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Response of Spermatozoa to Cooling and Theory of Cell Damage

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When sperm in suspension are cooled, frozen and then thawed a number of critical physical changes occur. Damage to sperm as a result of cryopreservation can include; loss of progressive motility, morphological and sperm chromatin alterations, membrane permeability changes, membrane destabilization and generation of reactive oxygen species (ROS). Some of these changes are non-lethal but can lead to premature capacitation of sperm so that when thawed many of the sperm have a shortened time period for which they retain the functional ability to bind and fertilize oocytes. Successful cryopreservation with retention of fertilizing ability requires that these changes are minimized. It is therefore critical to thoroughly understand the nature of these changes in order to design successful cryopreservation protocols.

Sperm are sensitive to rapid temperature changes from 35C to near 0C and damage that occurs during this phase is termed "cold shock". At body temperature sperm membranes are composed of lipids and proteins arranged as a bilayer with the hydrophilic ends of the lipids external and the hydrophobic ends internal. The predominant lipids in sperm membranes are phospholipids and cholesterol. (Figure 1) The ratio of phospholipid

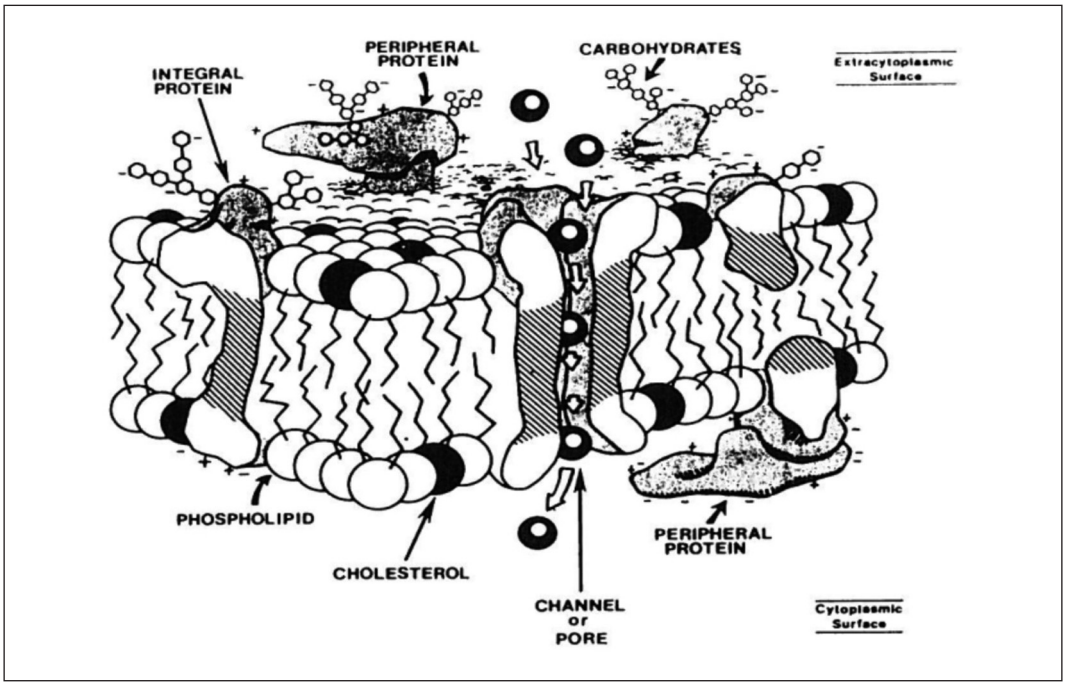

Figure 1. *From Amann and Pickett, JEVS, vol. 7, No. 3, 1987.*

to cholesterol in sperm membranes along with other membrane characteristics differs from species to species and these differences affect how susceptible sperm are to damage from cold shock. Proteins make up 50% of the weight of most membranes and are interspersed throughout the membrane. Some proteins are embedded through both layers of the membrane (integral) and others are only partially embedded or adsorbed loosely to the membrane surface (peripheral). Integral proteins form channels or pores through which small molecules and ions can pass. Many proteins in the sperm membrane contain carbohydrate side chains that are negatively charged and extend from the surface of the membrane. The negatively charged carbohydrates tend to attract and bind other proteins and glycoproteins and therefore the surface of the plasma membrane is very complex and unstable.

Cold shock is characterized by changes in sperm membrane fluidity during which lipids undergo a phase change from liquid-crystalline to gel phase, become more rigid and lose their ability to move laterally within the membrane bilayer. This creates microdomains of non-bilayer forming lipids and modified protein environments which results in changes to membrane permeability for water and solutes. Aggregates of proteins within the lipid bilayer will often remain after rewarming of the cells and result in increased membrane permeability. This increased membrane permeability to calcium and other molecules can trigger the cascade of events characteristic of normal capacitation and an acceleration of the events leading to the acrosome reaction and rapid cell death. The severity of damage from cold shock can be minimized by controlling the rate of cooling from 20C to 5C and diluting the semen in extenders containing protective components such as milk and egg yolk. Although the mechanism is unclear, it is believed that the protein fractions of milk and the low density lipoprotein and phospholipid components of egg yolk protect sperm from cold shock through close association but not alteration of the sperm membrane. While reducing the temperature of sperm from 37C to 5C greatly reduces sperm metabolism, it does not halt it and therefore prolonged storage of sperm requires that they be cooled to approximately -130C at a temperature below which thermally driven chemical reactions do not occur. It is also evident that maintaining viable sperm at liquid nitrogen temperature (-196C) is not the problem but rather cooling and warming sperm through sensitive temperature ranges of about -15C and -60C is where most damage occurs.

Damage to sperm that occurs during freezing and thawing is primarily due to two main causes; ice formation (primarily intracellular) and exposure to severe hyperosmotic conditions (solution-effect injury). Successful cryopreservation protocols must achieve a balance between damage due to large internal ice crystal formation and damage due to solution effect injury.

When sperm in a suspension are cooled below 0C, intracellular and extracellular water remains unfrozen (supercooled) due to the fact that the freezing point of the extender and intracellular liquid is below 0C. At -5C to -10C, extracellular ice crystals begin to form and the sperm are increasingly restricted to channels of unfrozen extender that now contains an increased concentration of solutes. Because the plasma membrane acts as an osmometer, unfrozen water within the cell moves across the membrane to balance the osmotic gradient caused by extracellular ice formation. As the temperature is further reduced, more extracellular ice is formed, more water moves out of the cell and the cell dehydrates until the temperature reaches a point where any remaining unfrozen water and ice reach equilibrium. If the cooling rate is moderate, the cell does not dehydrate sufficiently and because the cooling rate is not fast enough, large intracellular ice crystals are formed. Large intracellular ice is probably physically damaging to sperm membranes however very rapid cooling results in small internal ice crystal formation which is less damaging. If the cooling rate is very slow, the sperm dehydrates to a great extent and as a result the decrease in cell volume causes the membranes to fold as the surface area of the cell is decreased. Regardless of the cooling rate, some cellular dehydration occurs and

likely results in folding of the membranes. This folding creates a situation where common layers of the bilayer are brought together in close proximity (inner halves for exvagination folding and outer halves for invagination folding). Either situation may result in fusing or interactions of surface components that permanently alter the membrane structure when sperm are thawed and the original cell volume is restored. Prolonged exposure to high solute concentrations is damaging to sperm as a result of denaturation of macromolecules and extreme cell shrinkage. The ideal cooling rate for sperm must be slow enough to allow partial dehydration and prevent large intracellular ice formation but rapid enough to limit exposure of sperm to high solute concentrations.

Cryoprotective agents (CPA's) are added to semen extenders to protect sperm and minimize freeze-thaw damage. The most common CPA added to freezing extenders for sperm is glycerol and is typically included in extenders for stallion sperm at concentrations of 2 to 5%. Alternative CPA's including ethylene glycol, propylene glycol, DMSO and amides such as methyl formamide and dimethyl formamide have also been used to successfully cryopreserve stallion sperm. Although glycerol (and the other CPA's mentioned) can penetrate sperm membranes they probably have their major effect as external CPA's by stimulating osmotic cell dehydration and decreasing the amount of intracellular water available for ice formation. Sugars such as lactose and manose do not penetrate membranes and can also act as external CPA's by altering the phase transition properties of membranes, raising the percentage of unfrozen water at a given temperature and reducing salt concentration in unfrozen water. Glycerol also has beneficial actions as a penetrating CPA by replacing water from the dehydrated cell thereby limiting damage to increased intracellular solute concentrations and maintenance of cellular volume. It also decreases the freezing point of intracellular water and interacts with ions and macromolecules. All penetrating CPA's are toxic to sperm at high concentrations and therefore should only be included at low concentrations sufficient to protect the cells. Penetrating CPA's may be damaging to sperm primarily during thawing due to the effect of cell volume changes related to rehydration of the sperm. When sperm are in equilibrium with freezing extender containing glycerol, the concentration of glycerol is the same on both sides of the cell membrane. Upon thawing, extracellular ice melts and water moves back into the cell to balance the solute concentration. When thawed sperm are placed in an environment without glycerol such as non-glycerol containing extender or the female reproductive tract for insemination an osmotic gradient for glycerol is established. Because of the difference in membrane permeability between water and glycerol, water moves into the cell at a much faster rate than glycerol can diffuse out. This causes a rapid increase in cell volume as water moves into the cell to balance the glycerol concentration and then slowly returns to normal after the glycerol has left the cell. Some alternative CPA's such as ethylene glycol have increased membrane permeability rates and theoretically would result in less damage due to these cell volume changes although in practice glycerol still seems to perform better than the others for cryopreservation of sperm. Damage from this rapid volume change may be minimized by serially diluting CPA's from sperm after thawing and prior to insemination.

In addition to lethal damage from freezing and thawing, latent sperm damage or alterations take place that cause changes in membrane permeability and function as described above. The normal process of capacitation followed by the acrosome reaction involves many biochemical and physical changes that are similar to those observed from sperm following cryopreservation. Alterations of membrane permeability and ionic deregulation, particularly to calcium ions, removal of loosely adsorbed decapacitation factors from the surface of the plasma membrane, changes in membrane structure and composition and increased generation of ROS are normal events leading to fertilization. They are also all possible results of sub lethal cryoinjury.

Fertilization is a complex process and requires a number of functional attributes of both sperm and oocyte. In natural mating, billions of sperm are ejaculated from the stallion directly into the mare's uterus yet only thousands reach the site of fertilization in the oviduct and are available for fertilization and only a single spermatozoon fertilizes the oocyte. Sperm maturation is a lengthy process that starts in the testicle with spermatogenesis, continues as the sperm transit the epididymis where they acquire motility and fertilizing potential and then proceeds within the female reproductive tract as they undergo capacitation and the acrosome reaction in response to signals from the female reproductive tract. Only sperm that have survived this journey and encounter a viable oocyte at the exact right stage of maturation will successfully fertilize. The stallion ejaculate consists of a very heterogeneous population of sperm with respect to this maturation process. Some sperm are in advanced stages of "readiness" to undergo capacitation upon entering the female tract and can quickly capacitate and undergo the acrosome reaction with the proper stimulus so that fertilization of an oocyte already resident in the oviduct at the time of mating can take place. Additionally many sperm are at various pre-capacitation states and through interaction with the oviductal epithelium are able to be maintained within the female tract for several days. Sperm are then gradually released from the oviductal epithelium, capacitate and acrosome react so that fully mature sperm are available for fertilization even when a mare ovulates many days after mating. After freezing and thawing more sperm may already be at an advanced maturation state resulting in reduced binding to the oviductal epithelium and a shorter period after insemination that successful fertilization can take place. This may explain the general tendency for higher pregnancy rates with frozen-thawed semen when insemination takes place closer to ovulation.

The challenge of successful cryopreservation of stallion sperm is to interrupt a carefully and exquisitely designed biological system, retard its progress through temperature reduction and then upon returning the sperm to physiological temperature and deposition in the female reproductive tract hope that a sufficient number of functional characteristics have been retained to allow efficient resumption of the process.