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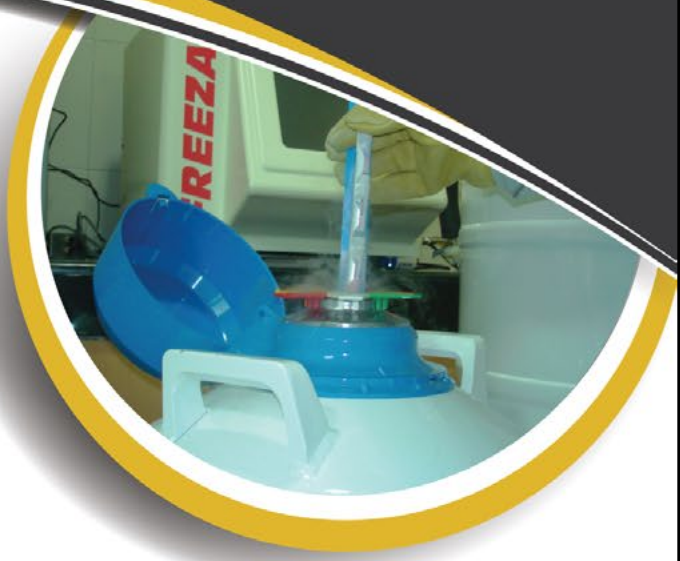
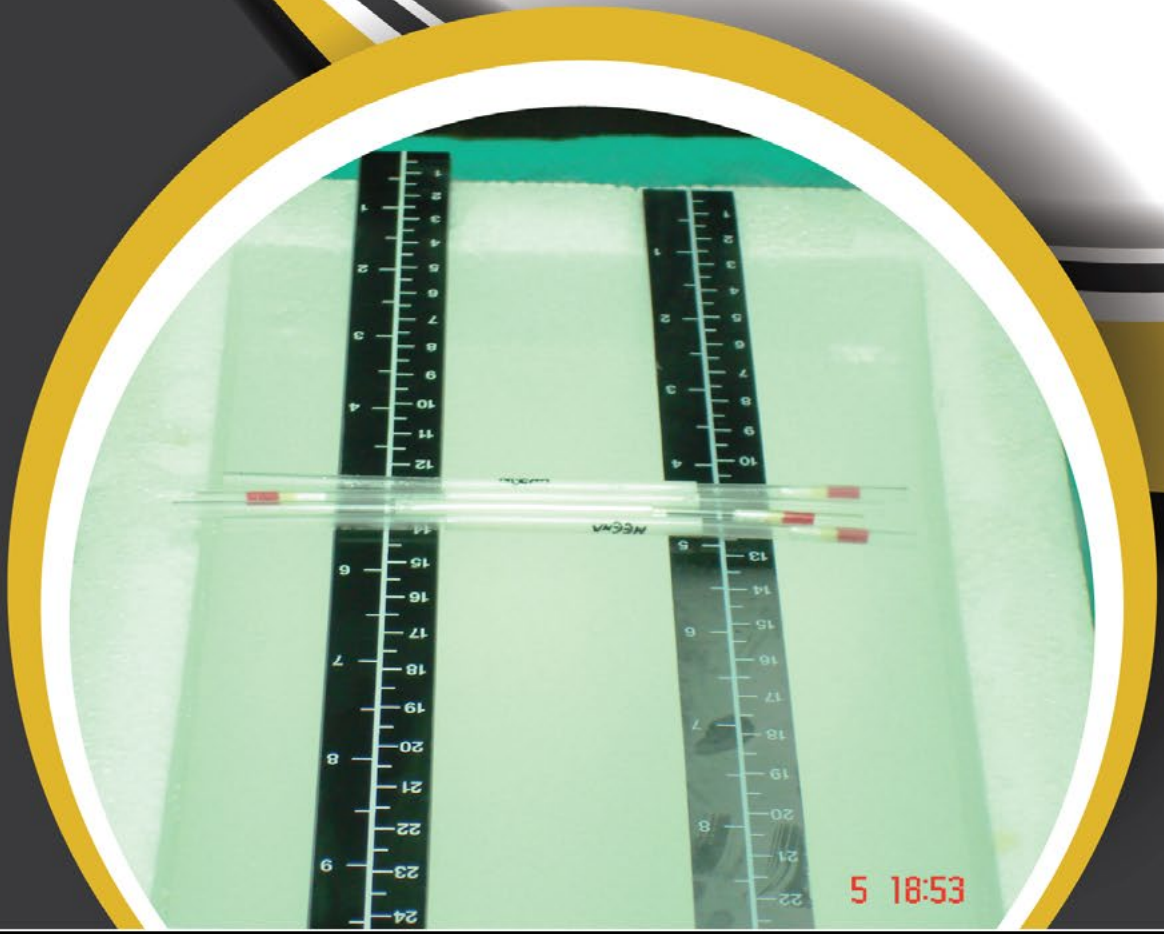
NEXUS

Indian Fertility Society &
ORIGIO India Initiative



Semen Freezing
Past, Present & Future



We are aware that sperms can be frozen for future use for artificial insemination or other ART procedures. Semen can also be donated for the use of males having testicular failure and Cryopreservation of spermatozoa is a widely used technique to preserve the male fertility too.

Semen has been frozen, thawed and successfully used for infertility treatment for an indefinite period, although not all sperm survive the freezing process. In long-term follow-up studies, no evidence has been found either of an increase in birth defects or chromosomal abnormalities in children conceived from cryopreserved sperm related with the normal population.

In this issue, we discuss the nuances of semen freezing and we are sure that the bulletin would benefit andrologist and lab directors alike.

It is a great privilege and pleasure to write this message for the 4th E-bulletin of IFS-Nexus. I also sincerely thank “ORIGIO India Private Ltd” for participating with us in this academic endeavor.

Dr. Sohani Verma
President-IFS

This is an honor for me to write best wishes message for this very special Nexus E-bulletin on “Sperm freezing”.

Sperm cryopreservation or semen freezing is a method for men to preserve their semen in an ART bank for future use in varied indications. Many medical treatments, including cancer therapies, can damage the sperm integrity and functionality, making it necessary for us to bank the semen sample pre-treatment.

Although there is no information about how long frozen sperm can remain viable, a three-decade old sample has been used successfully in achieving pregnancy. Semen freezing allows the sample to be used in the future for fertility treatments, such as Intrauterine insemination (IUI) or In vitro fertilization (IVF).

Optimization of freezing protocols, Cryoprotectant concentrations used, and correct semen preparation techniques are necessary to ensure successful application of cryopreservation for semen preservation.

This bulletin would cover various steps involved in this procedure and would help the ART centers to improve their techniques and improve the results.

Indian fertility Society feels proud and congratulates the editors on the launch of the 4th edition of Nexus E-Bulletin. It would not only help to disseminate scientific & ethical content but also constantly update everyone with new researches and developments across the world.

I wish the editorial team best of luck in this endeavor.

Dr. K.D. Nayar
Secretary General-IFS

At the very onset, the editorial team would like to thank all of you for positively appreciating our previous E-bulletins of Nexus. Team 'Nexus' sincerely hopes to bring out such teaching material for you regularly. The bulletin has been named NEXUS - which means building bridges.

Such bulletins are the call of the day and enormously bridge the gap between the existing knowledge and recent advances. Our present edition is focused on simplifying the process of semen freezing and covers all essential details with nice algorithms. Part 1 deal with commonly asked questions and part 2 with bench side work. I am sure it will immensely benefit you all.

Cryopreservation refers to the maintenance of cellular life in the cells at subzero temperatures for a protracted period of time. Cryopreservation of human spermatozoa is a fundamental part of assisted reproduction technologies and has aided us to autologous or donor banking for myriad indications. Standardization of the freeze-thaw protocols is the need of the day. The type of Cryoprotectants and their concentrations used decide the efficacious utilization and end result of the procedure.

The spermatozoa are exposed to a diverse ultrastructural cryoinjuries during the procedure that may not be noticeable during semen analysis using light microscopy examination. Defined measures should be embraced to minimize the damage to the spermatozoa during freeze-thawing because of apoptosis of the cell and DNA damage.

The aim of this bringing out this bulletin is educating ourselves about the finer aspects of semen freezing and banking.

Our motto is "knowledge empowers" and we sincerely hope that you would enjoy reading this write-up. Feel free to communicate with us at any point of time and contribute critically. Your comments would be published in the next bulletin, which is titled "QA-QC of an ART Centre".

We would also like to place on record our truthful thanks to Origio India limited who are helping us in the publication of this bulletin and off course I promise that there is no conflict of interest at any level. Wish you happy reading and yes don't forget to file this issue.

I would formally like to thank my friend Dr. PRANAY GHOSH from Elixir Fertility Centre, New Delhi who has worked un-relentlessly towards bringing out this issue from conception to end.

Prof (Dr) Pankaj Talwar
Joint Secretary-IFS
Editor NEXUS

The woods are lovely, dark and deep,
But I have promises to keep,
And miles to go before I sleep,
And miles to go before I sleep.

Stopping by Woods on a Snowy Evening,
1923 ,by Robert Frost
(26 March 1874 – 29 January 1963)

INTRODUCTION

Cryopreservation refers to the maintainence of cellular life in the form of cells or tissues at subzero temperatures for an extended period of time . Prolonged storage of sperm and other reproductive cells and tissues is possible only by retarding the cellular reactions by lowering the temperature until the cellular activity ceases. Storage in LN_2(-196ºc) has become the standard for sperm freezing since early days of sperm banking. **Prolonged storage** at -196ºC does not affect absolute cryosurvival since at this temperature there is virtually no movement of atoms or molecules. **Cryopreservation** of human spermatozoa is an integral part of assisted reproduction technologies.

Ques. 1 History of sperm freezing. When did it all begin?

Freezing and thawing techniques have been used to cryopreserve semen since 1776. Refinements in the outcome of freeze-thaw cycles have been made possible by the discovery of better cryoprotectants and with advancements in the equipment, disposables, and awareness about sterility.**(Table 1)**

Table 1. History of sperm freezing

Table 1

Year	Scientist	Breakthrough
1780	**Lazzro Spallanzani**	Performed first successful artificial insemination. Observed the effects of freezing on human sperm and subsequent recovery of motility on warming.
1866	**Montegazza**	Proposed the idea of semen banking for soldiers going off to battle to ensure their continued lineage.
1930	**Shettels & Jahnel**	Observed that sperm survives at temperatures as low as -269ºC.
1949	**Polge**	Accidental discovery that glycerol could act as a cryoprotectant.
1953	**Bunge and Sherman**	Reported three pregnancies following the use of sperm that had been treated with glycerol and frozen in dry ice.
1963	**Sherman**	Demonstrated that sperm could be stored for longer duration if kept at -196ºC in liquid nitrogen (LN_2).

Ques. 2 How does the cryobiology help in sperm banking?

Prolonged storage of sperm and other reproductive cells and tissue is possible by retarding the cellular activity by lowering the temperature until the cellular reactions cease. **LN_2 (-196ºC)** storage has become the standard for sperm freezing since early days of sperm banking. Prolonged storage at -196ºC does not affect cryosurvival since at this temperature there is virtually no movement of atoms or molecules. The atoms and molecules have a tendency to move above -130ºC, while temperatures above -90ºC permit ice crystal growth following even brief exposure. The sole potential damage to the cells maintained at -196ºC is degradation of deoxyribonucleic acid (DNA) caused by background radiation. The spermatozoa can maintain their genetic integrity for approximately 200 years when stored at -196ºC (based on normal background radiation of 0.1 rad/year).

The **main objective** of any cryopreservation protocol is to avoid intracellular ice crystal formation, control cell volume during the procedure and to minimize the membrane damage following exposure to subzero temperatures. Solutes in the medium in which the cells are suspended lower the freezing point to -10 to -15ºC below that of water (0ºC) at which the water in the extracellular environment freezes, hence increasing the solute concentration which generates an oncotic pressure, resulting in a solvent flow across the membrane from inside to outside the cell. This causes a decrease in cellular volume and then, dehydration. However, the degree of dehydration depends mainly on the cooling rate. **Rapid cooling** of cells can lead to incomplete dehydration and intracellular ice crystal formation. On the other hand, **slow cooling** of the cells may result in excessive dehydration. **Intense dehydration** may render the cell unsalvageable (at approximately 40% of the original cell volume).

During thawing, the water is drawn back into the cells, hence restoring the intracellular volume. At this point, recrystallisation injury may ensue due to intracellular ice crystal formation. Hence, thawing should be rapid in order to avoid the formation of ice crystals.

Cellular survival following freeze/thaw depends on a fine balance between an intermediate cooling rate that is fast enough not to cause excessive dehydration but slow enough to bypass the problem of intracellular crystallization, and a rapid thawing rate.

Factors affecting the outcome of a freeze-thaw cycle are:

- The cryoprotectant in which the cells are suspended.
- Cooling rate.
- The temperature at which the sample is plunged into liquid nitrogen.
- The temperature at which the sample is stored.
- Warming rate.
- Cryoprotectant removal after thawing.

Human sperm plasma membrane, like all other mammalian species, is composed of a **phospholipid bilayer** and associated **proteins.** The lipid bilayer is a thin polar membrane composed of a polar head group along with hydrocarbon tails. The sperm plasma membrane plays an active role in sperm fertilization capacity and in sperm–oocyte cross-talk. The two leaflets in the membrane of the cap region, overlying the acrosomal vesicle, constitute the area sensitive to the capacitation stimuli. When various steps of capacitation increase the membrane fluidity in this region, a fusogenic process initiates between this membrane and that of the outer acrosomal vesicle. The ultimate event is the formation of pores that allow a dispersion of the acrosomal enzymes. During cryopreservation, the sperm plasma membrane undergoes **lipid phase separation,** solute effects, and osmotic stresses associated with ice crystallization. On cooling, a reordering of the membrane components occurs, hence increasing sperm membrane viscosity and decreasing fluidity. Though high concentrations of cholesterol and polyunsaturated fatty acids (PUFA) provide more fluidity to the sperm plasma membrane at lower temperatures, the cooling at subzero temperature causes a phase transition of membrane lipids, resulting in a more rigid membrane structure. These undesirable outcomes associated with cryopreservation can be prevented by controlling the rate of cooling and by the addition of cryoprotective agents. **(Table 2 and 3)**

Table 2. Principles of cryopreservation

Table 2

Avoid	Phase of freeze-thaw cycle	Principle
Ice crystal formation	On cooling below the freezing point, water solidifies in a crystalline structure called ice. Since ice is lighter than water, ice crystals occupy a greater volume than does the liquid water. As the water begins to solidify, expansion of ice crystals exerts pressure and shearing forces on intracellular organelles, causing considerable damage.	Ice crystal formation should be prevented for successful cryopreservation.
Osmotic shock	As water freezes into ice, any solutes in the liquid phase are excluded from the solid component. This causes lowering of the freezing point of the remaining unfrozen solution. The concentration of solutes & electrolytes can reach very high levels as the temperature drops.	The disparity in osmolarity gradient may damage the cell and release reactive oxygen species. The osmotic shock should be avoided at every step of semen freezing.
Recrystallization & solution effects	During thawing, the ice melts decreasing the osmolarity of the solution. Recrystallization of water may occur leading to sperm damage.	

Table 3: Guidelines for better recovery

Table 3

S.no.	Preventive strategies	Guidelines
1.	**Freezing initiation**	To avoid cytotoxic effects (reactive oxygen species) release from immotile/dead spermatozoa, it is suggested that the semen sample is prepared and frozen soon after collection. Raw sample may also be banked immediately after liquefaction.
2.	**Accidental thawing**	The sperm's plasma membrane is very sensitive. Careless handling of the frozen semen straw will injure the spermatozoal plasma and acrosomal membranes resulting in a low sperm survival rate after thawing.
3.	**Cryoprotectant removal after thawing**	Glycerol as a cryoprotectant is toxic for sperms if exposed for long period at room or higher temperatures. Thus post-thaw survival should quickly be assessed and the sperm sample washed to remove all traces of glycerol.

Ques. 3 What are cryoprotective agents? How do they act?

Cryoprotectants are substances used to protect biological tissue from freezing damage. Despite having varying chemical compositions, all CPAs are highly water soluble but possess a concentration-dependent toxicity. They lower the freezing point of the solution, displace water from intracellular to extracellular environment and hence change the solute concentration in the liquid phase. There are different types of cryoprotectants, and usually, a combination of these is used in routine cryobiology

Glycerol is the most common CPA used for human sperm cryopreservation. Other CPAs have been tried subsequently with little success, because of potentially deleterious effects on human sperm. A final concentration of 6.0-7.5% (v/v) seems to be optimum. Glycerol mediates its protective effects by virtue of its colligative properties, freezing point depression, alteration of cell membrane properties by inducing changes in lipid packing structure, and the consequent lowering of electrolyte concentration in the unfrozen fraction. To optimize the cryosurvival rates, more complex diluents containing other non-permeable CPAs like glycine, zwitterions, citrate and egg yolk have been introduced. Almost all CPAs for human sperm cryopreservation contain **glycerol** (to protect against thermal shock); **sugars** (which provide the sperm with energy and optimise osmolarity and pH); **egg yolk** (which improves the fluidity of the plasma membrane, provides structural and functional protection and safeguards sperm integrity); and antibiotics (to protect against microorganisms). **Glycerol egg yolk citrate (GEYC)** was amongst the earliest and best-known extenders. Other commonly used cryoprotective buffer is a zwitterion buffer system containing N-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) and tris-(hydroxymethyl)-aminomethane **(TRIS).** This **TES-TRIS** combination (abbreviated as **TEST**) is most often used along with egg yolk, citrate and glycerol as the permeating CPA. **(Table 4)**

Table 4. Types of cryoprotectants

Table 4

TYPE OF CRYO-PROTECTANT	MECHANISM OF ACTION	EXAMPLES
Permeating	These are compounds that readily permeate cell plasma membranes. Their movement across cell membranes follows osmolarity gradient. They form hydrogen bonds with water molecules and prevent ice crystallization.	**Dimethyl sulfoxide (DMSO), ethylene glycol, propylene glycol (PROH), glycerol**
Non-permeating	They are large molecules that remain extracellular. Create osmolarity gradient by drawing water from within the cell. These can be toxic to cells at higher temperatures, and after prolonged exposure. Are used in combination with permeating CPAs to decrease cytotoxicity.	**Sucrose, Raffinose, Trehalose**

Ques. 4 What are the principles of cryopreservation?

The basic tenet of achieving success with freeze-thaw cycles is to optimize cooling and warming rates. As the extent of dehydration depends mainly on the cooling rate. rapid cooling of cells will lead to incomplete dehydration and intracellular ice crystal formation. Conversely, slow cooling of the cells may result in intense dehydration rendering the cell unsalvageable. Similarly, warming rates should be high to prevent recrystallization injury owing to intracellular ice formation.

Ques. 5 What are the common indications of semen cryopreservation?

Semen can either be cryopreserved by an individual for autologous use in the future or by fertile donors following screening for heterologous use. Due attention should be paid during donor semen banking with regards to the phenotype/blood group matching besides matching physical characteristics, race, hair color and eye color. **(Table 5 and Table 6).**

Table 5. Indications of semen cryopreservation (Adapted from WHO 2010)

Table 5

Donor semen	Semen from healthy donors known or presumed to be fertile may be stored for future use. These donors may be recruited by a clinic or sperm bank and their spermatozoa used anonymously. Donor spermatozoa can be used for AI, IUI, IVF or ICSI: • For the partner of an infertile man with **no live spermatozoa** or elongated spermatids suitable for ICSI, or where treatment has failed or is too costly • To prevent transmission of an **inherited disorder.** • To prevent fetal hemolytic anemia from **blood group incompatibility.** • After **recurrent miscarriages,** where donor insemination may result in a successful pregnancy. • For women who wish to conceive, but **do not have a male partner.** (Local and national legislation regarding genetic and infection screening should always be complied with).
Fertility preservation	Semen may be obtained and stored before a man undergoes a procedure or exposure that might prevent or impair his fertility, such as: • **Vasectomy** (in the case of a future change in the marital situation or desire for more children) • Treatment with **cytotoxic agents or radiotherapy,** which is likely to impair spermatogenesis permanently • **Active duty in a dangerous occupation,** e.g. in military forces, in countries where posthumous procreation is acceptable.
Infertility treatment	Spermatozoa may be stored for treatment of the man's partner by artificial insemination by husband's semen (AIH), IUI, IVF or ICSI, in cases of: • **Severe oligozoospermia** or intermittent presence of motile spermatozoa in the semen (as the backup for ICSI). • Treatment of infertility that may not persist, such as surgery for **genital tract obstruction** or gonadotrophin treatment for **hypothalamic-pituitary hypogonadism** • The need for special collection, such as assisted ejaculation for patients with **spinal cord injury,** spermatozoa from **retrograde ejaculation** in urine, or surgical collection from the genital tract. • Men who are unable to provide fresh semen on the day of an ART procedure.
Minimizing infectious disease transmission	For men with HIV controlled by antiretroviral therapy, samples with an undetectable viral load may be stored for IUI, IVF or ICSI, to attempt conception while reducing the risk of transmission of HIV to the female partner.

For fertility preservation or infertility treatment, enough normal specimens should be stored for **10 or more inseminations,** to ensure a good chance of pregnancy.

Table 6: Current approaches to semen banking

Table 6

Clinical presentation of the male requiring semen banking	Method of sperm harvesting	Method of semen banking
Male with normal/or low semen count and desires autologous semen banking	Semen obtained by masturbation/ electroejaculation	Raw/prepared semen freezing
Azoospermic males in reproductive age group:		
Obstructive (LH, FSH within normal limits)	Sample extracted from the epididymis: Percutaneous epididymal sperm aspiration (PESA)	Freezing of the epididymal aspirate either unprepared or after a gentle wash and swim up
Nonobstructive (FSH may be raised)	**MESA:** Microsurgical epididymal sperm aspiration **TESE:** Testicular sperm extraction **TESA:** Testicular sperm Aspiration.	Freezing of the testicular tissue is done after gentle teasing in a sterile dish using fine needles. Tissue is frozen either unprepared or after density gradient wash
Cancer patients prior chemotherapy or radiotherapy:		
Prepubertal boys	Testicular tissue (multiple samples)	Freezing of the testicular tissue (Experimental)
Pubertal	Masturbation	Semen banking

Ques. 6 What are different techniques for sperm cryopreservation?

The 2 main conventional freezing techniques used in sperm cryopreservation are slow freezing and rapid freezing, apart from the recent emergence of cryoprotectant free vitrification.

i. Slow freezing

This involves progressive sperm cooling over a 2-3 hour period in various steps, either manually or automatically using a semi-programmable freezer. The **manual method**-This involves direct contact between the straws and liquid nitrogen vapors (LNV) for 8-10 mins, followed by plunging the straws in LN_2 at -196°C. Initially, the semen sample is mixed with equal volume of CPA in a dropwise manner, and the mixture is then loaded in either straws or cryovials and left to incubate at 4°C for 10 minutes. The straws/vials are then placed 15-20 cm above the level of LN_2 for 15 mins and finally immersed in LN_2. Involves the stepwise addition of CPA while simultaneously decreasing temperature and finally plunging the samples in LN_2. Since reproducibility of this technique was an issue, **programmable freezers** were investigated. These use software data logging to obtain cooling from 20°C to -80°C at a rate of 1.5°C/min and then at 6°C/min. At the completion of freezing, the straws are plunged into LN_2 at -196°C. However, it has been argued that conventional slow freezing, either manual or automated, leads to considerable damage to the sperm due to ice crystal formation.

ii. Vitrification

The cooling and warming processes during slow and rapid freezing associated with the intermediate zone of temperature (-10 to -60°C), which the cells must traverse twice (once during cooling and then during warming), can be lethal to the sperms. Vitrification method does not require either the use of specially devised cooling programs or CPAs and is much faster, simpler and cheaper. The method is based on cooling of sperms by direct immersion into LN_2, thereby avoiding intracellular ice crystal formation. Optimal cooling rates are obtained with the following specifically designed packaging systems: Open pulled straws, the Flexipet denuding pipette, micro-drops, electron microscope copper grids, the Hemi-straw system, Cryotop, Cryoleaf, Cryotip and other carrier devices. Another modification of vitrification is direct dropping of spermatozoa suspension in LN_2. **(Table 7)**

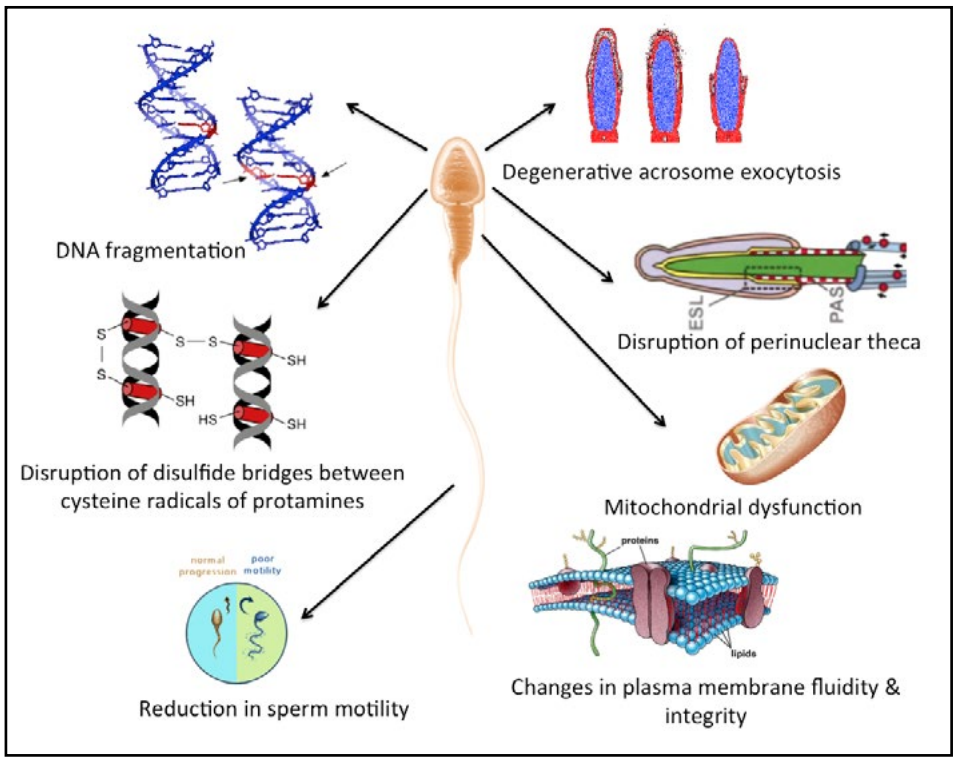
Table 7: Methods of semen freezing

Methods	Principles	Storage
Vapor-phase cooling	1. The procedure is carried out manually 2. LN2 is always vaporizing due to its low boiling point and this vapor phase that naturally exists around liquid nitrogen tank is utilized for the desired cooling 3. The cryovials/straws are placed at predetermined heights above liquid phase for predetermined periods so that the desired cooling curve is attained	Sample is stored either in the vapor phase in the LN2 container or dipped in the LN_2 after gradual cooling
Programmable freezing machine	1. Not essential for human sperm cryopreservation since vapour phase cooling gives us reproducible good reults on thawing . 2. Sample is loaded in the straw or the vial These are then cooled using a programmable machine and then dipped in liquid nitrogen	Sample is stored in the liquid phase

Table 7

Ques. 7 What are the common injuries sustained during sperm freezing?

Even though spermatozoa seem to be less sensitive to cryodamage as compared to other cell types because of their membrane fluidity and the low water content, cryopreservation does render significant structural and functional damage to the sperm. A combination of thermal shock, osmotic shock, cellular dehydration and intracellular ice crystal formation is responsible for the cryoinjuries sustained during freeze-thaw process. As previously discussed, the cryopreservation procedure has a detrimental effect by changing the carbohydrate composition of the glycocalyx, and hence it impairs the function of membrane proteins responsible for ion transport and metabolism thereby impairing the fertilizing ability. Cryopreservation significantly affects post-thaw sperm motility due to membrane swelling and acrosome degeneration. Fatty acids present in the sperm plasma membrane are vulnerable to lipid peroxidation resulting in loss of intracellular enzymes and inhibition of oxidative phosphorylation. The mitochondrial membrane is susceptible to damage at low temperatures, and an alteration in membrane fluidity can cause the release of reactive oxygen species (ROS). The ROS damage may involve single- or double-stranded DNA breakage. Though the detrimental effects of cryopreservation on the fertilization capacity, motility, morphology, and vitality are well established, there is little consensus on whether cryopreservation induces DNA damage or not. Donnelly et al. studied the pre-freezing and post-freezing sperm DNA integrity in both semen and prepared samples, and found that sperm frozen along with seminal plasma had improved post-thaw DNA integrity, probably due to the presence of abundant antioxidants in seminal plasma.



PIC 1. Various forms of cryoinjuries inflicted by the freeze-thaw procedure

Sperm freezing and DNA damage

Several authors conclusively believe that overall sperm quality deteriorates after freeze-thawing, including sperm DNA damage, as assessed by sperm chromatin structure assay (SCSA) or Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Thomson et al. proposed that the cryopreservation-induced sperm DNA damage is mediated predominantly by oxidative stress rather than apoptosis.

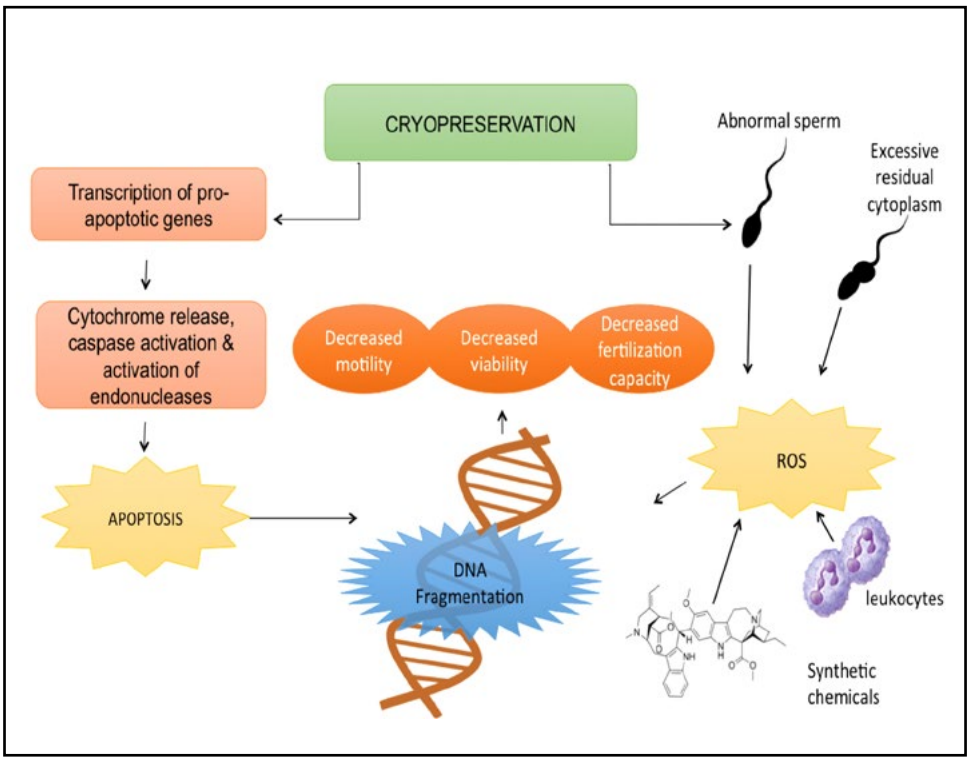
Few others believe that freeze-thawing does induce sperm DNA damage, but only in infertile men that have a greater incidence of irregular chromatin organization and show significantly decreased resistance to thermal denaturation as compared to spermatozoa from fertile men. Kalthur et al. evaluated sperm morphology and DNA damage before and after cryopreservation, and reported that the susceptibility of morphologically abnormal sperm to DNA damage during cryopreservation is significantly higher as compared to sperm with normal morphology.

In contrast to the above two, the third line of thought believes that freeze-thawing is not associated with a detrimental effect on sperm DNA integrity. Duru et al. noted in their study that though cryopreservation altered the plasma membrane symmetry and was associated with translocation of phosphatidylserine, the DNA integrity remained intact. Similarly, Isachenko et al. concluded that the integrity of DNA is unaffected by cryopreservation while comparing the effects of slow-freezing and vitrification on sperm DNA integrity.

Sperm freezing and apoptosis

Apoptosis, or programmed cell death, has been proposed to play a role in causing cryoinjury to sperm DNA by increased activation of various aspartic acid-directed cysteine proteases, called caspases. Activation of **caspases** leads to morphological changes and characteristics of apoptotic cells. Initiator caspases include caspases-2, 8, 9 and 10, that activate effector caspases (3, 6 and 7). This results in cleavage of several substrates and culminates in apoptosis. Besides the presence of caspases in spermatozoa as a marker of apoptosis, the externalization of phosphatidylserine on the sperm membrane is considered as a relatively early apoptotic marker. This exposed phosphatidylserine is then amenable to phagocyte-mediated lysis. Apart from this, a family of ligands known as **Fas-FasL** also plays a significant role in activation of effector caspases. Fas receptor is present only in <10% of healthy ejaculated spermatozoa, as compared to >50% of ejaculated spermatozoa in oligozoospermic men.

DNA fragmentation is considered a late-stage marker for apoptosis in spermatozoa, and can partially be caused by activation of caspase-3. The triggers for activation of apoptosis result in the permeabilization of the outer mitochondrial membrane, first by activation of **BAX and BAK** proteins followed by the release of **cytochrome-c**. This results in caspase-9 activation along with **APAF-1** thereby forming an apoptosome, finally initiating the apoptosis cascade. Chlamydia and mycoplasma infections, and various other microbiological toxins have been shown to increase apoptosis in human spermatozoa; the resultant high DNA fragmentation levels are amenable to treatment using specific antibiotic therapy. The other triggers for induction of apoptosis may be neoplasia (esp. patients with Hodgkin's disease and testicular cancer), and environmental toxins.



PIC 2. Mechanism of DNA Injury during cryopreservation

Apoptosis markers (caspase activation, DNA damage, and phosphatidylserine externalization) have been linked to male infertility in numerous studies. Spermatozoa positive for active caspase-3 demonstrate phosphatidylserine externalization and DNA fragmentation more frequently than normal controls. Moreover, low-motility spermatozoa demonstrate a higher level of apoptosis markers as compared to high-motility spermatozoa. Various studies have shown that these apoptosis markers tend to increase in spermatozoa following the freeze-thaw process. Normozoospermic semen samples are more resistant to the damage induced by freezing and thawing as compared to oligozoospermic samples. Verza et al. reported that motile sperm could be recovered even after five freeze-thaw cycles in normozoospermic men, while motility could be salvaged after only two freeze-thaw cycles in oligozoospermic samples. The extent of damage is correlated to the degree of oligoasthenoteratozoospermia. Moreover, cryopreserved spermatozoa from cancer patients were also found to be having a higher level of DNA fragmentation as compared to healthy controls.

Cryopreservation and mitochondrial damage

Transmission electron microscopy (TEM) studies have shown an alteration in the ultrastructure of the mitochondria and plasma membranes, and have confirmed that mitochondrial destruction is secondary to widespread cellular destruction. Changes in **mitochondrial membrane potential (M ΔΨ)** are assessed using a fluorescent cationic dye, 5,5', 6,6' –tetrachloro-1-1', 3, 3'-tetraethylbenzamidazolocarbocyanin iodide (commonly know as **JC-1**). Uncoupled mitochondria are suggestive of unhealthy spermatozoa, and hence determination of mitochondrial membrane potential is useful to assess post-thaw sperm survival. In intact mitochondria, M ΔΨ is unaltered and the JC-1 dye aggregates inside the non-damaged mitochondria and fluoresces red. In damaged mitochondria, the M ΔΨ is broken down and the JC-1 dye disperses through the entire cell and fluoresces green. **(Table 8)**

Table 8. Sperm freezing injuries

Table 8

Mechanism of cryoinjury	Cellular Effects
DNA damage	Irregular chromatin organization leads to decreased resistance to thermal denaturation, increased DNA fragmentation index (DFI >30%) and increase in reactive oxygen species (ROS).
Apoptosis	Externalization of phosphatidylserine to the outer leaflet of plasma membrane, caspase activation, impairment in mismatch repair genes, excision repair genes, a decrease in sperm count, motility and vitality, decreased fertilization rates.
Mitochondrial damage	Decreased sperm motility and vitality, decreased fertilization rates, decreased sperm capacitation, altered zona pellucida induced acrosome reaction.

Ques. 8 How do we cryopreserve small numbers of spermatozoa or rare sperm?

Conventional methods of sperm cryopreservation are not suitable for preserving very small numbers of spermatozoa, such as epididymal or testicular spermatozoa obtained after surgical sperm retrieval. Hence, various novel methods have been devised to store limited numbers of such spermatozoa in a small volume. Though both biological and non-biological carriers have been used for this purpose, no prospective randomized trials have been conducted to show the superiority of one technique over the other. Hence, novel techniques for storing a small number of spermatozoa need to be further explored.

Spermatozoa have been successfully cryopreserved using empty zona pellucida by various groups and this has the advantage of reducing the time in screening to locate motile sperm but carries a potential risk of biological contamination. Similarly, others have used microdroplets for freezing spermatozoa. This method avoids sperm loss due to adherence to the vessel but potentially carries the risk of cross contamination. Other methods used for storing a limited number of spermatozoa include ICSI pipette, Volvox globator spheres, Alginate beads, Cryoloop, Agarose microspheres, and straws.

Ques. 9 Does sperm cryopreservation effect fertility outcome?

Due to the wide range of cryoinjuries incurred to the spermatozoa, the fertilization potential of sperm is reduced and hence, the pregnancy rates following intrauterine insemination and conventional in vitro fertilization (IVF) are lower as compared to insemination with fresh sperm. Hence, cryopreservation of sperm before intrauterine insemination or conventional IVF is not recommended. However, the results with testicular frozen spermatozoa when used for ICSI are comparable to fresh spermatozoa from the same subject. No differences in fertilization rate, cleavage rate, embryo quality, clinical pregnancy rate and ongoing pregnancy rates are noted with the use of cryopreserved testicular spermatozoa as compared to fresh sperm. Even with the use of ejaculated spermatozoa, the fertilization rates are comparable between fresh and frozen groups.

CONCLUSION

Sperm banking has become a widely accepted mode of infertility treatment, and in recent times mooted as having a new role as fertility insurance. Once looked upon with improbability, this practice has established to be a successful technique of keeping the anticipation of a family alive for countless families. The motives for storage are as diverse as humans themselves. So far, no limit has been established for how long human semen can be frozen when maintained and stored in appropriate liquid nitrogen storage. Scientific literature shows conclusively that sperm motility, viability and morphology are not affected by proper long-term cryopreservation. Cryo-thaw semen pregnancies have been reported after 2 to 3 decades of semen banking.Appropriate screening should be carried out before semen banking as per available guidelines.

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Part : 2

Semen freezing, hands on....

Index

Ques. 10 How do we prepare the laboratory and semen sample for freezing?

Organize the bench table, media and disposables.
The common things required are enumerated in **Table no 9, 10 & 11.**
Please also ref to **Fig 1-12.**

Table 9. Equipment and disposables for semen freezing

Table 9

Equipment	Principles
Liquid nitrogen storage can	LN_2 storage can, roller base, box rack & vial boxes. Aluminum canes, canisters for holding canes, cryogenic gloves, vapor shipper.
Control rate freezer	Low-level alarms, liquid nitrogen ruler
Microscope	-Makler chamber or hemocytometer. -10x, 20x, 40x and 100x objectives. -Trinocular head with camera port.
Centrifuge	Digital speed indicator, time indicator, swing out head, auto lock on the lid to avoid accidents during operation, built-in alarm to indicate end of cycle.
Disposables	-Slides, cover slips. -IUI consumables and disposables used in semen preparation process (toxicity tested). -Gamma sterilized and individually wrapped plastic ware. -Sterile volumetric pipettes. -14 ml conical bottom test tubes. -Sterile specimen container. -Scissors. -Dispensing pipettes and tips. -Round bottom test tubes.
Workstation 4' x 2' x 2'	-HEPA filters having the capacity to retain particles of 0.3 microns and higher. -Filtering efficiency: 99.997% -Full body made of powder coated steel with stainless steel work table. -Illuminated by fluorescent tube lights. -Fitted with static pressure indicator (manometer). -Inner chamber sterilization through UV-germicidal tubes.
Incubator	-Double-walled, the outer chamber made of MS with glass wool insulation between 2 walls. Inner wall made of SS. -Heating elements placed in the ribs of the bottom and sides for uniform heating. -Fitted with air circulating fan for better maintenance of ambient temperature inside the incubator. -Thermometer fixed at the front of the oven. -Temperature controlled by PID (proportional-integral-derivative controller) with an accuracy of 0.1°C. -Perforated shelves.

Fig. 1

FIG 1. 1.8 ml capacity High-density Polypropylene cryovials with screw caps for semen packaging. Vials are designed for exceptional ease of use and sample integrity in ultra-low temperature storage. Closure seals without gasket or O-ring, minimizing risk of contamination. The deep skirt of the closure allows single-handed aseptic technique without exposing fingers to vial contents. Ribs around the bottom of the vial interlock with wells in the Cryovial racks so the vial will not turn when opening or closing.
Bottoms of vials are skirted; thus the vial stands without a holder.
Sterile vials and closures are pre-assembled and radiation-sterilized. A white marking area, fill line and graduations are printed on the side. Certified sterile, non-cytotoxic and non-pyrogenic.

Fig. 2

FIG 2. Cryovials from different manufacturers vary in size and placement of cap rings. Cryocell vials have an external gutter for LN_2. NUNC cryovials have an internal threading's. These are mounted on the aluminum vial holders by plugging mechanism**.**

Fig. 3

FIG 3. The **CBS High Security Tube** which comes without a cap and a sealable tip eliminates concerns related to cross-contamination and sample degradation related to oxidation and evaporation. Once sealed, the CBS High Security tube can be stored in widely available storage boxes and current storage systems. The High Security resin material is compatible with cryogenic storage temperatures from -80°C to LN_2. These have the option of Colored inserts for simple identification in cryogenic environments, RFID cryoresistant chips integrated inside inserts, 2D data matrix coded inserts and is Compatible with cryoresistant labels.

Fig. 4

FIG 4. High security sperm straw (manufactured by cryobiosystem). The special design and the mode of filling and sealing of CBS High Security straws for cryopreservation of human biological samples makes them fully hermetic, especially in liquid nitrogen.
Manufactured from biocompatible materials, they are used in medically assisted procreation techniques and particularly for sperm preservation. The CBS straw is a clear, flexible tube made of ionomeric resin. Length: 133 mm, Internal diameter: 2.5 mm and is Sterilized by irradiation.
These are available with different colors of cotton plug so as to aid in easy identification.

Fig. 5

FIG 5. High security cotton-plugged sperm straws (0.5ml). The CBS High Security sperm straw is filled by aspiration using a micro-aspirator according to a specific protocol. The impermeability of the seal is only guaranteed if the CBS straws are sealed with the **SYMS** or **SYMS III** sealing systems.

Fig. 6

FIG 6. Goblet with visotubes and 0.5 ml sperm straws. Different colours help in easy identification of the sample. CBS High Security straws are placed in goblets for storage in freezers. Goblets come in 8 different colors and their sub-compartments (called visotubes) in 12 different colors. In the Daisy configuration, goblets hold 12 visotubes of different colors and can hold a maximum of 168 CBS straws of 0.3 ml or 0.5 mL

Fig. 7

FIG 7. Micro-aspirator (manufactured by Cryobiosystem) used to manually fill sperm straws. The micro-aspirator is used to manually fill straws without any risk of contamination.
Very simple to use:

- Hold the aspirator in a horizontal position.
- Carefully push the straw into the adaptor and aspirate the liquid. The tube length should be about 10 cm.
- Slowly turn the thumb wheel towards the adaptor until reaching the correct filling height.
- Remove the straw just underneath the adaptor.

Fig. 8

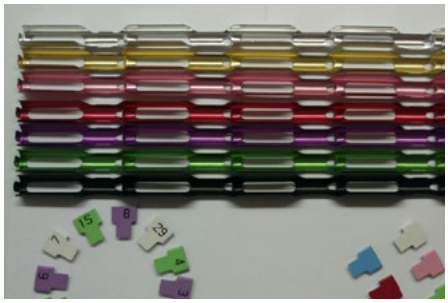
FIG 8. SYMS III sealer for high security straws (manufactured by Cryobiosystem). The **SYMS III** thermally seals 0.3 mL, 0.5 mL and 2 mL CBS™ High Security straws as well as CBS™ High Security Vitrification straws and CBS™ High Security Tubes for top quality cryopreservation of biological samples. A color touch screen that can be used while wearing laboratory gloves is present.
It has a specially adapted pre-programming for each CBS™ product's sealing temperature and time.

Fig. 9

FIG 9. Label printer (manufactured by Brady, UK) for printing labels for cryovials and straws. Labels are designed to withstand extreme laboratory environments. It is strongly recommended to overlap the label on the straw or a vial.

Fig. 10

FIG 10. Manual sealer for CBS™ High Security straws. Filled and labeled straws are heat sealed using a heat sealer. This makes the packaging relatively aseptic. It is a thermal pulse-sealing device for impermeable sealing of Security straws. The sealing device is electronically operated by a push-button or foot pedal as desired. Setup, maintenance and downtime time are approximately 5 minutes per day.
Potentially contaminated parts of the system can be cleaned with ethanol or non-corrosive decontamination fluids.



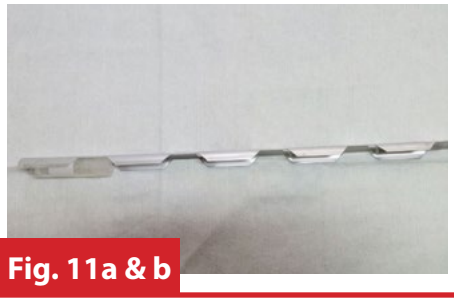


Fig. 11a & b

FIG 11a&b. OOSAFE® COLOR CANES Oosafe® Color Canes are designed for safely storing cryo vials and straws inside goblets. Unlimited and unique ID combinations can be made for stored samples together with different colors of goblets and printed tags. This great feature helps to differentiate each patient's samples in LN_2 tanks which minimizes mix-ups. The whole range of canes is made of aluminum and safe to use for IVF and other cryo applications. There are different options to be used with different sizes of goblets and 1-2 ml cryo vials. **OOSAFE® TAGS FOR CANES** The whole range of color and printed ID Tags is made of aluminum and safe to use for IVF and other cryo applications. Our prints on tags are durable and permanent, unlike hand writing on plaintags, with perma nent cryo marker pens. This feature makes the tags the safest choice for storage inside LN_2 tanks. The standard print on tags is the number from 1 to 35.

Fig. 12

FIG 12. Computer-controlled freezer: (Freeze Control temperature controller, cryochamber and cryobath)

The design ensures symmetrical heat transfer from all specimens to Liquid Nitrogen. The highly conductive material used for the chamber ensures a very high degree of temperature uniformity of each specimen whether Liquid Nitrogen levels are high or low. The conductive core of the chamber, and the tight thermal coupling between specimens and core, ensure that Latent Heat is efficiently removed during the nucleation process.

Specimen temperature is monitored continuously, and is measured by a precision Platinum temperature sensor permanently mounted in the core. Specimens can be safely accessed for manual seeding, without exposing them to temperature fluctuations.

A range of FREEZE CONTROL® cryochambers is available to accommodate different applications, with cooling rates appropriate for most protocols. Chambers are interchangeable because each chamber is separate from, and plugs into the controller.

Ques. 11 How do we finally package the sample for freezing?

We use vide variety of containers for the packaging. They all have their pros and cons.

Table 10 : Packaging of the semen sample

Table 10

	Material	Advantages	Disadvantages
Straws	Ionomeric resin CBS High security (Cryo Biosystem, Paris, France)	Straws are available in a variety of colors suitable for the easy identification of samples, and many hundreds can be stored in plastic goblets in canisters within liquid nitrogen containers.	Maximum capacity of approximately 0.5 ml only. Overfilled straws are prone to cracking and expelling the powder sealing plugs into the liquid nitrogen. Labeling and filling difficulties. A high surface/volume ratio which makes the sample very susceptible to warming shock damage resulting from exposure to ambient temperatures during handling.
Cryovials	Polypropylene with screw caps	These are easy to fill and stores nearly 1.5 ml of the semen plus cryoprotectant mixture.	Storage on aluminum canes is not dependable as they lose their memory and cryovials may jump off the holder. Screw-top vials do not maintain their seals. They have the potential of exploding upon thawing because liquid nitrogen trapped in the vials expand to many times its volume when it converts to gaseous nitrogen. The low surface: volume ratio and thick wall of the cryovial increase the time required for samples to reach critical temperatures, and thus increase the risk of damage from brief exposure to ambient temperatures.

Ques. 12 Do We have any commercial media available for semen freezing? Which is a better brand. (Fig. 13)

Yes, a large number of brands are available. You may pick up any. Ensure that the cold chain is maintained and the company adheres to strict manufacturing guidelines. Quality control testing should be checked and the following test are mandatory before releasing the media in the market for commercial use.

Sterility tested (Ph. Eur. USP) pH tested (Ph.Eur. USP) Endotoxin tested ≤ 0.1 EU/mL (Ph.Eur., USP) Not Mouse Embryo Assay (MEA) tested Sperm Survival tested. The results of each batch are stated on a Certificate of Analysis. Fungal Screen and Bacterial Screen should be negative. Sperm Freezing contains only a low concentration of sodium bicarbonate and should not be gassed with CO_2. Always store the media Store in an original container at 2-8°C, protected from light.

Semen freezing media components	Commercial semen freezing media contains: $CaCl_2$, Gentamicin sulfate, glucose, glycerol, HEPES human albumin solution, Milli Rx water, $MgSO_4$, Phenol red, KCl, $NaHCO_3$, NaCl, Sodium L- lactate, Na_3PO_4, SSR (synthetic serum replacement), sucrose

A

B

C

Fig. 13

Fig 13. Sperm Freezing Media is Egg yolk-free media and contains glycerol and sucrose as the cryoprotective agents.

Ques. 13 What is the approximate Pricing of the semen freezing media and disposables?

We are commonly asked about the pricing of the SFM and disposables for semen banking. The price depends upon the technique of freezing and quality of products we use. Approximate prices are mentioned in **(Table 13)**

Product	Price per unit with VAT(in INR)	Remarks
Aluminium Cryocane	70-100/-	Any suggested vendors. For oosafe products consult intermedics India limited.
Cryovials	100/-	Nunc /Nalgene/Tarson
Cryotubes	104/-	CBS
Sperm straws	48/-	CBS
Manual aspirator	9000/-	CBS
SYMS sealer III	3.6 lac/-	CBS
Semen freezing media	2000/-	Irvine/Origio/Cook/Vitrolife

Ques. 14 How is the Sample collection done for semen freezing?

- The sample collection is achieved by masturbation in healthy individuals.
- The patient should be counseled about adequate abstinence (2-7 days) and provided with a sterile specimen collection container with ample time and privacy to do so.
- Lubricants (soap, jelly, and saliva) should be avoided as they are spermatotoxic.
- If no ejaculate is produced by the patient, then post-ejaculate urine analysis should be performed to rule out retrograde ejaculation.
- In a case of retrograde ejaculation, alpha agonists may be administered in an effort to convert retrograde to antegrade ejaculation. Alkalinization of urine should be carried out before attempting to harvest the sperms from the urine sample.
- Audio-visual aids may be provided to enable patient stimulation and arousal.

- If the patient is unable to provide the sample, consideration should be given to vibratory stimulation or electroejaculation.

Ques. 15 What are the different methods for semen freezing? (Fig 14-28)

a. Liquid nitrogen vapor cooling and thawing method.

- Confirm the identity of the sample and the reason for cryopreservation. Keep the sample at room temperature for 30 minutes for liquefaction. Meanwhile, label the cryovials/cryo straws and enter the procedure details in the logbook. Weigh the semen collection container after obtaining the sample, and estimate the volume of the semen obtained. Take out an equal volume of the sperm freezing media (SFM) from the bottle (kept at 4-8°C) and bring it to room temperature.

- After liquefaction, carry out semen analysis as per WHO 2010 guidelines. A normozoospermic sample can be frozen raw, whereas oligozoospermic samples may be concentrated prior to freezing to enhance post-thaw recovery.

- Once it is ensured that both the semen sample and the SFM have been brought to room temperature, dilute the semen 1:1 (v/v) with the SFM. The medium should be added drop by drop to the semen and the solution should be carefully mixed after each additional drop of SFM to reduce the toxicity associated with the cryoprotectant. The mixture is then left at room temperature for 10-15 mins to equilibrate.

- The diluted semen is then loaded into cryo straws or cryovials according to the manufacturer's recommendations. Either cryovials or cryo straws can be used, but each has its own pros and cons **(Table 10).** It is important to leave some air space to allow expansion of the solution during freezing. If using straws, the straws are then sealed by using SYMS sealer/ultrasound sealer.

- The straws/vials are then kept at 4°C for 10 minutes and then suspended 4-5 cm above the surface of liquid nitrogen for 20-30 minutes.

- Finally, the straws/vials are transferred into LN_2 and stored at -196°C.

Thawing procedure

- The straws/vials are removed from LN_2 after ascertaining the identity of the patient and washed under running water for few minutes. They are then placed at room temperature until the time the external sweating is no longer appreciable and the sample has completely liquefied. In the case of cryovials, the cap is then carefully loosened slightly.

-The vials are wiped totally dry, the cap is opened in accordance with the manufacturer's guidelines and the thawed semen is removed.

- Dilute the semen with sperm preparation medium (1:1) to reduce the toxic effect of glycerol.

- Evaluate the post-thaw sperm survival, and thaw additional straws/vials if necessary.

- Immediately prepare the sample by the density gradient method or single wash technique to remove the cryoprotectant.

- Dilute the final sample to achieve a concentration of 20 million/ml or above.

b. Controlled-rate freezing method

Computer-controlled freezer:
(a) LN_2 vapor filled chambers (e.g kryo-10, planer, sunbury, UK).
(b) Nicole models, Air liquid, Bussy-Saint-Georges, France.
(c) Cooled metal blocks- Cryologic, Australia are widely used cryoplanars.

Protocol:

- Ensure that both the semen sample and sperm cryopreservation buffer are at room temperature.
- Mix equal volume of the buffer to the semen sample (1:1).
- Leave the mixture at room temperature for 10 minutes.
- Label the straws with relevant patient information.
-Load the sample into a freezing straw/vial and seal according to the manufacturer's guidelines.

i . Protocol with straws

Load straws into the freezing machine and initiate the freeze program having parameters as described below:
- Start temperature is +20°C
- Cooling rate of -6°C/min until -80°C
- At -80°C plunge the straws into liquid nitrogen

ii. Protocol with cryovials

Load vials into the freezing machine and initiate the freeze program having parameters as described below:

- Start temperature is +20°C
- Cooling rate of -0.05 °C/min until +5°C
- At 5°C, cooling at rate of -1°C /min to +4°C
- At 4°C, cooling at rate of -2°C /min to +3°C
- At 3°C, cooling at rate of -4°C /min to +2°C
- At 2°C, cooling at rate of -8°C /min to +1°C
- At 1°C, cooling at rate of -10°C /min to -80°C
- At -80°C, hold for 10 min
- Plunge the cryovials into LN_2

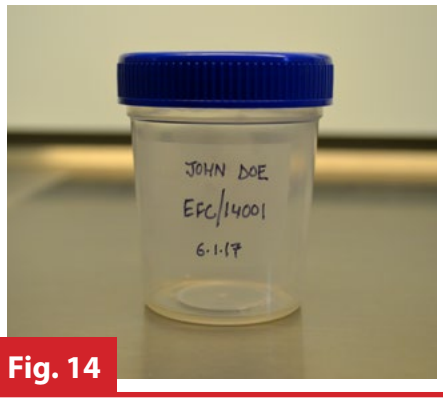


Fig. 14

FIG14. Semen sample is kept at the room temperature for liquefaction for 30 min-1 hr. Semen sample is then analysed as per WHO 2010 criterion. Depending upon the count we decide about the number of vials to be frozen. The post thaw recovery should be around 20 million active sperms per ml. Remember that only 50-60 % sperms survive cryo thaw cycle.

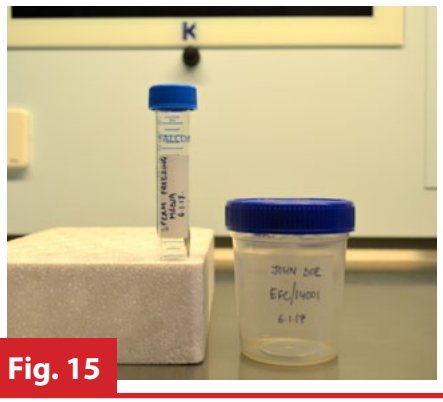


Fig. 15

FIG 15. Semen sample and the sperm freezing media kept at room temperature in equal volumes for equilibration.

Fig. 16

FIG 16. Add the Cryoprotectant media drop by drop over a period of 10 minutes to the equal volume of the semen sample raw or unprepared. The solution should be carefully mixed after each additional drop of SFM to reduce the toxicity associated with the cryoprotectant. The mixture is then left at room temperature for 10-15 mins to equilibrate.

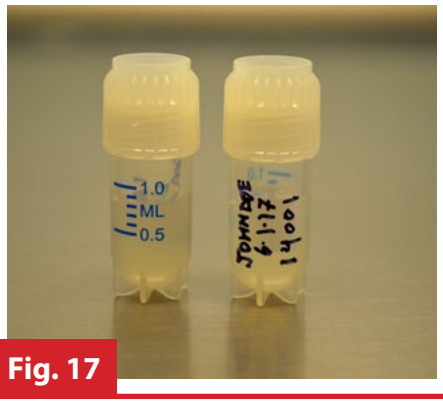


Fig. 17

FIG 17. Keep the vials at the room temperature for a period of 10-15 minutes.

Fig. 18

FIG 18. If we are using CBS cryostraws ,we have to Load the cryovial on SYMS III sealer.

Fig. 19

FIG 19. Sealing of cryotube using SYMS III sealer has been done and the cryotube is hermitically sealed.

Fig. 20

FIG 20. The cryovials/crytubes are loaded on the aluminium cryocanes and Kept at 4ºC temperature for a period of 15-20 minutes in regrigerator.

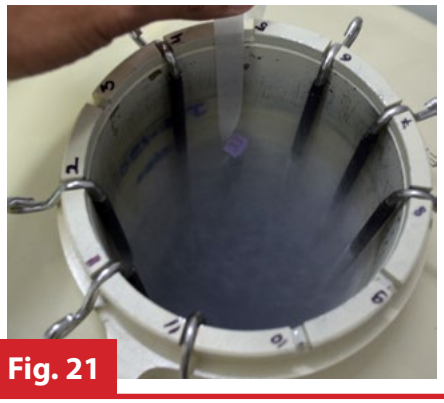


Fig. 21

Figure 21. If we are doing vapor phase manual freezing , place the sample vial in the vapor phase for final step of cooling for minimum 20 minutes in a cryocan for gradual cooling to approximately - 100ºC.

Fig. 22

FIG 22. Programmable freezer for carrying out slow-freezing. Nicole models, Air liquid, Bussy-Saint-Georges, France.

Fig. 23

FIG 23. If you prefer straws for semen freezing, load semen sample into the labeled high security cryostraws using a **manual aspirator.**

Fig. 24

FIG 24. Filled and labeled straws are heat sealed using a heat sealer. This makes the packaging relatively aseptic.

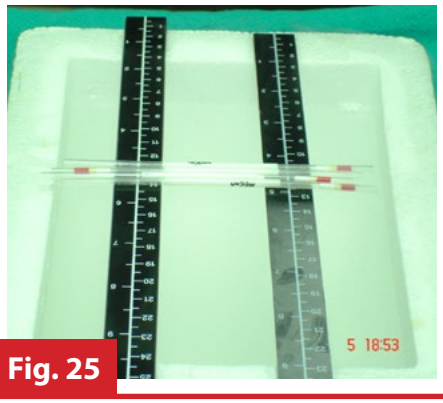


Fig. 25

FIG 25. Vapour phase non-equilibration cooling of the loaded semen straws. Like for the vials, place the straws 2-3 cm above the surface of liquid nitrogen for 20-30 minutes in a styrofoam box.

Fig. 26

FIG 26. The straws/vials are removed from LN_2 after ascertaining the identity of the patient and washed under running water for few minutes or Keep vials at room temperature for 5 minutes for thawing. They are normally placed at room temperature until the external sweating is no longer appreciable. In the case of cryovials, the cap is then carefully loosened slightly. Prepare the sample after the sweating has stopped and the contents have warmed up.

Fig. 27

FIG 27. Gradually add the sperm preparation media to the thawed suspension to avoid sudden osmotic stress injuries. Thawed semen sample and the sperm preparation media should be at the same temperature to avoid thermal shock.

Fig. 28

FIG 28. Prepare the semen sample at low centrifugation speed and collect swim-up after 20 minutes.

Suggested Vendors

Vendor	Contact Person	Phone	Email
Origio India Pvt. Ltd.	Mr. Rajeev Kayestha	8450994447	rkayestha@origio.com
Vision Diagnostics Pvt. Ltd.	Mr. Punit Khatnani	9910188771	punit@vision-groups.com
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