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REVIEW

Cryopreservation of Human Spermatozoa

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Since the first attempts to freeze human semen,[1] there have been many improvements in cryopreservation. The ability to freeze human spermatozoa has been possible for many years.[2] It is widely used in assisted conception to preserve male gametes and provide the opportunity for future fertility, for example cytotoxic chemotherapy, radiation therapy and some kinds of surgical treatment may lead to testicular failure or ejaculatory dysfunction. Freezing of spermatozoa before treatment provide patients with fertility insurance. It is also useful for azoospermic patients who have undergone testicular sperm extraction or percutaneous epididymal sperm aspiration, avoiding the need for repeat biopsies or aspiration.[3] In this article, we will review about benefits of sperm cryopreservation, methods for evaluation of sperm cryopreservation, biochemical optimization of sperm cryopreservation, physical aspects of freeze-thawing procedure and future aspects of sperm cryopreservation.

Table 1. Benefits of sperm cryopreservation[4-6]

Self preservation
1. Preserve male gametes and provide opportunity for future fertilityesp in treatment of malignancy.
2. Preserve male gametes in azoospermic patients who have undergone testicular sperm extraction or percuta neous epididymal sperm aspiration.
3. Increase in convenience and availability at time required for insemination.
Donor preservation
1. Increase in the convenience and availability of donor semen at time required for insemination.
2. Allow detailed screening of donors for infection such as HIV and hepatitis B prior to release of spermatozoa for insemination.
3. Increase possibility to use semen from donors having characteristics preferred by the couple using artificial donor insemination.

Methods for evaluation of sperm cryopreservation

Although the primary end points which we would like to evaluate after sperm cryopreservation are "take home baby rate", there are several confounding factors involving pregnancy from sperm cryopreservation, especially female factor. Several arameters which are the direct end points of sperm cryopreservation have been used for evaluation of the quality of sperm cryopreservation

Recovery of motility

Sperm motility is the most frequent observed parameter which is investigated in many published papers.[2,4,6] The relationship between sperm motility and fertilizing ability has been assessed over a long period and a motility of >30% is generally regarded as the criterion for the suitability of donated semen for cryostorage. However, the link between fertilizing ability and motility is not so strong.[7] With the development of objective assessment of motility through computer assisted sperm assessment, some trials have been performed to better determine which objective parameter of motility may be a more reliable index of cryopreservation. One study has focused in the prediction of sperm cryosurvival based on straight line velocity and linearity, and established an equation based on prefreeze kinetics variables.[8]

Acrosome integrity, and damage to the plasma membrane and subcellular structure

We will distinguish approach in ultrastructural approach and other methods of evaluating membrane integrity.

1. Ultrastuctural studies

Morphological assessment is performed from stained slide with magnification.[9] Ultra-structural damage has been highlighted to plasma membrane, acrosome, mid-piece as well as flagella of spermatozoa after freezing and thawing, and is indicated by membrane swelling, acrosome swelling, acrosome loss and pulling back of the mitochondrial sheath.[7]

2. Other methods of assessing membrane integrity

The use of flow cytometry and fluorescent probes has been used to determine plasma membrane integrity and mitochondrial function.[10] A different method of membrane assessment used the hypo-osmotic medium test either to evaluate membrane damage.[11] Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) was also used to assess the acrosome reaction.[12]

Damage to the nucleus after freezing and thawing

1. Chromosomal damage

Cytogenetic investigation of the effects of cryopreservation on human spermatozoa, using the hamster oocyte test to reveal sperm chromosome, has reported no alteration in the frequency of chromosomal abnormalities or sex ratio in human spermatozoa after freezing.[13]

2. DNA/nuclear protein interactions

The DNA status of individual spermatozoa could be determine using a modified alkaline single cell gel electrophoresis (comet) assay.[9] When sperms are frozen in semen, there is a significant decrease of 20% in DNA integrity.[3] From the published study DNA of semen and prepared spermatozoa from fertile men was found to be unaffected by cryopreservation. In contrast, spermatozoa from infertile men were significantly damaged by freeze-thawing.[9]

Biochemical optimization of sperm cryopreservation

Cryoprotective substances

Significant survival of cryopreserved cells became a reality only after the discovery and use of cryoprotective agents.[14] Cryoprotectants are generally classified as either permeating or nonpermeating according to whether or not they enter cell. The majority of cryoprotectants currently used are permeating agents. Two important procedures related to the use of permeating cryoprotective agents are (I) the addition of a cryoprotective agent to the cells before freezing, and (ii) the removal of the cryoprotective agents from the cells after freezing and thawing. In the first case, when a cell is placed in

a solution that is hyperosmotic with respect to the permeating agents (e.g. glycerol) but was isotonic with respect to the impermeable salts, it first shrinks because of the osmotic efflux of intracellular cell water and then increased in volume as the solute (glycerol) permeates and as water concomitantly reenters the cell. Subsequently, in the second case, when cells with a high intracellular concentration of cryoprotectant are exposed to an isotonic solution, they will swell because of an osmotic influx of extracellular water and then decrease in volume as glycerol diffuse out of the cells and as water concomitantly move out. As a result, the cells experience osmotic stress which may cause significant 'osmotic' cell injury.[15]

There was a published paper which found a need for cryoprotectants for optimal cryosurvival, with little or no survival after freezing in the absence of glycerol.[16] Although glycerol is the most commonly used cryoprotectant for spermatozoa. It has been shown to have toxic effects in this cell type. The detrimental effects of glycerol decreases as the temperature of glycerol addition decreases and reducing the concentration used.[16] From the fundamental cryobiology, human spermatozoa are least permeable to DMSO. Ethylene glycol permeates human spermatozoa at the fastest rate of cryoprotectants. It is an optimal cryoprotectant when using optimal protocol, standard cooling and warming rates (3˚C/min from 25 to -5˚C; hold at -5˚C for 10 min; after 3 min at -5˚C,sample were seeded; 10˚C/min from -5˚C down to -80˚C; samples plunged into liquid nitrogen).[17]

Choice of extender

Extender is a dilution medium which is added in cryopreservative medium. Many studies have evaluated various media including zwitter-ions, citrate, egg-yolk or milk, fructose, TEST citrate-yolk buffer,[18] yolk citrate dextrose glycine,[19]egg yolk citrate[20] and human sperm preservation medium.[16] The addition of certain factors (dithiothreitol (DTT), cryoseed) to TEST yolk citrate was reported to be of some benefit for poor quality human semen, possibly through the prevention of oxidation of sulhydryl groups.[21] Direct addition of pentoxyfylline to the seminal plasma before cryopreservation improved sperm motility and acrosome reaction.[12]

Physical aspects of the freeze-thawing procedure

Sperm concentration

Some studies have reported the possibility of improving cryopreservation efficiency by concentrating ejaculates before cryopreservation, particularly for low quality semen. This has been achieved using either a continuous step-density gradient[22] or swim-up.[23] Swim-up method using a washed sperm preparation before freezing also offers the possibility of selecting spermatozoa with better acrosome integrity and ability to undergo acrosome reaction.[24]

Cooling and warming

Two procedures have been proposed: a rapid method using uncirculated vapour in liquid nitrogen and a slow method using a semi-programmable freezer. Briefly, the rapid method consists of exposing straws to nitrogen vapour for 8-30 min, before putting them directly into liquid nitrogen. The slow, computerized method consists of decreasing the temperature in two or three steps, the first one at a slow rate to between 5 and -9˚C, and the following steps at a higher rate to between -40˚C and -80˚C, before plunging the straws into liquid nitrogen.[7] The first method was first proposed by Sherman.[2] The respective advantages of each method have been debated with reported results favouring both the cheaper manual cooling method[25,26] One study has focused on the necessity of optimizing the warming method with the cooling method, thawing at 37˚C being better when performed following rapid vapour freezing, and thawing at 22˚C following the slower, computer-controlled freezing.[26] Such interaction between cooling and warming rates were confirmed by another study which indicated maximal survival rate occurred at 10˚C/min cooling rate for the rapidly warmed sperm and at 1˚C/min for

the slowly warmed sperm.[10]

Future aspects of sperm cryopreservation

With the advent of ICSI, theoretically only one sperm is required per retrieved oocyte, allowing men with severe oligozoospermia the opportunity to reproduce. Because of limitations associated with cryopreserved sperm from this population, there are many methods developed to improve the outcome. Using cryoloops to perform ultra-rapid freezing (samples of sperm on cryoloops held in liquid nitrogen vapour for 5 min prior to submerge into liquid nitrogen) is feasible.[27] Another method is cryopreservation of single human spermatozoa which is established by Jacques Cohen. This is achieved by injecting a single or a small group of human spermatozoa into cell-free human, mouse or hamster zonae pellucida before the addition of cryoprotectant.[28]

In conclusion, although the cryopreservation of human sperm has been available for more than 40 years, a true rational approach to defining optimal conditions for freezing and thawing spermatozoa has been introduced only recently. With the advance of gamete micromanipulation, the necessity of freezing a single or small group of spermatozoa are required, including testicular and epididymal spermatozoa. Thus there is still room for improvement in the methodology of sperm cryopreservation.

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