SPERM CRYOPRESERVATION

Introduction

- Upto 50% of infertility cases represent male factor infertility
- In the majority of these cases, patients may opt to freeze their sperm for later use for procreation

- Spermatozoa are ideal to cryopreserve because:
- (1) they are the smallest human cells
- (2) they have a relatively small volume
- (3) a large surface area
- (4) they contain very little cytoplasm
- (5) less total intracellular water compared to other cells
- (6) they exist individually, allowing effective dehydration

- Cryopreservation of spermatozoa is the best option available for having a biological child for these patients
- It involves freezing of sperm at extremely low temperatures using liquid nitrogen (LN2)
- This process is carried out in the presence of cryoprotectants
- Cryprotectants play a critical role in protecting sperm during freezing
- In the absence of cryoprotectant spermatozoa can be damaged because of the swelling of the plasma membrane as water expands during freezing, causing acrosomal leakage and breakdown

- Glycerol has been used as an effective primary cryoprotectant for freezing spermatozoa
- The function of glycerol is to remove or reduce the water content and help minimize intracellular ice formation during freezing

- Osmotic equilibrium is reached as the cryoprotectant penetrates the cell and displaces the intracellular water and the sperm returns to almost its original volume
- There are a number of factors that may influence and induce abnormal changes in spermatozoa as a result of cryopreservation

- These include failure to maintain the optimum temperature and use of diluents that might have negative effects on sperm quality, including reduced semen parameters such as vitality and motility and increased sperm DNA fragmentation

- Physicians recommend preservation of gametes especially for adults and young adolescents diagnosed with cancer before they start treatment for cancer
- These treatments interfere with the normal functioning of the gametes as well as the process of gametogenesis

- To ensure high success rates of the assisted reproduction via sperm cryopreservation, it is necessary to understand the physiology and cryobiology of the sperm
- Unlike embryos, sperm cells are smaller in size and have a larger surface area
- These characteristics help them to maintain viscosity and the glass transition temperature prevents their cytosol from cryodamage
- The role of cytosol is to provide protection from lipid peroxidation and DNA fragmentation
- Cooling of sperm cells at lower temperatures ceases all physiological processes and extends their life span

Sperm Cryopreservation

- Cryopreservation is the process of stabilizing the cells at low temperatures (cryogenic temperature) by applying the principles of cryobiology
- Freezing of sperm cells for extended periods is possible by arresting their metabolic processes
- This can be achieved by storing these cells at –196 °C in LN2
- At such a low temperature, the cells dehydrate as a result of water loss due to the formation of ice crystals
- The energy of the cells to carry out physiological cycles is reduced⟶ it becomes easier to store them for longer periods of time

- The addition of a cryoprotectant is usually accompanied by a reduction in temperature
- As the cooling process continues and the temperature reaches –5 °C to –15 °C, extracellular ice formation occurs
- This induces the development of the extracellular solid phase
- The freezing point of water can be reduced to –42 °C by preventing the process of nucleation – that is, interaction between water droplets to form ice crystals

- During the process of cryopreservation, the solutions are cooled below their freezing point without a change in their phase from liquid to solid
- This phenomenon is termed **supercooling**
- The formation of ice crystals during this process can be avoided by rapidly reducing the temperature
- This starts with forming an ice nucleus in the extracellular space
- Solutes are excluded from the ice formed, which increases the concentration of solutes outside the cell
- This causes solutes to enter the cell through the plasma membrane and causes them to lose intracellular water, resulting in cell dehydration

- During the freezing process, sufficient time should be allowed for sperm cell dehydration and osmotic equilibration
- Very slow cooling causes dehydration of the cell while very fast cooling might result in intracellular ice formation → temperature plays a crucial role here and needs to be controlled accurately

- In contrast, when the cells are thawed, the process is reversed
- There is a continuous influx of water in the cell, which causes the cells to swell
- After a certain period, the cells bursts because of the disruption of the cell membrane

- This damage caused by cryopreservation is called cryodamage

- The thawing process is as important as the freezing process
- The cells should be allowed to restart their physiological activities
- Rapid warming can cause heat shock to the cells and damage the cell membrane
- Usually 37 °C is used, and higher temperatures are not recommended due to risks associated with cell damage
- Cells that have been frozen slowly should also be warmed slowly during thawing, while cells that were frozen rapidly should be thawed rapidly

- Although glycerol is a widely used cryoprotectant, it might have negative effects on sperm when added at higher concentrations
- The toxicity of glycerol is noted above the concentration of 6%vol./vol
- In addition, glycerol also has direct osmotic effects
- It tends to cross the plasma membrane comparatively slower than water
- For these reasons, addition and removal of glycerol changes the volume of the cell
- If the change in volume crosses the osmotic tolerance level of the cell, it causes shrinking or swelling of the cells

Indications for Sperm Cryopreservation

- 1. **Cancer**: Men diagnosed with malignant cancer, including testicular cancer, Hodgkin's and non- Hodgkin's lymphoma, leukemia, prostate cancer, and many other types of cancer, prior to radiation therapy for the treatment of cancer

- 2. **Surgery**: Physicians recommend freezing of spermatozoa before undergoing surgeries for vasectomy, vasectomy reversal, or ejaculation failure
- Patients with other medical conditions, including varicocele, spinal cord injury, and infections from Zika virus, hepatitis B, or HIV, are also recommended to preserve their fertility

- 3. **Obstructive or nonobstructive azoospermia**: Sperm are absent in the semen of patients suffering from azoospermia, which may be obstructive or nonobstructive► Sperm cryopreservation is a relevant option for them to preserve fertility

- 4. **Male factor infertility**: When the infertility is caused due to problems in the male while the female has no abnormal parameters, it is recommended to preserve a semen sample to avoid sterility in a later phase

- 5. **Autologous or donor sperm cryopreservation**: Autologous sperm banking or client depositor refers to the individual who preserves spermatozoa for future use with his partner, while a sperm donor is an individual who is serving as a surrogate father for an infertile couple or single female
- He may be a directed or anonymous donor; It is necessary for all donors to undergo clinical assessment before sperm donation to avoid infections

- 6. **Occupational**: Occupations with higher exposure to chemicals such as phthalates, pesticides, polychlorinated biphenyls, etc. might affect the fertility in men by increasing oxidative stress and reducing semen parameters► It is recommended to preserve fertility in such cases

- 7. **Traveling husband/military assignments**: In these cases, it becomes difficult to time intercourse with the process of ovulation; Thus, it is necessary to preserve the semen sample from the husband for future use through assisted reproduction

- 8. **Gender reassignment**: Approximately three times more individuals opt for male-to-female gender reassignment as compared to female-to-male; These individuals can preserve their sperm before going through gender reassignment and achieve parenthood in the future

Techniques of Sperm Cryopreservation

- **Slow Freezing**
- The principle for this protocol is the formation of ice crystals from extracellular water

- This differentiates the two phases:
- ice crystals formed from extracellular water
- and water in the liquid phase

- The liquid phase consists of cryoprotectants, salts, and sugars
- The sperm cells are cooled progressively in several steps over 2–4 hours
- This can be carried out manually or with a semi-programmable freezer

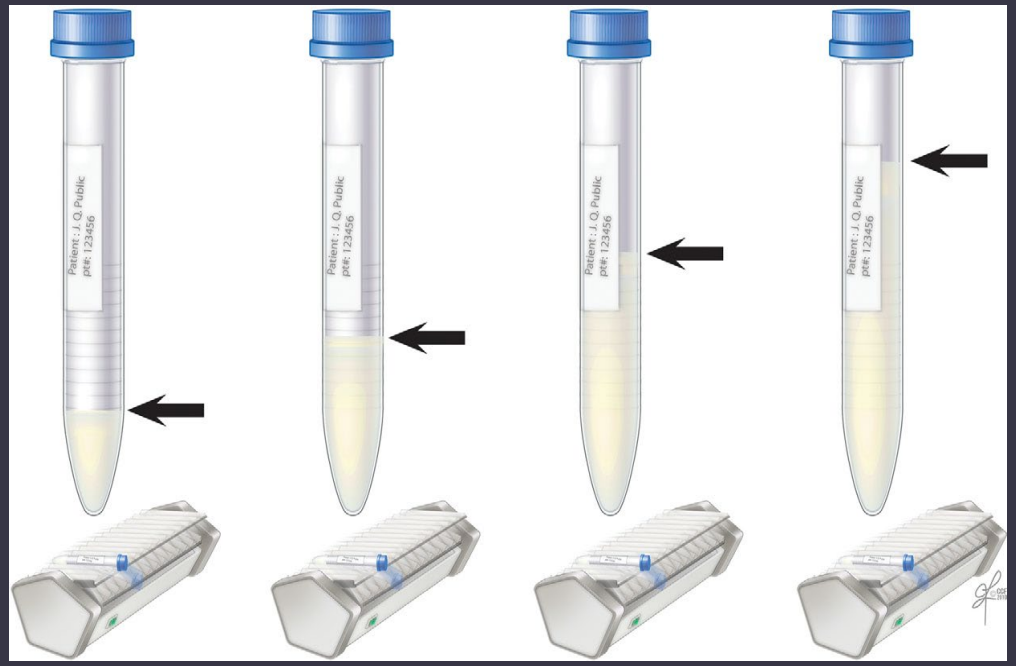
- The loss of water during this process increases the osmolality of the solution.
- This causes the cell membrane to shrink
- In the andrology laboratory, the commonly used cryoprotectant is modified TES, TRIS and egg yolk buffer (TEST-yolk buffer)
- The TEST-yolk buffer is an excellent extender that helps maintain sperm viability
- Addition of egg yolk helps maintain sperm viability during cryostorage TES, TRIS improves membrane fluidity
- Egg yolk-free buffers have also been introduced to avoid potential allergenic reactions and reduce exposure to animal derived products

- Zwitterion buffers also help in the recovery of motile sperm due to their ability to bind free hydrogen and hydroxyl ions in the surrounding medium and to aid in the dehydration process
- TEST-yolk buffer contains glycerol
- Glycerol has a role in lowering the concentration of salts at an extracellular level by increasing the level of unfrozen water
- This decreases the osmotic effects
- TEST-yolk consists of low-density lipoproteins which are responsible for protecting the sperm membrane
- Sperm suspensions are diluted 1:1 vol./vol. with TEST-yolk buffer, resulting in a final glycerol concentration in the frozen sample of 6 percent
- The freezing medium consist of TEST-yolk, glycerol, and gentamicin

- In the slow-freezing technique, after complete liquefaction, the freezing medium (TEST-yolk buffer) is added dropwise to the liquefied semen by addition of 25 percent of the semen volume four times, with 5-min interval of slow mixing until a 1:1 sample–medium ratio is achieved
- The sample is transferred into the cryovials and frozen at –20 °C for 8 min followed by exposure to the liquid nitrogen vapors at –80 °C
- After 2 h of incubation in the vapors, it is preserved in LN2 at –196 °C

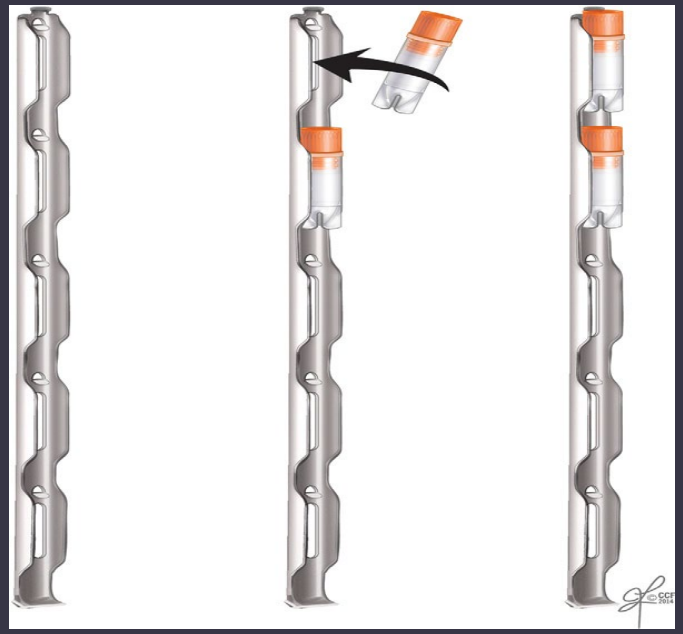
Incubator set at 37 °C and semen sample collected for liquefaction

Mixing of the semen sample and TEST-yolk on a test tube rocker for 5 min



Step-wise addition of TEST-yolk equal to one-quarter volume of buffer to patient sample to give a final 1:1 volume

Distribution of cryodiluted sample into cryovials using a sterile serological pipette



Correct loading of cryovials into cryocanes

Cryovials placed in a cryocane and covered with cryosleeve placed upright in –20 °C freezer for 8 min

Proper loading of cryocanes with cryovials upright into the cryotank canister

- For thawing, the sample is removed from the LN2 and warmed either slowly at room temperature for 30–40 min or rapidly at 37 °C for 290 min
- The conventional slow-freezing protocol performed manually or automated can cause physical and chemical damage to the sperm cell

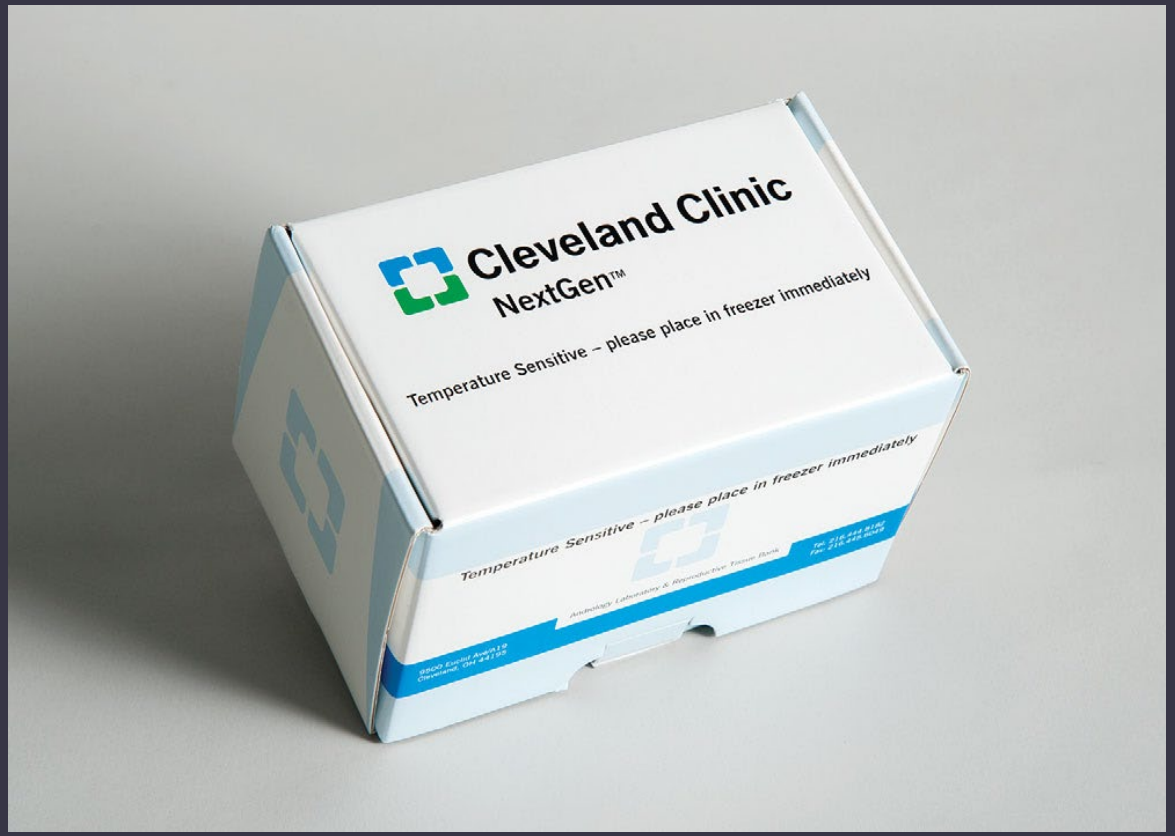
- **Rapid Freezing**
- In the rapid-freezing protocol (less than 15 min), the sample is directly exposed to the nitrogen vapors at –80 °C
- It is mixed with the cryoprotectant in a dropwise manner and incubated at 4 °C for 10 min
- The straws are first placed at a distance of 15–20 cm from the level of the LN2 for 15 min for vapor exposure, and later immersed in liquid nitrogen at –196 °C
- Rapid freezing is not used widely because of its low success rate and reproducibility

- Controlling the variation in temperature is also difficult
- This difficulty can be reduced by using automated programmable freezers, which are easy to use while handling the specimens at different temperatures
- The samples are loaded on a cryoplate and placed in the freezer
- Automated systems frequently use a cooling rate of –0.5 °C /min from room temperature to –5 °C and a freezing rate of –10 °C/min from –5 °C to –80 °C or –90 °C, followed by immersion in LN2

Home Sperm Banking

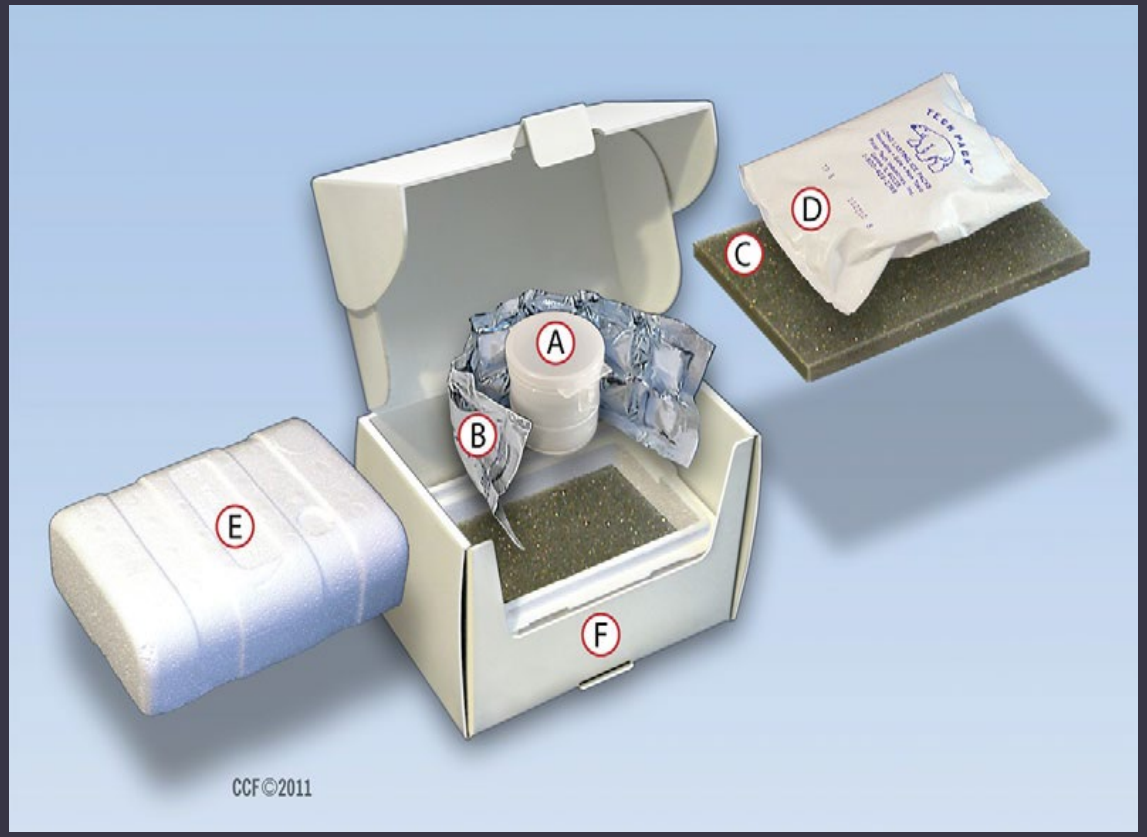
- Home sperm banking is a novel approach for collecting the semen specimen at home
- For many patients, collection of a semen specimen is a challenge because of various factors such as discomfort and stress
- Sperm banking facilities are not available in many cities and patients may have to travel from distant places to a sperm banking facility
- This may also cause emotional trauma and anxiety
- The home sperm banking process is a novel approach for such men that helps overcome the issues related to privacy and anxiety

The NextGen kit is a novel, specially designed kit developed by the Cleveland Clinic Andrology Center for sperm collection and transport



View of the NextGen kit optimized for overnight shipping of semen samples

Components of the NextGen
kit: refrigeration media and (A) collection cup, (B) cooling sleeve, (C) foam insert, (D) ice pack, (E) ice brick, and (F) NextGen box



- The kit is composed of a collection cup and transportation media, ice sleeves, foam inserts, ice packs, Styrofoam packing box, and the outer box
- Sample collection and shipping instructions are included in the kit
- On receipt of the kit, patients are instructed to place the collection cup, ice packs, freezing sleeve, and refrigeration media in a freezer for at least 12 hours
- On the day of collection, before collecting the semen, the refrigeration medium and collection cup are removed from the freezer and allowed to thaw at room temperature for 60 min

- The semen sample is deposited by masturbation only, avoiding the use of lubricating gels
- After sample collection, the entire content of the refrigeration media (5.0 ml) is added to the collection cup, the cup is sealed securely and gently swirled to mix the contents.
- The cup is then placed in the kit, along with ice bricks, which are placed on the outside of the foam layers
- Finally, the kit is sealed and shipped overnight

- After receiving the sperm sample with the kit, the cryopreservation protocol is carried out as per the slow cryopreservation technique

- The effects of overnight transport of the semen specimen on sperm motility using the home sperm banking kit have been examined
- Prefreeze and postthaw sperm motility, total motile sperm, and percentage cryosurvival rates were compared between samples collected from infertile men on-site at the Andrology Center (n = 10) and samples collected from infertile patients at home (off-site; n = 9), and shipped by NextGen to the laboratory
- A second group (n = 17) consisted of 10 semen samples from cancer patients collected on-site, which were compared with seven semen samples from cancer patients shipped by the NextGen

- In the infertile men, percentage cryosurvival rates were similar with NextGen compared with those of on-site collection (53.14 ± 28.9 vs 61.90 ± 20.46 percent; p = 0.51)
- Similarly, in the cancer patients, all four parameters were comparable between the on-site and NextGen collections
- Cryosurvival rates were also similar between NextGen compared with those of on-site collection (52.71 ± 20.37 vs 58.90 ± 22.68 percent; p = 0.46)
- Cancer patients can bank sperm as effectively as men banking for infertility reasons using the NextGen kit

Sperm Vitrification

- Sperm vitrification is an emerging technique for improving reproductive outcomes
- The first successful live birth of a human by the vitrification process was documented by researchers at the State University of Iowa in 1953
- Sperm vitrification involves solidification of the specimen at ultralow temperature by elevating its viscosity with a high cooling rate of 15 000–30 000 °C/min
- Cooling cells at such ultracool temperatures creates a glasslike appearance without formation of ice crystals
- The glass formation occurs efficiently when the cryoprotectant composing the vitrification solution includes the combination of dimethyl sulfoxide, which is a strong glass former, and ethylene glycol, acetamide, and formamide, which are weak glass formers

- The vitrification process with cryoprotective agent (CPA) requires exposing the cells to high concentrations of cryoprotectants at room temperature
- The sperm wash media for vitrification contains 5% HSA (human serum albumin) and sucrose
- Vitrification is not effective with permeable cryoprotectants as this increases the osmolarity and causes human sperm cells to suffer from osmotic shock when exposed to the hypertonic environment
- This results in morphological defects such as coiled tails

- This problem can be solved by using isomolar vitrification media which uses nonpermeable cryoprotectants
- The sperm suspension is transferred to the cryovial
- The cryovial is then placed at the bottom of the cryocane and is exposed to the liquid nitrogen tank

- This technique is efficient in maintaining the motility and DNA integrity
- It results in higher fertilization and pregnancy rates during the ART procedure compared to the slow-freezing technique

- A study conducted on 33 semen samples from humans showed the same outcomes for slow freezing and vitrification
- Vitrification is fast, easier, and costeffective
- It has no deleterious effects on sperm quality because of its low toxicity
- Sperm vitrification has been performed successfully with and without CPA, but has failed to demonstrate superiority over the conventional sperm-freezing method
- In one study, results of conventional slow freezing and vitrification of 105 human semen samples demonstrated that slow freezing yields better sperm motility and vitality

- In contrast to this, vitrification showed better results for sperm morphology
- Li et al., in their systematic review, concluded that vitrification is a superior technique compared to conventional slow freezing
- They reviewed 2428 studies and showed that total motility and progressive motility of post-thaw semen sample were well preserved by vitrification as compared to conventional slow freezing
- It is important to note that these results varied on the protocol and the sample size

- A new technique called cryoprotectant-free vitrification has been introduced
- It is carried out in the absence of cryoprotectants
- Vitrification results of 35 human semen samples using the cryoprotectantfree approach showed that high membrane potential and low DNA damage were observed in samples
- On the contrary, another study failed to show significant differences in postthaw motility between conventional slow freezing and cryoprotectant-free vitrification
- This study was further supported by Aizpurua et al.
- This approach yields a greater number of live sperm and maintains the acrosome with reduced DNA fragmentation
- Sucrose yields better results in the absence of cryoprotectants

- Use of sucrose during vitrification has been correlated with better results in postthaw motility
- The plasma membrane and acrosome integrity are also maintained
- Sucrose is a sugar and acts like a solute with high viscosity
- The increased viscosity during vitrification makes it easy for the cells to achieve a glassy state
- Sperm have been shown to be particularly sensitive to exposure to high concentrations of CPAs used routinely in oocyte and embryo vitrification
- The sperm suspension is directly exposed to the LN2; however, the non-penetrating cryoprotectants inhibit the flow of water outside the sperm cells and thereby it may cause osmotic damage

- In addition to freezing, there is a need for extreme care during the process of thawing
- The velocity of thawing plays a challenging role in the cryosurvival rate of spermatozoa
- Despite high-speed freezing of spermatozoa prefreeze, the thawing velocity should be high as well
- The reason for this is that the water in the sperm cells must move from the glassy state to the liquid phase
- This process should occur without forming ice crystals
- Sperm samples show higher motility when thawed at 42 °C
- Vitrification of neat ejaculates is associated with several effects on sperm parameters and DNA integrity, suggesting that seminal plasma does not have the same protective effects during sperm vitrification

Sperm Storage Techniques

- Before proceeding with ART, it is recommended to store the sperm cells
- The process of cryopreservation damages sperm health on some levels
- As a result, the quality of the sperm is reduced in terms of motility
- To reduce the amount of damage caused to spermatozoa, it is recommended to store them for only a short interval

- In cases of infertility with obstructive and nonobstructive azoospermia, sperm are surgically retrieved and patients are recommended to cryopreserve their samples
- Cryopreserving spermatozoa after surgery through the slow-freezing protocol results in an approximately 1 percent recovery rate
- Among all the ART procedures, sperm recovery for ICSI is noted to be difficult
- There are several methods to cryopreserve spermatozoa, including biological and nonbiological methods

1 Sperm Storage Inside Zona Pellucida

- When the spermatozoa are few in number, they can be stored in the empty zona of human or animals
- The process of storing the sperm in zona is critical and requires the stripping of the oocytes
- The sperm are aspirated using the ICSI pipette and then transferred to the empty zona
- Small holes are created using laser-assisted techniques in the zona membrane to allow the sperm to enter
- In the case of the large-sized holes, there are chances for the sperm to leak out and the DNA of the host might enter the oocyte
- Smallsized holes may result in entrapped DNA, which causes it to adhere to the sperm
- Following the insertion of sperm into the zona, they are frozen using slow freezing
- This process has shown a significant improvement in sperm recovery during postthaw

2 Volvox Globator Spheres

- This method is based on the use of an algae named Volvox globator
- These algae have spherical colonies
- Sperm cells are mixed with the cryoprotectants and introduced into the spheres
- This process is followed by slow freezing of the spermatozoa
- The success rate of this process is noted to be 100 percent
- FDA restricts the use of this method as the sperm are exposed to algae

3 Straws and Pipette

- The sperm sample can be stored using straws
- These straws are distinguished into many types, including open, mini, and open pulled
- The open pulled straws use capillary action to help protect samples from mechanical damage
- They are highly recommended for the process of vitrification
- However, there is a high risk of contamination as the system is open
- Mini straws, which are smaller in size, are used when the volume of the sample is comparatively lower
- The sample is loaded inside with cryprotectants and the ends of this straw are closed
- The disadvantage of this type of straws is that the sperm might stick to the walls

4 Preserving Spermatozoa Using Microdroplets

- The sperm sample, along with cryoprotectants, is rapidly cooled in the presence of dry ice
- This process forms microdroplets
- These microdroplets are exposed to LN2 at –196 °C
- This method has resulted in six clinical pregnancies (33 percent)

5 Cryoloop

- A study was conducted by Schuster et al. in which they froze the spermatozoa using slow-freezing and ultrarapid- freezing techniques
- The cryoloop method uses a nylon loop that traps the low-volume sample by capillary action
- The recovery rate obtained by this process is up to 73 percent
- The disadvantage of this process is that it contains a higher risk of contamination because of its open system

6 Cryotop Method

- The cryotop method is an open system
- This method is used for individual sperm or a low number of sperm
- Sperm cells are transferred to the cryotop strip using the ICSI pipette
- It is placed at 4 cm distance from the surface of the LN for 2 min following direct exposure to the LN

7 Cell Sleeper Method

- This method is a closed nonbiological system that consist of a cryovial, which is used as a container
- A tray is placed in the cryovial, which can be sealed by a screw cap and mounted on a cryocane, just like a regular cryovial
- Approximately 2 µl of spermatozoa droplets prepared by swim-up are transferred on the sleeper cell tray with the help of the ICSI pipette and a micromanipulator
- The tray is placed in the cryovial and cooled in LN vapors for 2.5 min, followed by immersion in LN
- Sleeper cells have been extremely efficient for cryopreserving small numbers and single sperm

Freezing Spermatozoa

- cryopreserving spermatozoa at extremely low temperature might cause damage to the DNA and adversely affect the semen parameters, especially motility
- In addition to this, the process of freezing and thawing can be detrimental to sperm health
- There are several thawing techniques used, including thawing the semen specimen at room temperature for a period of 10 minutes and incubating it at 37 °C for more than 10 minutes
- Samples can also be thawed in a water bath at 37 °C for 10 minutes or only thawed at room temperature for 15 min

1 Cryopreservation of Surgically Retrieved Spermatozoa

- **Testicular Spermatozoa**
- In male infertility cases such as obstructive and nonobstructive azoospermia, the use of testicular spermatozoa is preferable
- The absence of spermatozoa in the ejaculate is the primary reason for retrieving them from the testis
- A study by Friedler et al. showed that when the spermatozoa were extracted from the testis and cryopreserved, there was no significant difference observed in the quality of spermatozoa parameters such as fertilization rate, embryo quality, and implantation rate

- These results were based on ICSI cycles that compared fresh and cryopreserved spermatozoa retrieved from the testis
- The retrieval process for testicular spermatozoa includes open testicular biopsy and testicular sperm aspiration (TESA), and micro-testicular sperm extraction (micro-TESE)
- Micro-TESE, in comparison with TESE, has a higher retrieval rate
- Similar results were obtained in patients diagnosed with obstructive azoospermia
- Testicular sperm can be retrieved and cryopreserved for further use

- **Epididymal Spermatozoa**
- The retrieval of epididymal spermatozoa can be done with either MESA (microsurgical epididymal sperm aspiration) or PESA (percutaneous epididymal sperm aspiration) and is performed on the patients with an obstruction at a site near to the epididymis
- In contrast to PESA, sperm retrieval is higher with MESA
- These spermatozoa are mature and motile, and thus the sperm selection process becomes easier for ART procedures

Freezing of a Single or Low Number of Spermatozoa

- Some patients suffering from nonobstructive azoospermia may have an extremely low number of permatozoa and hence the sperm retrieval rate in these patients is comparatively lower than for patients with obstructive azoospermia
- As a result, cryopreserving a single or a few spermatozoa is a cumbersome process
- Single sperm can be cryopreserved by vitrification using sleeper cells or cryotop, both of which are nonbiological closed systems
- In most cases, for a successful pregnancy the storage techniques of empty zona or cryoloop have also been used

Fresh vs Frozen Epididymal Spermatozoa

- Studies have compared the results of ICSI with fresh semen samples and cryopreserved samples
- A study by Tournaye et al. reports that the rate of pregnancy in the ICSI cycle was comparable between fresh and frozen spermatozoa from the epididymis
- The study consisted of 53 ICSI cycles with 40 cycles of fresh spermatozoa and 13 cycles of a frozen sample
- This study was further supported by the study of Cayan et al., which showed that there was no significant difference between fertility rates with fresh and frozen samples

- Opposing this study, Shibahara et al. showed that there is a significant difference between the fertility rate of fresh and frozen epididymal samples
- Investigators have shown that the implantation rate can be higher with fresh sperm samples, and that it is more relevant in the case of obstructive than nonobstructive azoospermia

Fresh vs Frozen Ejaculated Spermatozoa

- The two major studies showing the results of ejaculated spermatozoa include one by Kuczynski et al., which consisted of 118 ICSI cycles with fresh spermatozoa and 122 ICSI cycles with frozen spermatozoa
- The study showed no significant difference in the results of the cycles. The authors also state that the rate of pregnancy is significantly higher in ICSI with human samples as compared to other species
- The other study, by Ragni et al., consisted of 79 ICSI cycles that were performed on fresh and
- frozen samples
- They reported no significant differences
- In contrast to this, a sample with low sperm motility showed that the fertilization rate with the fresh sample is higher compared to that of a frozen sample
- This finding supported the hypothesis that the freezing–thawing process can cause damage to the sperm health and decrease its quality

Detrimental Effects of Freezing on DNA Integrity

- Despite the fact that sperm cells are comparatively less susceptible to cryodamage due to low hydration and high membrane fluidity, they may have some detrimental changes
- It is noteworthy that semen cryopreservation increases the oxidative stress in sperm cells
- Evidence suggests that freezing triggers caspase activity
- This requires caspase inhibitors to be added to the medium
- Addition of caspase inhibitors maintains motility, both prefreeze and postthaw

- In normal conditions, sperm requires healthy DNA, acrosomal reaction, and high motility in order to fertilize an egg
- These features of sperm should be preserved even after the postthaw period for successful fertilization during the ART procedure
- The amount of reactive oxygen species produced during cryopreservation may also reduce motility and viability, and cause DNA damage by increasing sperm DNA fragmentation
- Reports also show changes in the proteome due to cryopreservation

- This, in turn, reduces the fertility potential of spermatozoa
- Sperm motility is more susceptible to the damage caused by cryopreservation i.e motility is reduced up to 50 percent postthaw
- On the other hand, the damage caused to DNA integrity may result in genetic defects
- Some studies report significant changes in the DNA integrity after cryopreservation
- Contradicting these studies, reports also show no significant damage to the DNA integrity postthaw
- These differences may be attributed to differences in methodology, cryoprotectants used, and the tests used to analyze the integrity of the DNA

- In contrast, the study by Donnelly et al. shows that there was a significant improvement in the DNA integrity of postthaw
- This can be because of the presence of high antioxidant levels in seminal plasma
- Evidence shows that cryopreservation damages the mitochondrial membrane potential but does not have any adverse impact on DNA integrity
- In addition to this, the study by Donnelly et al. also states that sperm DNA from infertile men is more susceptible to cryodamage compared to that from fertile men
- Postthaw, there was a significant decrease in DNA integrity of samples from infertile men, while there was no change in the DNA integrity of fertile men

- Another study, by Zribi et al., reported that despite the increase in ROS production during cryopreservation, there is no association between DNA integrity and oxidation
- They stated that DNA fragmentation during cryopreservation is not caused by oxidative stress, but by other pathways involving changes in DNA repairing enzymes
- However, this hypothesis was rejected by Thomson et al., who reported that DNA fragmentation is a result of an increase in oxidative stress
- Certain antioxidants, such as genistein, are found to have lipid peroxidation and SDF-reducing properties
- There is increased caspase activity, which can be caused by the cryodamage to the sperm DNA
- Based on the literature, the results of DNA damage associated with cryopreservation remains controversial

Pitfalls and Challenges in Sperm Cryopreservation

- There are studies reporting the risk of contamination caused by the leakage of samples in the LN
- Guidelines are in place for practicing sperm cryopreservation which are helpful in preventing the risks associated with cryopreservation
- Cryopreservation of semen samples results in reduced semen parameters such as viability and sperm motility
- It is also associated with increased ROS, which causes oxidative stress
- Not only sperm motility is affected; cryopreservation can also impair sperm morphology
- Sperm damage occurs postthaw, when it is exposed to higher temperatures after long-term storage

- Sperm cryopreservation also affects acrosome activity. Studies show that cryopreservation results in a decrease in acrosome activity and acrosome cap, and this is responsible for reduced sperm penetration
- Moreover, preserving spermatozoa in LN is a safety hazard
- The cryopreservation of spermatozoa is not only time consuming, but also expensive
- There is an increase in the damage caused by the cryoprotectants; they might induce some toxic effects when added in high concentrations
- This causes changes in the mitochondria by inducing osmotic changes

- If the concentration of cryoprotectants are not maintained, it can cause excessive dehydration and ice formation
- Freezing of spermatozoa also involves ethical issues
- The number of offspring that can be given birth by a single donor is the most common ethical issue due to the unintended blood relation of a donor to the child
- Also unresolved is the question of discarding the sperm sample or using it for research in the case that the funding period expires or the couple has a successful pregnancy

Future Directions

- There should be increased awareness among physicians, oncologists, and also patients regarding the importance of cryopreserving sperm for ART procedures
- Sperm cryopreservation causes reduced sperm parameters such as motility and viability
- Thus, there should be increased use of advanced techniques such as vitrification
- Also, the use of opensystem carriers might cause cross-contamination
- Hence, better options should be considered for storing spermatozoa
- Vitrification can also be done using the sleeper cell technique, which preserves a smaller number of sperm
- This is preferable as the system is closed and there is a reduced risk of contamination

Conclusion

- Sperm cryopreservation by slow freezing is the most widely used technique for preserving spermatozoa or cancer patients, before treatment for malignant diseases, vasectomy, traveling husbands, or gender reassignment
- Sperm cryopreservation is an important component of fertility management, and its successful application affects the reproductive outcome of ART
- Patients who do not have access to sperm cryopreservation facilities have the option of freezing their sperm utilizing the NextGen kit
- Sperm vitrification is another option available for freezing low sperm numbers of surgically retrieved perm
- Vitrification of sperm means less acrosomal or DNA damage while maintaining sperm motility

- The effects of cryopreservation on cells are well documented; the effects on sperm DNA integrity are unclear
- Therefore, utmost care should be exercised to provide the maximum protection to the male gamete using techniques aimed at minimizing sperm DNA fragmentation and improving sperm cryosurvival
- Although there are several disadvantages of sperm cryopreservation, it is the only option for a man to preserve his fertility and father his biological child