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Review Article

Effect of Cryopreservation on Rat Sperm DNA and involvement of elevated Oxidative Stress, Apoptosis – A Review

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ABSTRACT

Current article explains about the effects of cryopreservation on testicular sperm DNA. Conservation of sperms has several purposes in artificial reproductive technologies (ART), species conservation and clinical medicine. Despite of various advances in cryopreservation methodology, the recovery rate of functional post thawed spermatozoa remains mediocre, with sperm motility being significantly decreased after freezing. Sperm cryopreservation and storage facility currently require liquid nitrogen method for long or short term storage. The combinations of sperm storage involved are temperature, cooling rate, chemical composition of the extender, cryoprotectant concentration, reactive oxygen species (ROS), seminal plasma composition and hygienic control factors that affect the life-span of spermatozoa. The lack of structural integrity to sperm and damage to DNA of sperm shall have a significant negative impact on oocyte fertilization, embryo development rate, and live-birth rate. Hence the research conducted as on today conclude that cryopreservation of testicular spermatozoa may reduce DNA life expectancy; therefore scientists are yet in a position to rectify this troubleshoot for ultimate shell of life of DNA for prolonged periods without damage. This review describes about the effect of oxidative stress and apoptosis as one of the possible mechanisms involved in sperm cryoinjury.

Keywords: Cryopreservation, Sperm, DNA, Oxidative stress, Apoptosis

INTRODUCTION

Cryopreservation of spermatozoa has been possible for many years. The cryopreservation of mammalian sperm is a complex process that involves balancing of many factors in order to obtain satisfactory result. To ensure in minimal success, in cryopreservation proper cryoprotectant, cooling and thawing rates are required. One absolute requirement for the use of cryopreserved sperm is that the cryopreservation process must not induce alterations that would impair progeny development. Many years ago, spermatozoa of many mammalian species were cryopreserved successfully. It is widely used in assisted conception units to preserve male gametes and provided the opportunity for future fertility, for example in the treatment of malignancy and in nonmalignant diseases like autoimmune disorders. Cryopreservation techniques and invitro fertilization (IVF) protocols are useful in genetic research and in the production of transgenic animals. Small part of spermatozoa from fertile men also has detectable levels of DNA damage. Factors that cause the DNA damage include protamine deficiency, apoptosis, chemotherapy, ROS, cigarette smoking and varicoceles. In addition to these it is known that the sperm DNA damage is clearly associated with male infertility. To avoid the population growth due to infertility the sperm cryopreservation has emerged and during sperm cryopreservation the formed intracellular ice crystals, osmotic and chilling injury induces sperm cell damage, cytoplasm fracture and effects on the cytoskeleton or genome related structures [1].

In mouse sperm cryopreservation the problems that were associated on sperm were sensitivity to cold and osmotic effect [2]. The most commonly described adverse effect of freeze–thawing on mouse sperm was dramatic and sharp in decrease in sperm motility and plasma membrane integrity [3-4]. The freezing rate of sperm must be slow enough to allow water to leave the cells by osmosis and preventing intracellular ice formation which was a cause of irreversible damage to sperm cell's membrane [5]. Due to inefficiencies in collecting, freezing and inseminating of frozen ram semen, the sheep industry has not been able to utilize many of assisted reproductive technologies on live stock [6]. Based upon this scientific history, the developments of successful mammalian sperm cryopreservation methods were established. It is critical to realize, only a very few mammalian species sperm can be effectively cryopreserved. Even in those cases, the success as measured by post thawed motility, routinely was 50 percent or less than that of the prefreeze motility. Successful cryopreservation varies highly among species, individuals within species, and even within ejaculates of individuals, which are largely attributed to the differences in biophysical characteristics among cell types [7]. In this review we provide an overview of effects of cryopreservation, which are at risk of viability of semen, seminal oxidative stress, sperm DNA damage and extensive apoptosis.

NEED OF CRYOPRESERVATION

Cryopreservation is a process in which cells or whole tissues are preserved by cooling to low sub-zero temperatures, such as −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. The DNA analysis of sperm in our laboratory has revealed that there exists damage to DNA in normal to 0^{o}C of storage [8]. The IVF results after use of cryopreserved testicular sperm are comparable with

those obtained from the fresh specimens. Lack of sperm motility before cryopreservation does not exclude favorable outcome and therefore testicular sperm freezing is feasible whenever there are enough sperm cells in the extracted testicular tissue [9]. Cryopreservation is a valuable technique used to assist in the genetic improvement of cultured stocks as well as providing a continuous supply of good quality sperm for artificial insemination. Globally, the cryopreservation method includes temperature reduction, cellular dehydration, freezing and thawing [10]. The lowering of temperature from normal (37^{o}C) to 4^{o}C reduces cellular metabolic activity and increases the life span of sperm cells. Hence cryopreservation stops cells cellular activity, and restarts its normal functions after thawing [11].

Cryopreservation is a way of preserving germplasm that have applications in agriculture, aquaculture, biotechnology and conservation of threatened species [12]. In agriculture, germplasm cryopreservations are used for genetic improvement of domestic species, preserve rare breeds (well adapted to environmental changes), and in international germplasm exchanges [13]. Germplasm cryopreservation has essential applications in the production and conservation of transgenic species and in biomedical research, specifically in the areas of immunology, virology, neurobiology, toxicology and pharmaceutical industry. At present semen banks are developed for both rare domestic breeds (cattle, sheep, goat and boar) and for non-domestic species. The concept of using of this facility is to facilitate the management in conservation and extensive promotion of endangered species [14]. Now a day, semen cryopreservation has many biotechnological applications. It can be used to solve problems of human infertility, life threatening diseases, preservation of semen from endangered species for long term use and for conservation of biodiversity.

SPERM CELL PARTICULARITIES

The sperm cell is haploid, almost devoid of cytoplasm, constituted by a large nucleus, with highly condensed chromosomes that impede transcriptional activity to replace proteins, an acrosome that enables the spermatozoa to interact and penetrate the oocyte at fertilization and a series of mitochondria located at the anterior region of the flagellum [15]. Mitochondria produce ATP, greatly consumed to maintain motility and a little endoplasmic reticulum or Golgi apparatus, contributes to maintain membrane integrity. The shape and the size of the sperm head could define its cryosensitivity. Comparative studies in the boar, bull, rat, rabbit, cat, dog, horse, and human, have shown negative correlations between the size of the sperm head and cryostability. The human spermatozoa are smaller and show greater cryostability [7]. Sperm cells have little biosynthetic activity and it depends greatly on catabolic function to sustain its life.

CRYOPROTECTANTS

A cryoprotectant is a substance that is used to protect biological tissue from freezing damage due to ice formation. Cryoprotectant is included in a cryopreservation medium of cells to minimize the physical and chemical stresses resulting from the cooling, freezing, and thawing of sperm cells. These are classified as either penetrating or non-penetrating. A penetrating cryoprotectant is membrane permeable and acts intra and extracellularly. Penetrating cryoprotectants are solutes, which cause dehydration of spermatozoa due to the osmotically driven flow of water, which varies according to compound. After short periods of time the cryoprotectant and water equilibrate and result in equilibrium in intracellular and extra cellular concentrations [16]. As the sperm cell now has less intracellular water, the freezing point of the cell is decreased and less intracellular ice formation will occur which is beneficial, because intracellular ice formation results in cell death, and consequently reduce fertility of the semen sample.

The penetrating cryoprotectants, in addition to above, can cause cause membrane lipid and protein rearrangement, which results in increased membrane fluidity, greater dehydration at lower temperatures, and therefore acquire an increased ability to survive from cryopreservation [17]. A non-penetrating cryoprotectant cannot cross the sperm plasma membrane and therefore, only acts extracellularly. Therefore, a non-penetrating cryoprotectant may modify the plasma membrane of a cell, or act as a solute and lower the freezing temperature of the medium. Many membrane-permeable cryoprotectants (glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, and propylene glycol), and their combinations, have been tested with buck sperm, [18-19] but the most frequently used penetrating cryoprotectant is glycerol. The addition of glycerol as media using may be performed in a 1-, 2- or 3-step methodology at either 37 °C or 5°C [20-21]. The final concentration (v/v) of a penetrating cryoprotectant in a medium varies, but is determined by the toxicity of the chemical, and its beneficial effect on the spermatozoa. Excessive addition of glycerol can induce osmotic damage to spermatozoa, but the extent of the damage varies according to the species. Among the cryoprotectants, Glycerol, dimethyl sulfoxide (DMSO), and ethylene glycol used in a range of 1–8%, greatest recovery of sperm post-thawing has been achieved with glycerol 3 %(v/v) [19].

Combinations of cryoprotectants, such as glycerol and DMSO, have been used, and yielded positive results. The use of both glycerol (6%) and DMSO (5.9%) achieves a synergistic effect of the cryoprotectants. The post-thaw motility using glycerol and DMSO media separately was 33% and 15%, respectively, while the combination of the cryoprotectants resulted in 45% progressively motile spermatozoa [22]. For the successful cryopreservation of stallion sperm, amides such as methyl formamide, methyl acetamide and acetamide are used as cryoprotectants with more remarkable improvement [23]. And in 2007 it is reported that computer assisted cryopreservation can do less damage to human sperm than direct vapour cryostorage [24]. Antifreeze proteins (AFPs) increase the velocity, linearity of movement and percentage of viable cells with the combination of 5% glycerol in Sparus aurata [25].

CRYOPRESERVATION FOR RAT MODEL

The rat has become a significant research tool in genetics and molecular biology allowing the study of many models for human diseases based on transgenic or targeted mutations [26]. Because of recent advancements in the development of novel gene modification techniques, the use of genetically modified rats in biomedical research is expected to increase significantly in the near future on the protection and preservation of sperm cells. A wide array of research opportunities are now open up especially in studies involving the laboratory rat [27]. Due to the difference in sperm membrane lipid composition and length of the tail the rodent sperm is differs from other mammalian sperms.

Rat sperm is extremely sensitive to a number of environmental changes, such as centrifugation, pH, viscosity, osmotic stress, effect of chilling on motility and acrosomal integrity of rat sperm in the presence of extenders [28]. The primary problem in rat sperm cryopreservation is that it becomes very sensitive to cold and osmotic effect [2] and the cellular damage occurs during cryopreservation is due to formation of intracellular ice [29].When rat epididymal sperms are frozen in an extender of raffinose with tricitrate-glucose egg yolk solution, the freezability of sperm was improved [30]. The biophysical changes brought about by the transition of liquid water to ice during the relatively slow cooling most often used, are the assumed main causes for sperm damage. Successful cryopreservation of gametes and embryos has great importance for genome banking of transgenic and mutant rat lines as well as for studies of rat sperm biology [31]. Efficient cryopreservation of rat sperm has been difficult, and few published studies have aimed at understanding the underlying physiologic and cryobiologic mechanisms for the low survival [31-32].Therefore, greater use of the cryopreservation of rat sperm may provide an essential resource to preserve and increase the number of valuable genetic strains for research and application.

EFFECT OF CRYOPROTECTANTS ON RAT SPERM DURING PRESERVATION

Cryoprotectants themselves can be toxic if used in high concentrations and these are included in cryopreservation medium is to reduce the physical and chemical stresses derived from cooling, freezing and thawing of sperm cells [7]. This encourages searching for other less toxic, effective cryoprotectants for sperm freezing. Since different extenders were used to freeze spermatozoa in those studies, whether extender type affects the efficiencies of these permeating cryoprotectants is yet to be proved. However, no studies have compared the effect of cryoprotectants other than glycerol on sperm freezing using various extenders at the same time. Glutathione-S-transferases are a family of multifunctional proteins involved in intracellular processes and detoxification. Studies show that infertile men have high oxidative stress and have low antioxidant levels in their semen [33]. It is found that Glutathione peroxidase levels were significantly lower in the infertile group compared to the fertile group. Hence antioxidants in combination with potent cryoprotectants may provide better sperm storage without doing damage to DNA. The effects of inclusion of various cryoprotectants (glycerol, 1, 2 propanediol, sucrose and trehalose) allowed to apoptosis to some extent, with negative effects on sperm morphology and DNA integrity. But addition of glycerol was more successful for maintaining sperm post thaw motility [19, 34].

PHYSICAL & BIOCHEMICAL CHANGES IN SPERM DUE TO CRYOPRESERVATION

Dehydration, Extracellular&Intracellular ice formation

In the process of cryopreservation the migration of water a stress due to ice formation and dehydration can cause damage directly on the cell. Because of it some organisms and tissues can tolerate to some extent extracellular and intracellular ice formation however the excessive formation of ice is almost always fatal to cells. The intracellular ice formation increases the osmotic potential inside and causes physical destruction of membranes, gas bubble formation and organelle disruption. Upon slow cooling of tissues water migrates out of cells and forms ice into the extracellular space. The dehydration is one of the first harmful consequences identified in cell cryobiology and later shown to cause a number of damaging events including changes in ultra structure of cell membranes, loss or fusion of membrane bilayers and organelle space disruption. Excessive extracellular ice formation can cause mechanical damage to the cell membrane due to crushing.

Mitochondrial DNA damage

The mitochondrion, organelles of the cell which contains the DNA, along with genes of female parent can be passed on to the child. The oxy radicals are formed during electron transfer in mitochondria. When this free radical formation in the mitochondria is high it can seriously hinder the development of first form of life. In this process the Glutathione system has a determinant role in the protection of sperm cells because it scavenges free radicals by binding to sulfur of glutathione [35]. Spermatozoa are particularly likely developing different forms of mitochondrial DNA (mtDNA) abnormalities including deletions, point mutations and polymorphism. These abnormalities have been associated with a decline in semen quality, motility and male fertility [36]. MtDNA also lacks histones, whose help protect against damage, therefore it is believed to contain only a base repair mechanism. Although the first study on mtDNA inheritance after intra-cytoplasmic sperm injection (ICSI) suggested that human embryos eliminate the mtDNA of the injected sperm [37], and another study has shown that abnormal paternal mtDNA transmission may not be uncommon when poor-quality gametes are used. It is also of interest that populations of human spermatozoa exhibiting evidence of mitochondrial dysfunction at high rates of nuclear DNA fragmentation [38]. Abnormal sperm samples study has revealed that high incidence of mtDNA damage, which confirms their role in male infertility [39].

Further mitochondria play a key role in the mechanism of apoptosis. The integrity of mitochondria is established by the presence of cytochrome C in the inner membrane space. High levels of ROS disrupt the inner and outer mitochondrial membranes. This results in the release of mitochondrial cytochrome C protein, which inturn activates the caspases and induces apoptosis. Studies in infertile men showed that high levels of cytochrome C in seminal plasma indicate significant mitochondrial damage by ROS. Considerable evidence exists that disruption of mitochondrial functions (e.g., loss of transmembrane potential, permeability transition, and release of cytochrome C leading to impaired electron transport) has important events in many apoptotic cell deaths. Four major factors that may be involved in the etiology of DNA damage in the germ line: are Oxidative stress (OS), dehydration, deficiencies in natural processes such as chromatin packaging and abortive apoptosis. Although extent of DNA damage is closely related to sperm dysfunction and male infertility [40], the origin of such damage is still largely controversial.

Oxidative stress

Oxidative stress is an imbalance generated in between the production of reactive oxygen species (ROS) and the existence of antioxidant defense in the body. The oxidative

stress in the testicular tissue can lead to sperm damage, deformity, and eventually male infertility. Virtually every human ejaculate is contaminated with potential sources of ROS [41], for the purpose of fertilization initiation. ROS are oxygen-derived molecules that act as powerful oxidants, and are such as superoxide anion (O_2•), hydrogen peroxide (H_2O_2), and the hydroxyl radicals (OH) formed as intermediary products in low concentrations in male and female genital tracts for minimal activities of sperm and ovum for their release movement and fusion [42]. Reactive oxygen species have the ability to react with any molecule and modify it oxidatively, resulting in structural and functional alterations. All cellular components including lipids, proteins, nucleic acids, and sugars are potential targets of oxidative stress [43]. They induce cellular damages when they pass this unpaired electron onto nearby cellular structures, resulting in oxidation of cell membrane lipids, amino acids in proteins or within nucleic acids [44].

Two factors that protect the sperm DNA from oxidative insult are: the characteristic tight packaging of the DNA; and the antioxidants - Superoxide dismutase, Catalase, Glutathione peroxidase (enzymatic) and α-tocopherol, β-carotene, ascorbate, urate, Transition-metal chelators, transferring, lactoferrin, and caeruloplasmin(non enzymatic) present in seminal plasma [45]. However, oxidative stress as a result may develop an imbalance between ROS generation and antioxidant scavenging activities [46]. In general, DNA bases and phosphodiester backbones present in spermatozoa are particularly susceptible to OS induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm also contains low concentrations of scavenging enzymes due to the large size occupation of nucleus.

The process of cryopreservation does seem to increase the level of ROS generation in spermatozoa [47-48], it is possible that oxidative stress is responsible for cryo-injury to sperm DNA. The freezing and subsequent thawing of sperm is a physically stressful process carried out during routine procedures in assisted reproduction, which results in a highly variable and unpredictable reduction of motile sperm. Subsequently, oxidative status can positively or negatively affect the motility, viability, and fertilizing capacity of thawed sperm. Many studies have reported a link between sperm DNA fragmentation and ROS [49-51]. Uncontrolled production of ROS that exceeds the antioxidant capacity of the seminal plasma leads to oxidative stress which is harmful to spermatozoa [52]. In general the occurrence of sperm DNA fragmentation during cryopreservation remains to be elucidated. The evidence suggests that sperm DNA fragmentation is associated with an increase in oxidative stress during cryopreservation, rather than the apoptosis activation [53].

Mechanism of ROS on sperm during storage

ROS was exclusively considered toxic to spermatozoa. However, evidences suggest that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities [54]. Low levels of ROS have been shown to be essential for fertilization, acrosome reaction, hyperactivation, motility, and capacitation [55]. Reactive nitrogen species (nitrous oxide, peroxynitrite, nitroxyl ion, etc.) are also a class of free radicals derived from nitrogen and considered a subclass of ROS. Virtually every human ejaculate is considered to be contaminated with potential sources of ROS. Leukocytes and spermatozoa have been shown to be the two main sources of ROS [56].

In general most mammalian cells can withstand low, non freezing temperatures. These conditions can affect several aspects of cell function. The cell membrane is a lipid bilayer structure with proteins spanning across it. In general the cell membrane is impermeable except where membrane proteins allow mass transfer to occur. At low temperatures, the lipid transforms into a gel phase, or a structure with low free energy. During this process, the cell membrane proteins become separated and lose their ability to control mass transfer. The membrane becomes more permeable and allows ions to transfer in and out of cells more easily. As a result, the ionic composition of the cells changes and damage occurs. It has been shown that lipid peroxidation and ROS are triggers and essential mediators of apoptosis [57-60].

Cold shock induces lipid peroxidation in sperm membrane during cryopreservation [61]. Therefore, it is apparent that long term freezing renders the hydrophobic domains more structured. As a result, a change in lipid-protein interaction is highly expected during the cooling as well as freezing of spermatozoa [62]. In humans, the movement of lipids in the sperm membrane also seemed to be restricted significantly after cryopreservation. Lipids in the bilayer aggregate together during cooling and freezing, and give a new conformation to the protein-lipid lattice [63]. Accumulation of lipid hydroperoxides during freezing or thawing could be the cause for the formation of crystalline domain in the sperm membrane. It is also possible that ROS production during thawing of frozen spermatozoa causes a perturbation in membrane fluidity and restricts the rotational behavior of lipid molecules.

It also demonstrates an elevation in the level of lipid peroxidation associated with an increase of the rigidity in the hydrophobic portion of the sperm membrane, a likely consequence of an increase in ROS production during cryopreservation. Excessive production of free radicals or reactive oxygen species (ROS) can damage sperm, and ROS have been extensively studied as one of the mechanisms of infertility. Reactive oxygen species are the product of normal metabolism in cell. Free radicals are highly chemically reactive because of unpaired electrons. Also ROS produced by leucocytes in phagocytic process. ROS can effect on sperm DNA integrity. High levels of ROS are detected in the semen of infertile men. The mechanism of ROS-induced damage to spermatozoa includes an oxidative attack on the sperm membrane lipids leading to initiation of lipid peroxidation (LPO) cascade [64]. One of the most common types of damage induced by free radicals is membrane lipid peroxidation (LPO). Spontaneous LPO has been well characterized in human semen samples [65].

The reactions proceed through three main steps-initiation, propagation, and termination. During initiation, the free radicals react with fatty acid chains and release lipid free radicals. This lipid free radical may further react with molecular oxygen to form the lipid peroxyl radical. Peroxyl radicals can react with fatty acids to produce lipid free radicals, thus propagating the reaction. One of the byproducts of lipid peroxidation is malondialdehyde. This byproduct has been used in various biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa. Results of such an assay exhibit an excellent correlation when examining the relationship between

impaired sperm function, discussed in terms of motility, and the capacity for sperm-oocyte fusion.

Apoptosis

Apoptosis is a mode of cellular death based on a genetic mechanism that induces cellular morphological and biochemical alterations, leading to the cell to commit suicide [66]. A programmed cell death is a mechanism, activated by the Caspases; these are cysteine proteases, regulate cell count and removes unnecessary cells that compromise survival in eukaryotes. Caspase activation, externalization of phosphatidylserine, alteration of mitochondrial membrane potential and DNA fragmentation are markers of apoptosis found in ejaculated human spermatozoa [67-69]. Apoptosis is a natural process that occurs throughout spermatogenesis but abortive apoptosis, in which the cells are not completely destroyed following the induction of DNA fragmentation by endonucleases, has been implicated in the generation of sperm exhibiting DNA damage in the ejaculate [40]. During cryopreservation, spermatozoa are exposed to physical and chemical stress, changes in lipid composition of sperm plasma membrane reduced head size and externalization of phosphatidylserine residues. Factors contributing to these changes include temperature, maturity of the cell, cryoprotectant used, and rate of cooling [70-71].

Cryopreservation and thawing is a procedure that inflicts irreversible injury on human spermatozoa. Apoptosis may play a role in cryo-injury to sperm DNA as the process of cryopreservation has been shown to increase the activation of particular aspartic acid-directed cysteine proteases, called caspases, in both human [72] and bull spermatozoa [73]. Caspases, particularly 1, 3, 8 and 9, are known to play a key role in the cellular apoptotic cascade and eventual cell death [74]. The exposed phosphatidylserine is recognized by various receptors on phagocytes for destruction. DNA fragmentation is a marker for late-stage apoptosis in spermatozoa. Although there are some reports of a correlation between the presence of activated caspases and sperm DNA fragmentation [75], the results are far from compelling, and there is not as yet any strong evidence to suggest a caspase/apoptosis-related increase in sperm DNA fragmentation during cryopreservation [73-74].

Cryoinjury is not limited to the freezing process but may also occur during thawing. Ice crystals melt during thawing and could result in damage of the sperm organelles. Osmotic changes during the cryopreservation process expose spermatozoa to changes in osmotic conditions resulting in cellular damage. In relation to cryopreservation, survival of human spermatozoa was decreased at hypoosmotic conditions due to cellular swelling leading to lysis. Spermatozoa were initially resistant to hyperosmotic conditions; however, significant cell damage occurred when returned to isosmotic conditions [76].

The integrity of the sperm membrane is affected during cryopreservation and thawing processes. Alterations in sperm membrane integrity have been well observed in ultra structural studies [77].The stability of the membrane is affected by changes in temperature, volume changes associated with the movement of water, cryoprotectants and osmotic stress due to increased salt concentration. During cryopreservation, the initial cooling process causes phase transitions of the membrane lipids and impairs the function of membrane proteins, which are responsible for ion transport and metabolism [78]. These changes compromise the membrane integrity and causes loss of function.

Cryopreservation of human spermatozoa is also known to have negative effects on sperm motility and velocity due to membrane swelling and acrosomal leakage and degeneration. It has been reported that irregular interaction between DNA and nuclear proteins can lead to impaired motion parameters in spermatozoa [79]. The alteration in the mitochondrial membrane fluidity that occurs during cryopreservation will lead to a rise in mitochondrial membrane potential and the release of ROS. The process of ROS generation during cryopreservation and thawing of spermatozoa has been well documented. It has been reported that ROS production by both human spermatozoa and seminal leukocytes increases on cooling to 4°C [80].

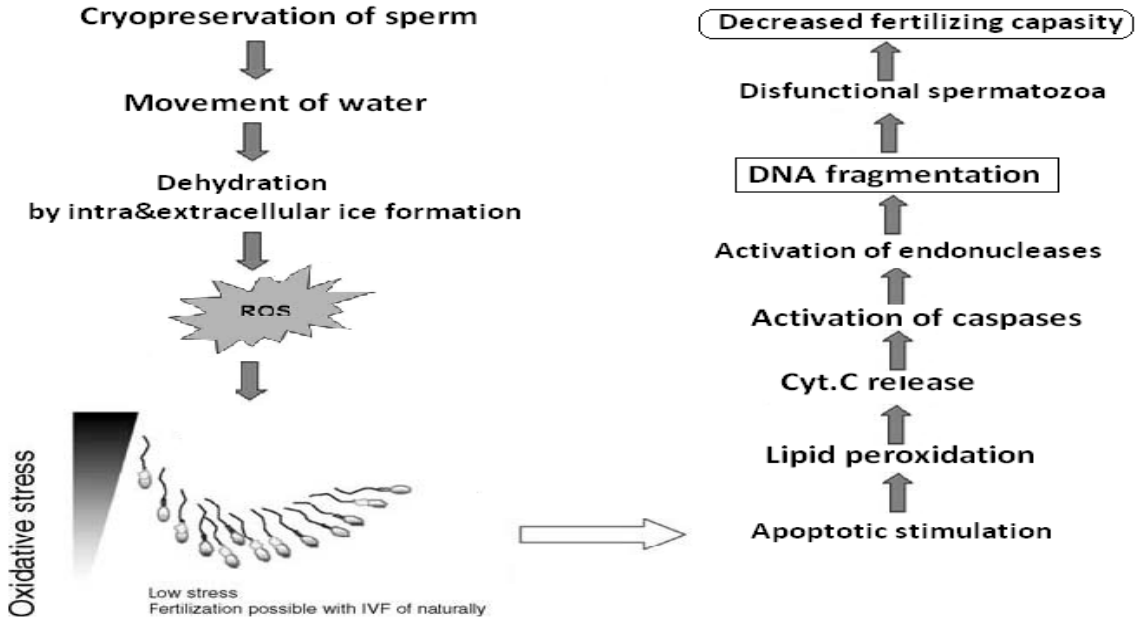

Figure: 1 Schematic representation of the effects of cryopreservation on sperm, associated with cell death.

Cryopreservation process has been shown to diminish the antioxidant activity of the spermatozoa making them more susceptible to ROS-induced damage [81]. Increased concentrations of ROS cause peroxidative damage to the sperm plasma membrane, leading to reduced membrane integrity. This can deleteriously affect sperm motility by damaging the axonemal structure of the spermatozoa [82]. Cryopreserved semen samples from cancer patients were also found to have higher DNA fragmentation compared with healthy donors [83]. It has been reported that men with oligozoospermia present with higher rates of sperm DNA fragmentation both pre- and post-cryopreservation compared with fertile men. Normozoospermic semen samples appear to be more tolerant to damage induced by freezing and thawing compared with oligozoospermic samples. It was reported that motile spermatozoa could be recovered after five refreeze-thaw cycles in normozoospermic rates, which indicates that too fast or too slow cooling rates can cause these parameters to be compromised [84].

Oxidative stress is created when the cellular generation of ROS overwhelms these anti-oxidant defences and this may occur during cryopreservation. In light of these considerations, it is important to ascertain whether the observed increase in DNA fragmentation following cryopreservation is due to an increase in the activation of an intrinsic apoptotic cascade or to an increase in ROS and oxidative stress.

FUTURE PERSPECTIVES

Sanger et al., (1992) said that the process of cryopreservation has resulted in an increase in DNA fragmentation, oxidative base damage and apoptosis in sperm. This suggests that DNA damage, oxidative stress and apoptosis induced by cryopreservation are features of the high quality sperm and simply the result of poor quality cells exhibiting vulnerability. It is well established that radiotherapy, chemotherapy, or even invasive surgery may lead to testicular failure or ejaculatory dysfunction [85]. Thus, by cryopreserving sperm as part of an assisted reproduction program, we can offer couples the option of having children in the future. Moreover, in situations of impaired male fertility, sperm storage will provide the necessary time for a reasonable amount of sperm to be obtained for successful artificial insemination or in vitro fertilization. Nevertheless, because of the damage associated with freezing, the motility of cryopreserved spermatozoa after thawing is statistically reduced with respect to prefreezing motility, and this factor also shows wide inter individual variability [86]. Sperm quality may also be affected by the subsequent slow-thawing process. This process induces further cell damage [87]. Further, the addition and removal of osmotically active cryoprotective agents (CPAs) during freezing and warming can induce lethal mechanical stress per se. Even other problems include the chemical toxicity of CPAs and possible negative influence on the genetic apparatus of mammalian spermatozoa [88, 89]. The protection of DNA has important implications in the use of freeze-thawed donor spermatozoa for insemination. This is extremely relevant for individuals who may have spermatozoa banked for long-term storage prior to chemotherapy or radiotherapy. It is critical to optimize the protocols that are used to prepare and freeze sperm samples from infertile men to protect their DNA. In support of the beneficial role of the sperm preparation protocols, a general improvement in nuclear maturity may be seen in post-swim-up samples [90]. Male factor infertility plays a role in approximately 50% of infertile couples.

Cryopreservation of testicular spermatozoa may reduce pregnancy rates although this will only be confirmed by a much larger multicentre trial [53]. Cryopreserved sperm serves as genetic bank or germplasm, which may help to ensure genetic diversity and reproductive success for population management strategies. From the literature it can be clear that dehydration induced oxidative stress being the major culprit involved in damaging the DNA, should be eradicated during cryopreservation process. Hence if we are in positions to rectify this troubleshoot ultimately the shelf-life of DNA can be prolonged to several years. How to safeguard this DNA even after cryopreservation is a million dollar question at present. One of the difficult compromises faced in cryopreservation is, let the DNA tests are stored at extreme temperature (-196 °C), the tests are getting damaged due to dehydration interference. When the DNA is cryopreserved, the liquid form of the test is almost converted to solid mass like structure which is immediately followed by the evaporation of moisture content in the eppendorf. So dehydration inhibition is of prime importance during the trials. If the dehydration is going to be stopped at sample storage level itself then there won't be any expectation of damage to DNA. In order to achieve this progress some automatically adjustable inert lid like substances which should not get interfere with the DNA sample should be prepared and layered as an inert surfactant on the DNA at the surface level itself.

CONCLUSION

Sperm cryopreservation is extensively used in ART programmes. Despite various advances in cryopreservation methods, the recovery rate of functional spermatozoa after thawing remains unsatisfactory. ROS are produced during the freezing and thawing of spermatozoa, which may be the cause for the decrease in sperm function after cryopreservation [48]. Cryopreservation induces many changes in sperm cells, including membrane disorders and cell death; it also acts as an inducer of apoptosis in sperm cells and in turn, it clearly facilitates DNA damage. Dehydration and re-hydration induces mechanical stresses on lipid membranes which have been shown to cause physical deformities in cells leads to damage to the offspring. Spermatozoa and oocyte also experience similar effects, though their membrane lipid constitution is quite different. So, if the dehydration is going to be stopped at sample storage level itself then there won't be any expectation of damage to DNA. In order to achieve this progress some automatically adjustable inert like substances, which should not get interfere with DNA sample should be prepared and layered as an inert surfactant on the DNA at the surface level itself, then ultimately the bulwark target of DNA can be successfully achieved that can be very much useful in the establishment of cryobank of DNA and cells of endangered species. Sperm cryopreservation is an important component of fertility management and much of its successful application seems to affect the reproductive outcome of assisted reproduction technologies (ART): appropriate use of cryoprotectants before and sperm selection technologies after cryopreservation seem to have the greatest impact on preventing DNA fragmentation, thus improving sperm cryosurvival rates [91].

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