CRYOINJURY TO MAMMALIAN SPERMATOZOA: AN OVERVIEW

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ABSTRACT

Despite many advantages of semen cryopreservation, there is an apparent contradiction between the concept of preservation and experimental findings that spermatozoa can be damaged by the preservation process itself. Sperm cryoinjury is caused by each step or a combination of the steps of cryopreservation. In this paper, various mechanisms of mammalian sperm cryoinjury (e.g., cryoprotective agents and osmotic injuries, hypothermic injury, cryoinjury during storage period, cryoinjury associated with the warming process etc) are briefly reviewed. In the light of this review, further understanding of fundamental cryobiology is needed to develop optimal cryopreservation conditions for sperm of individual species.

KEY WORDS: Sperm, Cryoinjury, Cryopreservation.

INTRODUCTION

Cryobiology is a multidisciplinary science to study the physical and biological behaviour of cells and tissues, including their interactions with environment at low temperatures. An important part in fundamental cryobiology is to reveal the underlying physical and biological mechanisms related to cryoinjury (Gao and Crister, 2000). The exact nature of sperm cryodamage still remains to be elucidated (Oehninger *et al.,* 2000). Further understanding of cryobiology is needed to develop optimal cryopreservation techniques for different mammalian species.

CRYOPROTECTION AND CRYOPROTECTANTS

A cryoprotectant is a substance added to living biological materials e.g. embryo, ova, spermatozoa; that is to be preserved in a viable state by freezing. The common substances are dimethyl sulfoxide (DMSO) and glycerol (Blood *et al.,* 2007). Sperm cryoprotectants are broadly classified into two categories viz. penetrating and non-penetrating cryoprotectants. Penetrating cryoprotectants include glycerol, methanol, ethylene glycol etc, while non-penetrating cryoprotectants include sucrose, fructose, raffinose (Holt, 2000). Glycerol is used at concentrations ranging from 2% to 10% to cryopreserve mammalian sperm and it has shown species specific and concentration-dependent toxicity (Katkov *et al.*, 1998).

PHYSICAL AND CHEMICAL EVENTS DURING CRYOPRESERVATION–THAWING

During the process of cooling, freezing and thawing, spermatozoa are subjected to a series of drastic changes in their environment. The initial cooling may cause phase transitions of the lipids in the plasma membrane and also impair the function of membrane proteins that are necessary for structural integrity or ion metabolism. Freezing may lead to extracellular ice nucleation, which in turn produces osmotic changes that cause efflux of water from the cells. These changes may lead to the loss of stability of the lipid bilayer (membrane leakiness). Alterations of cell-membrane components as a result of delayed reactions such as lipid peroxidation or the phase transition occurring during freezing may make the membrane more prone to early fracture at the stress points (Alvarez and Storey, 1992; Drobnis et al., 1993). Further consequences include denaturation of proteins, structural deformation of the cell organelles and eutectic crystallization of intracellular solutes leading to cell death.

MECHANISM OF SPERM CRYOINJURY

The challenge to spermatozoa during freezing is not their ability to endure storage at very low temperatures (-196°C); rather, it is the lethality of an intermediate zone of temperature (-15°C to -60°C) that spermatozoa must traverse twice - once during cooling and once during warming. Cryobiological research studies the

underlying physical and biological factors affecting survival of cells at low temperatures (during the cooling and warming processes). These factors and mechanisms of cryoinjury and its prevention are reviewed and discussed below.

Cryoprotective agents and osmotic injuries:

The efficacy of a given cryoprotective agent (CPA) for a specific cell type usually depends on the cellular permeability to that solute and its potential chemical toxicity (Fahy *et al.*, 1990). Too slow cooling causes severe volume shrinkage, long-term exposure to high solute concentrations and solidification of intracellular components. Too rapid cooling does not allow fast escape of intracellular water from the cell that leads to intracellular ice formation (Gao and Crister, 2000).

Hypothermic injury:

Slow cooling causes an irreversible damage to the spermatozoa. This is termed as 'cold shock'. Sensitivity to cold shock varies among species; bull and ram sperm being highly sensitive; dog and tom sperm moderately sensitive; and human and rabbit sperm relatively resistant (Watson, 1995). Cold shock destroys the selective permeability of sperm cell membrane to calcium, leading to excessive intracellular levels, which reduces motility and leads to necrosis (Robertson *et al.,* 1990). Plasma membrane is the primary site of injury associated with lipid phase transitions (LPT), which is in some cases irreparable even after cells are returned to physiological temperatures. Plasma membrane proteins like aquaporin transmembrane water channel proteins (AQPs) may also be affected by hypothermia which leads to glucose and fructose depletion from the extracellular environment into the sperm (Calamita *et al.*, 2001).

Cryoinjury during storage period:

Usually, the cryopreserved samples are stored at or below -70°C. Although the question is often asked as to the length of time cells can be kept in the frozen state without damage, the question is probably moot, if the storage temperature is less than -120°C and is certainly moot at -196°C (liquid nitrogen). At less than -120°C, chemical reactions cannot occur and at -196°C, thermally driven reactions are also suspended. The reactions that can occur are the slow accumulation of direct damage from ionizing radiation, but this accumulation would probably become significant only after centuries of storage (Mazur 1984).

Cryoinjury associated with the warming process:

Cells that have survived cooling to subzero temperature are still challenged during warming and thawing, depending on whether the prior rate of cooling has induced intracellular ice formation or cell dehydration. In case of intracellular ice formation, rapid thawing can rescue many cells, possibly because it can prevent the growth of small intracellular ice crystals into harmful large ice crystals, so called recrystallization (Mazur, 1984). For human spermatozoa, slower warming in 20°C or 35°C air results in more optimal sperm cryosurvival in terms of both motility and supravital staining (Gao and Crister, 2000). Slow warming was reported optimal for samples frozen slowly. However, there is a little effect of warming rate when a fast cooling rate is used, probably because the spermatozoa may be killed by the intracellular ice formation during prior fast cooling (Mahadevan, 1980).

Other causes of sperm cryoinjury:

Production of reactive oxygen species (ROS), such as H_2O_2 is known to arrest motility and block oxidative metabolism in sperm. ROS also decrease capacity for sperm-oocyte fusion, and reduce fertility due to plasma membrane lipid peroxidation. The major source of ROS production in the bull and ram semen is the activation of an aromatic amino acid oxidase following death of sperm (Upreti *et al.*, 1998). Some of the observations on the effect of cryopreservation on sperm motility and morphology have been reported by Connell *et al.* (2002) as given in the below Table.

Table: Effect of cryopreservation on human sperm motility and morphology

Parameter	Fresh semen	Freeze thawed semen	% change
Motile sperm (%)	40.2	24.8	-33
Progressively motile sperm (%)	22.9	13.6	-41
Average path velocity (VAP, µm/s)	51.2	35.3	-31
Straight line velocity (VSL, µm/s)	42.1	29.8	-29
Curvilinear velocity (VCL, µm/s)	75.5	51.5	-32
Normal morphology (%)	8.2	5.2	-37
Amorphous cells (%)	12.0	18.0	+50
Midpiece abnormalities (%)	10.0	19.0	+46
Loose head and tail (%)	8.0	11.3	+29
Tail defects (%)	6.0	9.3	+35

Values are medians n =50 (Connell ***et al.*, 2002)**

SPERM CELL DAMAGE DURING CRYOPRESERVATION

It includes damage to the plasma membrane, acrosome, nucleus, mitochondria and enzymes as summarized below.

Plasma membrane:

Plasma membrane is the primary site of cryodamage associated with lipid phase transitions (LPT). Spermatozoa of ram exposed to low temperatures followed by warming affect the plasma membranes over the principal-piece, mid-piece and head (Holt and North, 1994). Lipid phase transitions during cooling are inversely related to the proportion of cholesterol present in sperm. Bull and ram sperm have lower cholesterol levels and are more sensitive to cooling unlike rabbit and human sperm (Drobnis *et al.,* 1993). Cryopreservation causes disturbances in the lipid-lipid and lipid-protein interactions required for normal membrane function (Parks and Graham, 1992).

Acrosome:

Acrosome may show structural damage and degenerative changes like swelling, vesiculation and false acrosome reaction (Nizanski and Kuropka, 2005). Boar sperm are found to experience serious acrosomal damage with one-step addition of 3% glycerol. Bull and human sperm are able to withstand one-step addition of upto 5 to 8% glycerol. Mouse sperm cannot tolerate even 1.5% glycerol and thus, in some cases, this has forced the total elimination of glycerol from the freezing media (Koshimoto *et al.,* 2000).

Dog spermatozoon showing irregular space between inner and outer acrosomal membrane and between outer acrosomal membrane and plasma membrane – fuzzy appearance of acrosome (x 24000) (Nizanski and Kuropka, 2005)

Nucleus:

Acridine orange cytophotometric studies are useful in assessing the effects of cryopreservation on sperm damage. Spermatozoa with intact and damaged chromatin show green and orange fluorescence, respectively (Martins *et al.,* 2007).

Mitochondria:

Sperm mitochondria are localized in the mid-piece and serve as a source of ATP production by oxidative phosphorylation. Once the mitochondria loose their integrity the membrane proton gradient is lost, Thus, ATP production and respiration are blocked and mitochondrial dyes do not sense the inner mitochondrial membrane potential. Rhodamine 123 is used to measure the mitochondrial membrane potential and stain uptake indicates sperm with functional mitochondria. As mitochondria loose their internal structure they are more lightly stained than their unfrozen control (Connell *et al.*, 2002).

Enzyme leakage:

Dilution, glycerolozation, cooling, freezing and thawing cause variable physico-biochemical damage to the sperm plasma membrane, solute concentration and thereby leakage of intracellular material to the etracellular medium. Mostly the fertility and metabolism related acrosomal and mid-piece enzymes, viz. acrosin, hyaluronidase, esterase, transaminases, phosphatases, dehydrogenases, dysmutases etc. are leached out, thus altering the viability and fertilizing ability of spermatozoa. Improper use of semen extenders also causes dilution effect, increases membrane permeability and causes enzyme leakage (Dhami and Kodagali, 1990).

PREVENTIVE MEASURES

Pentoxifylline treatment before freezing improves the acrosome reaction to ionophore challenge in cryopreserved spermatozoa; moreover, pentoxifylline treatment appears to minimize sperm damage during the freeze-thaw process and may improve fertilization rates with assisted reproductive procedures such as intrauterine insemination or in-vitro fertilization (Esteves *et al.*, 1998).

Utilization of free radical scavengers (e.g., alpha-tocopherol and Ascorbate) in the freezing media prevents oxidative damage during cryopreservation (Beconi *et al.,* 1993). Various artificial stimulants have been studied with an aim to enhance sperm recovery following cryopreservation–thawing. Substances such as sodium nitroprusside, pentoxifylline, 2-chloroadenosine and 2-deoxyadenosine have been reported to enhance sperm cryosurvival (Sharma and Agarwal, 1997).

Selection of ideal Tris based buffer and inclusion of extender additives like enzymes, hormones, amino acids, surfactants, membrane stabilizers, antioxidants etc, are now being practiced in many semen banks for improving fertility by reducing cryodamage to the spermatozoa.

CONCLUSIONS

Most spermatozoa will die during cryopreservation unless proper concentrations of the cryoprotective agents are present. Spermatozoa may be damaged by cold shock and it is possible that conditions which allow the sperm plasma membrane to survive may not allow the survival of critical organelles inside. Cryopreservation as such is not a damaging process, However, sperm damage is due to the lethality of an intermediate zone of temperature (-15°C to -60°C) that cells must traverse twice once during cooling and again during warming. Efforts are therefore required to minimize cryodamage and maximize fertility of cryopreserved spermatozoa of all livestock species.

FUTURE THRUST AREAS

More research is to be carried out to determine whether the post-thaw survival of sperm can be improved by freezing selected sub populations rather than the whole semen. Understanding the mechanisms of sperm cryoinjury will make it possible to establish biophysical or mathematical models describing sperm cell responses to environmental changes during the cryopreservation process. Further understanding of fundamental cryobiology is needed to develop optimal cryopreservation conditions for sperm of individual species. The present programmable semen cryopreservation machines need to be updated with advanced technical and computer support.

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