field and in vitro assay of three methods for freezing ram semen. Theriogenology 60:1293–1308.
4. Bansal, K., G.S. Bilaspuri. 2008. Effect of manganese on bovine sperm motility, viability, and lipid peroxidation in vitro. Animal Reproduction CBRA 5: 90–96.
5. Barbas, J.P. and R.D. Mascarenhas. 2009. Cryopreservation of domestic animal sperm cells. Cell Tissue Bank 10:49–62.
6. Behrman, S.J. and Y. Sawada. 1966. Heterologous and homologous inseminations with human semen frozen and stored in a liquid-nitrogen refrigerator. Fertility and Sterility, 17:457–466.
7. Brick, A., R. Labouriau, P. Christensen. 2009. Dynamics of the induced acrosome reaction in boar sperm evaluated by flow cytometery. Animal Reproduction Science 115: 124-136.
8. Bucak, M.N., S. Sariozkan and P.B. Tuncer. 2010. The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. Small Ruminant Research 89:24–30.
9. Byrne, G.P., P. Lonergan, M. Wade, P. Duffy, A. Donavan, J.P. Hanrahan. 2000. Effect of freezing rate of ram spermatozoa on subsequent fertility in vivo and in vitro. Anim Reprod Sci. 62:265–275.
10. Chandler, J.E., C. Painter, R.W. Adkinson, M.A. Memon and P.G.

Hoyt. 1988. Semen quality characteristics of dairy goats. J Dairy Sci. 71:1638–1646.
11. Curry, M.R., J.D. Millar, P.F. Watson. 1994. Calculated optimal cooling, rates for ram and human sperm cryopreservation fail to confirm with empirical observations. Biol Reprod. 51:1014–1021.
12. De Leeuw, F.E., A.M. De Leeuw, J.H. Den Dass, V. Colenbrander. 1993. Effects of various cryoprotective agents and membrane stabilizing compounds on bull sperm membrane integrity after cooling and freezing. Cryobiology 30: 32–44.
13. Dorado, J., I. Rodriguez and M. Hidalgo. 2007. Cryopreservation of goat spermatozoa: comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. Theriogenology 68: 168–177.
14. Dorado, J., M. Hidalgo, A. Munoz and I. Rodriguez. 2009. Assessment of goat semen freezability according to the spermatozoa characteristics from fresh and frozen samples. Anim Reprod Sci. 112: 150–157.
15. Eiman, M.E.A. and T. Terada. 2003. Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. Biol Reprod. 69:1245–1250.
16. Evans, G. and W..M.C. Maxwell. 1987. Frozen storage of semen. In: Salamon's Artificial Insemination of Sheep and Goats. Butterworths, Wellington, pp. 122–141.
17. Evenson, D.and L. Jost. 2000. Sperm chromatin structure assay is useful for fertility assessment. Methods Cell Sci. 22:169–189.
18. Fabbri, R., P. Ciotti and B. Di Tommaso. 2004. Tecniche di crioconservazione riproduttiva. Rivista Italiana di Ostetriciae Ginecologia 3: 33–41.
19. Farshad, A. and S. Akhondzadeh. 2008. Effects of sucrose and glycerol during the freezing step of cryopreservation on the viability of goat spermatozoa. Asian-Aust J Anim Sci. 21: 1721–1727.
20. Fernandez-Santos, M.R., F. Martinez-Pastor, V. Garcia-Macias, M.C. Esteso, A.J. Soler, P. DePaz, L. Anel and J.J. Garde. 2007. Extender osmolality and sugar supplementation exert a complex effect on the cryopreservation of Iberian red deer (Cervus elaphus hispanicus) epididymal spermatozoa. Theriogenology 67: 738–753.
21. Fisher, P.S., L. Ainsworth, R.W. Fairfull. 1987. Evaluation of new diluent and different processing procedures for cryopreservation of ram semen. Theriogenology 28: 599–607
22. Funahashi, H. and T. Sano. 2005. Select antioxidants improve the function of extended boar semen stored at 10^0C. *Theriogenology* 63:1605–1616.
23. Gandini, L., F. Lombardo, A. Lenzi and F. Dondero. 2006. Cryopreservation and sperm DNA integrity. Cell Tissue Bank 7: 91–98
24. Gravance, C.G., C. White, K.R. Robertson, Z.J. Champion and P. J. Casey. 1997. The effects of cryopreservation on the morphometric dimensions of caprine sperm heads. Anim Reprod Sci. 49: 37–43.
25. Gravance, C.G., Z.J. Champion and P.J.Casey. 1998. Computer-assisted sperm head morphometry analysis (ASMA) of cryopreserved ram spermatozoa. Theriogenology 49: 1219–1230.
26. Hafez, E.S.E. 1993. Reproduction in farm animals. Pub. Lea and Febiger, philadelaphia, USA.
27. Hammerstedt, R.H., J.K. Graham and J.P. Nolan. 1990. Cryopreservation of mammalian sperm: what we ask them to survive. J Androl. 11: 73–88.
28. Holt, W.V., P.F. Watson, M. Curry and C. Holt. 1994. Reproducibility of computer-aided semen analysis: comparison of five different systems

used in a practical workshop. Fertil Steril. 62:1277–1282.
29. Isachenko, E. 2003. Vitrification of mammalian spermatozoa in the absence of cryoprotectans: from past practical difficulties to present. Reprod Biol. 6: 191–200.
30. Jafaroghlia, M., B. Khalilib, A. Farshad and M.J. Zamirid. 2011. The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. Small Ruminant Research 96: 58–63.
31. Jeyendran, R,S., H.H. Vander Ven, M. Peroz-Pelaez, B.G. Carbo, L.J.D. Zaneveldm. 1984. Development of an assay to assess functional integrity of the human membrane and its relationship to other semen characteristics. Journal of Reproduction and Fertility 70: 219-225.
32. Julian, S.M., J. Toledano-Dıaz, P.P. Antonio, D. Jesu´s, G.B. Amelia and L.S. Antonio. 2006. Effect of egg yolk concentration on cryopreserving Spanish ibex (Capra pyrenaica) epididymal spermatozoa. Theriogenology 66:1219–26.
33. Kakar, S.S. and S.R. Anand. 1984. Acrosomal damage and enzyme leakage during freeze preservation of buffalo spermatozoa. Indian Journal of Experimental Biology 22:5-10.
34. Karatzas, G., A. Karagiannidis, S. Varsakeli, P. Brikas. 1997. Fertility of fresh and frozen-thawed goat semen during the nonbreeding season. Theriogenology 48:1049–1059.
35. Khalili, B., A. Farshad, M.J. Zamiri, A. Rashidi and P. Fazeli. 2009. Effects of sucrose and trehalose on the freezability of Markhoz goat spermatozoa. Asian-Aust. J Anim Sci. 22: 1614–1619.
36. Le Grandois, J., E. Marchioni, M. Zhao, F. Giuffrida, S. Ennahar and F. Bindler. 2009. Investigation of natural phosphatidylcholine sources: separation and identification by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS2) of molecular species. J Agric Food Chem. 57:6014 –20.
37. Leboeuf, B., B. Restall and S. Salamon. 2000. Production and storage of goat semen for artificial insemination. Anim Reprod Sci. 62: 113-141.
38. Lightfoot, R.J. and S. Salamon. 1970a. Fertility of ram spermatozoa frozen by pellet method I: Transport and viability of spermatozoa within the genital tract of the ewe. Journal of Reproduction and Fertility 22:385-398.
39. Lightfoot, R.J. and S. Salamon. 1970b. Fertility of ram spermatozoa frozen by pellet method II: The effect of method of insemination on fertilization and embryonic mortality. Journal of Reproduction and Fertility 22:399-408.
40. Lindsay, G., G. Evans and W.M.C. 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 63: 445–457.
41. Liu, Z., Foote, R.H. and C.C. Brockett. 1998. Survival of bull sperm frozen at different rates in media varying in osmolarity. Cryobiology 37: 219– 230.
42. Mann, T. and C. Lutwak-Mann. 1981. In “Male reproduction function of semen”. Sprinager-Verlag, Berlin, Heidelberg, New York.
43. Martinez-Pastor, F., V. Garcia-Macias, M. Alvarez, P. Herrez, L. Ane and P. De Paz. 2005. Sperm subpopulations in Iberian red deer epididymal sperm and their changes through the cryopreservation process. Biol Reprod. 72: 316–327.
44. Matsuoka, T., H. Imai, H. Kohno and Y. Fukui. 2006. Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen thawed ram spermatozoa. J Reprod Dev. 52: 675–683.
45. Mazur, P., W. F. Rall and N. Rigopoulos. 1981. Relative contributions of the fraction of unfrozen water and of salt

concentration to the survival of slowly frozen human erythrocytes. Biophysical Journal 36: 653–675,
46. Medeiros, C.M, F. Forell, A.T. Oliveira and J.L. Rodrigues. 2002. Current status of sperm cryopreservation: why isn't better. Theriogenology 57:327–344.
47. Milovanov, V.K. and II. Sokolovskaja. 2000. The results and perspectives of scientific research. Zhivotnovodstvo No. 10, 48–50 (in Russian). Cited by Salamon and Maxwell, 1980.
48. Molinia, F.C., G. Evans, P.I. Casares and W.M.C. Maxwell. 1994a. Effect of monosaccharide and disaccharides in Tris-based diluents on motility, acrosome integrity and fertility of pellet frozen ram spermatozoa. Anim Reprod Sci. 36: 113–122.
49. Nogushi, N. and E. Niki. 1999. Chemistry of active oxygen species and antioxidants. In Antioxidant status, Det, Nutrition, and Health (edited by Papas, AM.) CRC Press. Washington, D.C. 3-20.
50. Nordstoga, A., L. Soderquist, T. Adnoy and H. Paulenz. 2011. Fertility results after vaginal deposition of frozen-thawed buck semen diluted with two different extenders using one- or two-step procedures. Reprod Domest Anim. 46: 82– 6.
51. Patil, R. and C.K.S.V. Raja. 1978. Effect of season on the semen characteristics of MalabariBucks. Indian Veterinary Journal 55: 761-766.
52. Pellicer-Rubio, M.T. and Y. Combarnous. 1998. Deterioration of goat spermatozoa in skimmed milk-based extenders as a result of oleic acid released by the bulbourethral lipase BUSgp60. J Reprod Fertil. 112: 95–105.
53. Pellicer-Rubio, M.T., T. Magallon and Y. Combarnous. 1997. Deterioration of goat semen viability in milk extenders is due to a bulbourethral 60 kDa glycoprotein with triglyceride lipase activity. Biol Reprod. 30:1023–31.
54. Purdy, P.H. and J.K. Graham. 2004 Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. Cryobiology 48: 36–45.
55. Purdy, P.H.2006. A review on goat sperm cryopreservation. Small Rumin Res. 63: 215–225.
56. Ritar, A.J. and S. Salamon. 1991. Effects of month of collection, method of processing, concentration of egg yolk and duration of frozen storage on viability of Angora goat spermatozoa. Small Ruminant Res. 4:29–37.
57. Ritar, A.J., P.D. Ball and P.J. O' May. 1990b. Examination of methods for the deep freezing of goat semen. Reprod Fertil Dev. 2: 27–34.
58. Ritar, A.J., P.D. Ball and P.J.O'May. 1990a. Artificial insemination of Cashmere goats: effects on fertility and fecundity of intravaginal treatment, method and time of insemination, semen freezing process, number of motile spermatozoa and age of females. Reprod Fertil Dev. 2: 377–384.
59. Rodriguez-Martinez, H. and A.D. Barth. 2007. *In vitro* evaluation of sperm quality related to in vivo function and fertility. Soc Reprod Fertil Suppl 64: 39–54.
60. Roof, D.J., S. Bowley, L.L. Price and D.J. Matsas. 2012. Comparison of two commercial extenders for cryopreservation of goat semen without sperm washing. Theriogenology 77: 412– 420
61. Said, T.M., A. Gaglani and A. Agarwal. 2010. Implication of apoptosis in sperm cryoinjury. Reproductive BioMedicine Online 21: 456–462,
62. Salamon, S and WM Maxwell. 1995b. Frozen storage of ram semen. Anim Reprod Sci. 38:1–36
63. Salamon, S. and WM Maxwell. 2000. Storage of ram semen. Anim Reprod Sci. 62: 77–111.
64. Santo, M.D., N. Tarozzi, M. Nadalini and A. Borini. 2012. Human Sperm

Cryopreservation: Update on Techniques, Effect on DNA Integrity, and Implications for ART. Advances in Urology Article ID 854837, 12 pages
65. Saraswat, S., R. Priyadharsini, S.K. Jindal, S. Yadav, N. Ramachandran, S.D. Kharche and A.K. Goel. 2012. Effect of Antioxidants Supplementation at Refrigeration Temperature on Sperm Motion Characteristics and Membrane Integrity of Sirohi Buck Semen. J Phys Pharm Adv. 2(1): 77-86.
66. Saraswat, S., S.K. Jindal, N. Ramachandran, S. Yadav and R.Priyardshini. 2012. Standardization of antioxidants fortification in frozen buck semen. Indian journal of Small Ruminants 18(1): 47-51
67. Saraswat, S., S.K. Jindal, R. Priyadharsini, N. Ramachandran, S. Yadav, P.K. Rout, S.D. Kharche and A.K. Goel. 2012. The Effect of Antioxidants Supplementation to Cryopreservation Protocol on Seminal Attributes and Sperm Membrane Characteristics in Sirohi Goat. J Phys Pharm Adv. 2(1): 49-58.
68. Saraswat. S. and S.K. Jindal. 2011. Effect of antioxidant fortification on post thaw sperm motility of the Sirohi buck semen. International Conference on frontiers in Reproductive Biotechnology & 21st Annual meeting of the ISSRF February 9-11, 2011, Karnal, India, 249.
69. Sariozkan, S., M.N. Bucak, P.B. Tuncer, U. Tasdemir, H. Kinet and P.A. Ulutas. 2010. Effects of different extenders and centrifugation/ washing on postthaw microscopic-oxidative stress parameters and fertilizing ability of Angora buck sperm. Theriogenology 73:316 –23.
70. Selvaraju, S., S. Nandi, P.S.P., Gupta and J.P. Ravindra. 2008. Effects of Heavy Metals and Pesticides on Buffalo (Bubalus bubalis) Spermatozoa Functions In Vitro. Reprod. Dom Anim, 46: 807-813.
71. Selvaraju, S., I.J.Reddy, S. Nandi, S.B.N. Rao and J.P. Ravindra. 2009. Influence of IGF-I on buffalo (Bubalusbubalis) spermatozoa motility, membrane integrity, lipid peroxidation and fructose uptake in vitro. Anim.Reprod Sci. 113: 60-70.
72. Sherman, J. 1990. Cryopreservation of human semen. In: Keel B, Webster BW, editors Handbook of the Laboratory Diagnosis and Treatment of Infertility. Webster, Eds., CRC Press, Boca Raton, Fla, USA.
73. Sias, B., F. Ferrato, M.T. Pellicer-Rubio, Y. Forgerit, P. Guillouet and B. Leboeuf 2005. Cloning and seasonal secretion of the pancreatic lipase-related protein 2 present in goat seminal plasma. Biochim Biophys Acta. 1686:169–80.
74. Sikka, S.C. 1996. Oxidative stress and role of antioxidants in normal and abnormal sperm function. Frontiers in Bioscience 1: 78–86.
75. Singh, M.P., A..K Sinha, B.K. Singh.1995. Effect of cryoprotectants on certain seminal attributes and on the fertility of buck spermatozoa. Theriogenology 43: 1047-1053.
76. Storey, B.T.1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Molecular Human Reproduction 3: 203-213.
77. Thachil, J.V., M.A.S. Jewett. 1981. Preservation techniques for human semen. Fertility and Sterility 35: 546–548,
78. Thurston, L.M., P.F. Watson, A.J. Mileham and W.V. Holt. 2001. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl. 22: 382–394.
79. Tuli, R.K. and W. Holtz. 1994. Effect of glycerolization procedure

and removal of seminal plasma on post-thaw survival and GOT-release from Boer goat spermatozoa. Theriogenology 42: 547–555.
80. Uysal, O.and M.N. Bucak. 2007. Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. Acta Veterinaria Brno, 76: 383–390.
81. Verstegen, J., M. Iguer-Ouada and K. Onclin. 2002. Computer assisted analyzers in andrology research and veterinary practice. Theriogenology 57: 149–179.
82. Voss, J.L., B.W. Picket and E.L. Squires. 1981. Stallion spermatozoal morphology and motility and their relationship to fertility. J Am Vet Med Assoc. 178:287–289.
83. Walters, E. and H. Men. 2005. Osmotic tolerance of mouse spermatozoa from various genetic backgrounds: Acrosome integrity, membrane integrity, and maintenance of motility. Cryobiology 50: 193-205.
84. Watson, P.F. 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod Sci. 60–61: 481–492.
85. Woelders, H., A. Matthijs and B. Engel. 1997. Effects of trehalose and sucrose, osmolality of the freezing medium and cooling rate on viability and intactness of bull sperm after freezing and thawing. Cryobiology, 35: 93–105.
86. Yanagimachi, R. 1994. Mammalian Fertilization. In the physiology of reproduction, Eds. E Knobil& J Neil. New York: Raven Press, 189-317.
87. Yildiz, C., A. Kaya, M. Aksoy and T. Tekeli .2000, Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. Theriogenology 54: 579–585.
88. Zuther, E., K. Buchel, M. Hundertmark, M. Stitt, D. Hincha, and AG Heyer. 2004. The role of raffinose in the cold acclimation response of Arabidopsis thaliana. FEBS Lett. 576: 169–173.

