

Mini Review

Fundamental Principles of Cryopreservation of *Bos taurus* and *Bos indicus* Bull Spermatozoa

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ABSTRACT

This essay illustrates the basic concepts of cryopreservation and the causes of cryoinjury to *Bos taurus* and *Bos indicus* bull spermatozoa. The possible roles of cryoprotectants, cooling/freezing and thawing rates are discussed. Advancement in freezing technology for sexed spermatozoa of bull is also briefly described.

Key Words: Cryopreservation; Bull; Spermatozoa

INTRODUCTION

Cryopreserved bull spermatozoa have been used since the 1950s for the artificial insemination of cattle (Foote & Parks, 1993). Semen cryopreservation of bull involves several steps each, which affects sperm structure and function (Garner *et al.*, 1999; Bailey *et al.*, 2003): extension, cryoprotection, cooling and freezing, storage and thawing (Bailey *et al.*, 2003) (Fig. 1). The plasmamembrane is the primary site of injury to cryopreserved spermatozoa and the principal damage occurs during freezing and thawing (Parks & Graham, 1992) resulting in substantial loss of viable spermatozoa (Bailey *et al.*, 2003). This vulnerability is due to lipids and proteins in the membrane that are not covalently linked and can move easily (Parks & Graham, 1992).

The success of *Bos taurus* or *Bos indicus* bull semen cryopreservation depends on numerous factors, like cryoprotectants, cooling/freezing and thawing rates. Therefore this essay deals with the events taking place during cooling/freezing and thawing of bull spermatozoa and the means to minimize the deleterious affects of cryopreservation.

Events during cooling, freezing and thawing. During the process of cooling, freezing and thawing, bovine spermatozoa are subjected to a series of drastic changes in their physical and chemical environment (Watson, 2000). The first change that the spermatozoa has to cope is with cooling from body temperature to near the freezing point of water (Watson, 2000; Medeiros *et al.*, 2002), which causes phase transitions of the lipids in the membranes of spermatozoa (commonly referred as *cold shock*) (Woelders, 1997). This vulnerability is due to lipids and proteins in the membrane that are not covalently linked and can move easily (Parks & Graham, 1992). It is found that different lipids have different phase transition temperature, some type

of lipid aggregate in domains of gel-like (*frozen*) lipid, thus excluding other lipid types that remain in the liquid-crystalline (*melted*) state (Woelders, 1997; Medeiros *et al.*, 2002). Membrane proteins are also excluded from these gel domains and consequently find themselves in a non-physiological lipid environment (*lateral phase separation*) (Medeiros *et al.*, 2002). This is believed to impair function of membrane proteins that are necessary for structural integrity (*cytoskeleton*) or ion metabolism (*ion pumps*) (Watson, 2000). Major changes in bull spermatozoa during this phase occur in the vicinity of 15 to 5°C and do not occur below 0°C (Watson, 2000).

A second change in the environment of *Bos taurus* or *Bos indicus* bull spermatozoa takes place when liquid water is converted into ice (commonly referred as *ice crystal formation*). Spontaneous ice nucleation usually occurs after the solution is *supercooled* to a temperature between -5 and -15°C (Woelders, 1997). This is due to the fact that small ice crystals have a large surface tension, which makes them thermodynamically un-stable. Therefore, spontaneous ice nucleation is a random process, occurring only, when by chance, enough molecules with lower than average kinetic energy get together and form a stable ice nucleus. Once this has happened, the ice crystals grow rapidly in all directions. The release of the *latent heat of fusion* then causes the sample to warm up abruptly, until the freezing/melting temperature of the solution (of the remaining un-frozen fraction) is reached (Watson, 2000). At this point, ice formation stops or proceeds at a rate governed by the rate at which the sample is further cooled and what remains is so called *un-frozen fraction* in which all solutes are confined. The concentration of the sugars, salts and cryoprotectants like glycerol and consequently, also the osmotic pressure of the un-frozen fraction increase rapidly. At the same time, the volume of un-frozen fraction decreases rapidly. The increase in the osmotic strength causes an efflux of water

from the cells. Therefore, the intracellular concentration of salts and glycerol also increases until the osmotic pressure inside the cells is as high as that outside the cells (Medeiros *et al.*, 2002). As the freezing continues to approximately -50°C (Kumar *et al.*, 2003), this phenomenon continues, until the viscosity of the un-frozen fraction becomes too high for any further crystallization (Holt, 2000). The hazardous effects of freezing phase on sperm structure and function due to the above mentioned physicochemical changes are (Woelders, 1997; Han & Bischof, 2004).

- The fast efflux of water causes a rapid decrease in the volume of spermatozoa, which leads to the structural deformation. The spermatozoa find themselves confined to very narrower channels of un-frozen solution, squeezed in between growing masses of ice, which could also lead to mechanical stress in cells.

- Extremely high concentrations of salts could be a cause of damage by itself, affecting the cell membrane or intracellular components. Also extracellular salts could leak into cells, changing the intracellular ion composition.

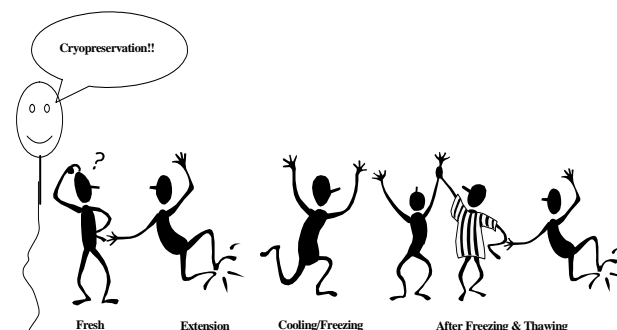
- *Eutectic crystallization* of solutes could take place and cause damage to the spermatozoa.

Thirdly, frozen bull spermatozoa are injured during the thawing process, which has been regarded as being due to recrystallization of ultra-microscopic ice crystals to form comparatively large ice crystals (Watson, 1995). The warming damage occurs when the spermatozoa pass through the critical zone of -50 to -15°C or up to -5°C (Mazur, 1984; Kumar *et al.*, 2003). Likewise, spermatozoa suffer osmotic stress, when the duration of thawing is insufficient for out-flow of excess cryoprotectants from the cell and the spermatozoa swells and lyses as the medium becomes abruptly diluted by the melting of extracellular ice (Pegg, 2002). While Mazur (1984) concluded from the osmotic studies that bull spermatozoa are damaged during thawing by the inflow of water rather than the outflow.

Role of cryoprotective agents. Cold shock to bull spermatozoa can be prevented by adding protective compounds to semen extenders, such as egg yolk (20%). The protective mechanism of egg yolk seems to rely on the phospholipids and low-density lipoproteins. Egg yolk phospholipids can lessen chilling injury on 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).

Freeze/thaw stresses can be minimized by the use of glycerol (4 - 8%), which is an osmotically active permeating cryoprotectant (Garner *et al.*, 1999; Curry, 2000). The physiological actions of glycerol in freezing and thawing of spermatozoa take place by replacing intracellular water necessary for the maintenance of cellular volume, interaction with ions and macromolecules and depressing the freezing point of water so that less ice forms at any given temperature (Holt, 2000; Medeiros *et al.*, 2002). Glycerol though has also been shown to have some adverse

Fig. 1. Pictograph of events during cryopreservation of bull spermatozoa



effects on spermatozoa. The deleterious effects are due to osmotic stress, changes in membrane organization, fluidity and permeability, as well changes in the lipid composition (Watson, 1995). The protective effects, however, outweigh the negative effects (Garner *et al.*, 1999).

Influence of cooling/freezing and thawing rates. Of considerable importance for the freezing regime is the cooling/freezing rate in the critical temperature range (approximately -5 to -50°C) that 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). Therefore, the survivability of the spermatozoa depends upon the optimum-cooling rate. During slow cooling, the dehydration of the spermatozoa can proceed to the point of osmotic equilibrium between intracellular and extracellular space i.e., cellular dehydration will be maximal. While raising the cooling rate too much, the dehydration is not fast enough to prevent occurrence of intracellular ice nucleation. However, if the cooling rate is within the required values ($50 - 100^{\circ}\text{C}$) this result in less excessive intracellular dehydration, less excessive intracellular solute concentrations and less shrinkage of the cells (Mazur, 1984; Woelders, 1997). Moreover, at optimum cooling rates, the bull spermatozoa remain vulnerable to the un-favourable conditions for a shorter period of time (Woelders, 1997).

Warming rates depend already on the freezing rate that was chosen. In slow thawing, small ice crystals formed during the freezing start to melt, turning into large crystals (recrystallization) that are harmful to the spermatozoa (Watson, 1995). During fast (optimum; at 37°C for at least 45 sec) thawing the time for recrystallization to occur is limited and this increases the survivability of spermatozoa.

Cryopreservation of sorted (X & Y chromosome-bearing) spermatozoa. There is no doubt that the ability to sort X and Y chromosome-bearing spermatozoa is a significant advancement in the field of assisted reproductive technologies (Agca & Crister, 2002). However, a limited number of bull spermatozoa are available for cryopreservation following sorting and fewer numbers of

the sexed bull spermatozoa are able to survive through the conventional freezing process. A new freezing method "Multi-Thermal-Gradient" (MTG) aims to overcome the problems of conventional freezing protocol for bull spermatozoa (Arav, 1999). This freezing technology is based on directional freezing; the spermatozoa are moved through a linear temperature gradient so that the cooling rate and ice front propagation are precisely controlled. Thus, the spermatozoa are preserved gently between horizontal columns of ice rather than subjecting it to the damaging effects of the random *ice crystal formation* in conventional freezing. The technique also prevents the *dehydration* of sperm inevitable in conventional freezing process and halves the level of glycerol needed. This method also enables the incorporation of controlled seeding into the freezing process (Arav *et al.*, 2002).

CONCLUSIONS

Cryoinjuries experienced by *Bos taurus* or *Bos indicus* bull spermatozoa, while passing through the intermediate zone of temperature during freezing and thawing process are; cold shock (15 to 5°C), ice crystal formation (-5 or -15 to -50°C) and recrystallization of ice crystals (-50 to -15 or -5°C). However, these cryoinjuries can be minimized with the use of cryoprotective agents like egg yolk and glycerol in semen extender. Also optimum freezing and thawing rates can reduce the vulnerability of spermatozoa to freeze/thaw stresses.

The laterally varying gradient used in MTG technology allows cooling to proceed at differing rates under varied temperature regimes. Therefore, dehydration of spermatozoa and random ice crystal formation in conventional freezing can be controlled by MTG freezing technology.

REFERENCES

- Agca, Y. and J.K. Cister, 2002. Cryopreservation of spermatozoa in assisted reproduction. *Semi. Reprod. Med.*, 20: 15-23
- Arav, A., 1999. *Method for Cryopreservation of Biological Samples*. US Patent
- Arav, A., S. Yavin, Y. Zeron, D. Natan, I. Dekel and H. Gacitu, 2002. New trends in gamete's cryopreservation. *Mol. Cell. Endocrinol.*, 187: 77-81
- Bailey, J., A. Morrie and N. Cormier, 2003. Semen cryopreservation: success and persistent in farm species. *Canadian J. Anim. Sci.*, 83: 393-401
- Curry, M.R., 2000. Cryopreservation of semen from domestic livestock. *Rev. Reprod.*, 5: 46-52
- Foote, R.H. and J.E. Parks, 1993. Factors affecting preservation and fertility of bull semen: a brief review. *Reprod. Fertil. Dev.*, 5: 665-73
- Garner, D.L., C.A. Thomas and C.G. Gravance, 1999. The effect of glycerol on the viability, mitochondrial function and acrosomal integrity of bovine spermatozoa. *Reprod. Dom. Anim.*, 34: 399-404
- Han, B. and J.C. Bischof, 2004. Direct cell injury associated with eutectic crystallization during freezing. *Cryobiology*, 48: 8-21
- Holt, W.V., 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. *Theriogenology*, 53: 47-58
- Kumar, S., J.D. Millar and P.F. Watson, 2003. The effect of cooling rate on the survival of cryopreserved bull, ram and boar spermatozoa: a comparison of two controlled-rate cooling machines. *Cryobiology*, 46: 246-53
- Mazur, P., 1984. Freezing of living cells: mechanisms and implications. *American J. Physiol.*, 247: C125-C142
- Medeiros, C.M.O., F. Forell, A.T.D. Oliveria and J.L. Rodrigues, 2002. Current status of sperm cryopreservation: why isn't it better? *Theriogenology*, 57: 327-44
- Parks, J.E. and J.K. Graham, 1992. Effects of cryopreservation procedures on sperm membranes. *Theriogenology*, 38: 209-22
- Pegg, D.E., 2002. The history and principles of cryopreservation. *Semi. Reprod. Med.*, 20: 5-13
- Watson, P.F., 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod. Fertil. Dev.*, 7: 871-91
- Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. *Anim. Reprod. Sci.*, 60/61: 481-2
- Woelders, H., 1997. Fundamentals and recent development in cryopreservation of bull and boar semen. *Vet. Quart.*, 19: 135-8

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