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Effect of sperm storage and selection techniques on sperm parameters

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Abstract

Sperm cryopreservation preserves the fertility of cancer patients undergoing chemotherapy, ensures sperm are available at the time of oocyte retrieval in assisted reproductive technology (ART) procedures and avoids the need for repeated sperm extraction surgeries in azoospermic patients. Conventional methods of cryopreservation involve storage in liquid nitrogen (LN₂), which causes a significant decline in sperm parameters such as motility and viability and results in DNA damage. Newer methods of sperm cryopreservation such as the LN₂ vapor method, vitrification, and experimental methods such as lyophilization, have significant advantages over the conventional methods in terms of cost effectiveness and ease of use. Density gradient centrifugation (DGC), swim up, and magnetic assisted cell sorting (MACS) can be used prior to or post-cryopreservation to improve post-thaw sperm quality. Cryopreservation in special carriers such as cryoloops and empty zona prevents the loss of small numbers of sperm during cryopreservation. This article will discuss these sperm preservation and selection techniques.

Introduction

Sperm freezing became possible more than 70 years ago. Today, a number of techniques are used to preserve the fertility of cancer patients undergoing chemotherapy and/or radiotherapy, ensuring the availability of sperm at the time of oocyte retrieval in assisted reproductive technology (ART) procedures and avoiding the need for repeated sperm extraction procedures in azoospermic patients. This article will discuss current sperm preservation and selection techniques and the advantages and disadvantages of each. It will also explain how cryopreservation affects semen parameters and highlight prospective research for improving these methods.

Freezing methods

Conventional cryopreservation techniques: slow, rapid, and programmable freezing

The most commonly used method of cryopreservation involves manual sperm storage in liquid nitrogen (LN₂). This can be done using fast freezing or slow freezing methods or with a programmable freezer. With all three methods, a low molecular weight cryoprotectant is added to a processed semen sample to prevent ice formation in sperm cells [Di Santo et al. 2012]. These chemicals optimize osmotic pressure and pH, provide extracellular energy to sperm, and prevent bacterial contamination (they include antibiotics) [Anger et al. 2003].

The most widely used cryoprotectant is glycerol, which is commonly mixed with egg yolk. This mixture protects sperm cell membranes and prevents osmotic stress within the cells [Anger et al., 2003]. However, glycerol can alter the acrosomal membrane and change the mitochondrial environment [Di Santo et al. 2012].

Manual slow freezing usually takes 2 to 4 hours to complete. With this technique, the sample is gradually cooled from room temperature to 5 °C at a rate of 0.5–1 °C/min. It is then frozen, and the temperature is further lowered from 5 °C to −80 °C at a rate of 1–10 °C/minute. Finally, the sample is plunged into LN₂ at −196 °C [Thachil and Jewett 1981]. The major drawback with this technique is that ice crystals can form within cells if the cooling rate is too fast. Additionally, cells can shrink due to osmosis if the cooling rate is too slow. Hence, cooling must be controlled at a certain rate [Said et al. 2010].

In the rapid freezing protocol, the sample comes into direct contact with nitrogen vapors at −80 °C after cryoprotectant is added to the sample [Sherman 1990]. The sample is loaded onto straws, which are stored 15 to 20 cm away from LN₂ (vapor phase) for 15 minutes and then plunged in the LN₂ itself (−196 °C). The nitrogen vapor phase has a thermal gradient due to the distance and volume of liquid below. Difficulty in controlling cooling rates with this protocol, might contribute to the variation in post-thaw sperm quality.

The lack of control over freezing rates in both the slow and rapid freezing methods can be overcome by the use of an automated programmable freezer. Here samples are loaded onto a plate in the freezer and a freezing program is chosen,
and the temperature is automatically adjusted. The main drawback of programmable freezers is that they are beneficial only when handling a large number of samples [Holt 2000]. Furthermore, programmable freezers are not as efficient as manual methods because heat (latent heat of fusion) is released from the samples themselves, which changes the chamber temperature considerably and delays cooling rates [Holt 2000]. This delay in cooling may be detrimental to sperm cells [Holt 2000].

The spermatozoa cellular membranes must be able to withstand the freezing and thawing stresses of cryopreservation in order for the technique to be suitable. The membrane should be able to withstand the response to the addition of hyperosmotic cryoprotectant and hence cell shrinkage, freeze-induced dehydration and temperature-induced changes in membrane phospholipids [Parks and Graham 1992]. This applies to mitochondrial membranes as well as plasma membranes.

Liquid nitrogen vapor freezing

With LN$_2$ vapor freezing, human spermatozoa are stored in LN$_2$ vapor during cryopreservation instead of LN$_2$ ($-196^\circ$C). Microbial cultures of LN$_2$ from storage tanks have yielded pathogenic microorganisms and hence, LN$_2$ cannot be considered sterile even though it is at a temperature of $-196^\circ$C [Fountain et al. 1997]. The presence of pathogenic microorganisms in storage tanks is a potential risk for the transmission of disease through stored semen samples. Even though the cryovials used for sperm storage are a closed system, the possibility of LN$_2$ seeping into the cryovials cannot be completely ruled out [Fountain et al. 1997]. This may also lead to the spread of contaminants/organisms between samples. Hence storing sperm samples in LN$_2$ vapor may prevent contamination as it avoids direct contact between samples and LN$_2$ [Saritha and Bongso 2001].

Studies comparing the viability and motility parameters of samples stored in vapor and LN$_2$ show similar results or slightly better results for the vapor method [Amesse et al. 2003; Saritha and Bongso 2001]. Sperm samples can be stored in the vapor for up to three months without inducing significant effects on sperm motility, viability, and morphology. DNA integrity and mitochondrial potential are similar for both vapor and LN$_2$ storage methods [Lim et al. 2010].

Because there is a progressive increase in temperature as one moves farther away from the surface of the LN$_2$, samples stored in vapor are generally higher in temperature than those stored near the LN$_2$. Sperm motility and viability differed significantly when samples were stored more than 17 cm from the surface of the liquid. This height corresponds to a temperature of $-161.7^\circ$C [Lim et al. 2010]. The optimal height for storage was found by storing the sample at different heights for a period of one week. Whether there is any significant change in sperm parameters when storing sperm for a longer period within the height of 17 cm from the surface has yet to be determined.

Currently, vapor storage can be considered as an alternative to storage in LN$_2$ as long as the samples are kept well within the height of 17 cm from the surface of the liquid for no more than three months. Further study is needed before this method can be recommended for long-term storage of sperm.

Effects of cryopreservation

Cryopreservation causes a significant decline in motility, viability, chromatin stability, and membrane integrity and causes significant morphological alterations. This loss in sperm quality is especially significant in patients whose sperm parameters are poor to begin with [Hammadeh et al. 1999; McLaughlin et al. 1992]. Motility is the most affected parameter [Donnelly et al. 2001a; Gandini et al. 2006]. O’Connell et al. [2002] proposed that the decrease in sperm motility is due to mitochondrial damage and physical changes to the sperm tail. Damage to mitochondrial membranes interrupts the process of energy production, leading to decreased ATP availability in the spermatozoa. Cryopreservation may induce an irreversible coiling of the flagellum, hindering the tail’s propelling motion [O’Connell et al. 2002].

Cryopreservation can also induce DNA damage. The packaging of DNA in a sperm nucleus follows a long process that begins in spermiogenesis where the histones are replaced by protamines [Boissonneault 2002]. The integrity of spermatozoa seems to be the determining factor in the success of fertilization in both natural and assisted reproductive methods [Gandini et al. 2006]. It has been noted that the decrease in post-thaw quality is mostly due to DNA fragmentation that occurs after cryopreservation. Such DNA damage may affect embryo quality and viability. Also, it has been noted that different sperm samples exhibit different levels of DNA damage. Such findings indicate the need to assess DNA integrity as a part of the routine infertility workup [Gosalvez et al. 2011].

It is also important to note that frozen samples from healthy men seem to be of higher quality post-thaw than samples obtained from infertile men. Several studies have shown that DNA damage is linked to changes in the morphology of frozen sperm samples, although this does not appear to be true according to studies done by Donnelly et al. [2001a]. Stanić et al. [2000] found that the use of slow programmable freezing was superior to vapor freezing and rapid programmable freezing for high quality semen samples. However, all of these freezing protocols produced notable changes in sperm cell motility as well as other parameters [Stanić et al. 2000]. In contrast, other studies have shown that the conventional rapid freezing technique is a better method for preserving sperm motility than the manual slow technique. The variable difference in motility results obtained could be explained by differences in sperm membrane phospholipid, glycolipid, and sterol content [Vutyavanich et al. 2010]. Such differences between studies may be caused by variability in freezing rates and freezing methods amongst laboratories.

Sperm selection techniques

Sperm selection techniques can be used before and after cryopreservation to significantly improve sperm quality (Table 1). Density gradient and swim-up are the most commonly used techniques for sperm selection. Newer techniques include magnetic assisted cell sorting (MACS),...
Selection techniques used pre-cryopreservation

- **DGC + MACS** [Said et al. 2005]:
  - Sample: 10 healthy donors
  - Cryopreservation after addition of seminal plasma to prepared sperm
  - DNA integrity – Lower (S)
  - Progressive motility – Higher (S)
  - Cryosurvival – Higher (S)

- **DGC + MACS** [Grunewald et al. 2006]:
  - Sample: 10 healthy donors
  - Cryopreservation after addition of seminal plasma to prepared sperm
  - DNA integrity – Lower (S)
  - Progressive motility – Higher (S)

- **Percoll Density gradient** [Donnelly et al. 2001a]:
  - Sample: 40 donors from ART program
  - DNA integrity – Lower (S)
  - Progressive motility – Higher (S)
  - Cryosurvival – Higher (S)

- **Swim-up**
  - Sample: Total motile sperm count – Higher(S)
  - Motility - Higher (S)
  - Viability – Higher(S)
  - Intact acrosome – Higher (S)

Selection techniques used post-cryopreservation

- **MACS** [Grunewald et al. 2001]:
  - Sample: 40 from 15 healthy donors
  - Vitality – Higher (S)
  - Apoptosis markers – Less (S)

- **Swim-up** [Spanò et al. 1999]:
  - Sample: 19 healthy donors
  - Progressive motility – Higher (S)
  - Sperm chromatin structure – Improved (S)

- **Zeta selection** [Kam et al. 2007]:
  - Sample: 9 donors from ART program
  - Total motility (NS)
  - Normal morphology – Increased (S)
  - Apoptosis and sperm necrosis – Decreased (S)
  - Intact acrosome – Increased (S)

- **Electrophoretic separation** [Ainsworth et al. 2007]:
  - Sample: 5
  - Motility – Higher (S)
  - Vitality – Higher (S)
  - Morphology – enhanced (S)
  - DNA damage – less (S)

- **PureSperm Density gradient vs. Swim-up** [Allamaneni et al. 2005]:
  - Sample: 9 donors from ART program
  - Longevity at 4 hours – Higher (S)
  - Recovery rate – Higher (S)

Table 1. Comparison of post-thaw sperm parameters when selection technique is used pre- and post-cryopreservation.

<table>
<thead>
<tr>
<th>Technique used</th>
<th>[Author year]</th>
<th>Sample size</th>
<th>Post-thaw parameters compared with cryopreservation without sperm selection</th>
</tr>
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<tr>
<td>Selection techniques used pre-cryopreservation</td>
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<tr>
<td>DGC + MACS</td>
<td>[Said et al. 2005]</td>
<td>n = 10</td>
<td>Motility – Higher (S)</td>
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<tr>
<td>DGC + MACS</td>
<td>[Grunewald et al. 2006]</td>
<td>n = 10</td>
<td>Cryosurvival – Higher (S)</td>
</tr>
<tr>
<td>Percoll Density gradient</td>
<td>[Donnelly et al. 2001a]</td>
<td>n = 40</td>
<td>DNA integrity – Lower (S)</td>
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<tr>
<td>Swim-up</td>
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<tr>
<td>PureSperm Density gradient vs. Swim-up</td>
<td>[Allamaneni et al. 2005]</td>
<td>n = 9</td>
<td>Longevity at 4 hours – Higher (S)</td>
</tr>
<tr>
<td>Density Gradient</td>
<td>[Counsel et al. 2004]</td>
<td>Healthy donors, n = 18</td>
<td>Normal sperm vs. Swim-up</td>
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<td></td>
<td>Cryopreservation after addition of seminal plasma to prepared sperm - DNA integrity – Improved (S)</td>
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<tr>
<td>Swim-up</td>
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<tr>
<td>Electrophoretic separation</td>
<td>[Ainsworth et al. 2007]</td>
<td>n = 5</td>
<td>Motility – Higher (S)</td>
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<td></td>
<td></td>
<td>n = 10</td>
<td>Vitality – Higher (S)</td>
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<td></td>
<td></td>
<td>n = 7</td>
<td>Morphology – enhanced (S)</td>
</tr>
</tbody>
</table>

zeta potential [Chan et al. 2006], and electrophoresis [Ainsworth et al. 2005].

Among the 44 studies we identified that discussed these advanced sperm selection techniques, only four evaluated the benefits of selection technique when used in a setting of sperm cryopreservation. The studies that integrated the selection techniques with cryopreservation protocols were divided into two groups, one in which cryopreservation was preceded by selection techniques and the other in which cryopreservation was followed by selection techniques. We excluded a large number of studies that were done on samples that did not undergo a cryopreservation protocol. Our aim was to determine which among the various selection techniques produce the best results.

### Swim-up and density gradient centrifugation

With the swim-up technique, washing media is placed over a semen sample inside a tube, which is incubated for approximately one hour. During that time, sperm that are highly motile swim into the top layer, which is removed. Density gradient centrifugation separates the sperm with the highest density and motility and best morphology from the rest [Sakkas et al. 2000].

Donnelly et al. [2001a, 2001b] studied the effects of density gradient and swim-up on sperm DNA damage. As expected, progressive sperm motility in the post-thaw samples that were cryopreserved after density gradient or swim-up was higher than that in the samples that were cryopreserved directly without the use of any selection technique. However, DNA damage was significantly higher in the samples that used these two selection techniques. This decrease in DNA integrity was improved when seminal plasma was added to the prepared sample. The prepared samples that were cryopreserved with seminal plasma had DNA damage similar to that in fresh frozen samples. It is hypothesized that antioxidants in the seminal plasma may protect sperm against DNA damage during the process of cryopreservation [Lewis et al. 1997].

- **Compared to prepared sperm frozen without addition of seminal plasma;**
- **Compared to cryopreserved sample without sperm preparation;**
- **Density gradient compared to Swim-up;**
- **Swim-up compared to density gradient; S: significant; NS: non-significant.**
There were no significant differences in sperm parameters between the samples prepared with density gradient and swim-up techniques. Use of either of the selection techniques for a second time on a cryopreserved sample did not yield any further improvement in sperm parameters [Donnelly et al. 2001a, 2001b].

A comparative study of post-thaw sperm parameters using swim-up and density gradient (using PureSperm) suggests that the latter is associated with a better recovery rate, total motile sperm count, and motile sperm count at four hours post thaw [Allamaneni et al. 2005]. The median total motile sperm count using PureSperm density gradient was 1.8 times higher than that obtained using swim-up, and the longevity at four hours also improved by approximately 1.5 times. The study also showed that recovery of sperm after cryopreservation is better with the density gradient technique (69% vs. 50%). Swim-up preparation prior to cryopreservation also significantly improved the number of sperm with an intact acrosome in the post-thaw samples [Esteves et al. 2000].

Magnetic activated cell sorting

MACS is a popular advanced technique for sperm selection (Table 1). Phosphatidylserine (PS) is a phospholipid that is present on the inner leaflet of the plasma membrane and it moves to the outer surface when the membrane is damaged [Vermes et al. 1995]. Externalized PS is a marker for apoptosis [Martin et al. 1995]. Hence, the binding of Annexin-V, which has high affinity to PS and cannot penetrate the sperm membrane, on the surface of a cell signifies that the membrane integrity has been breached and that the PS is externalized [Glander et al. 2002]. MACS uses colloidal super-paramagnetic microbeads conjugated with Annexin-V for cell separation. Cells are separated as they move through a magnetic field, which traps the magnetically tagged cells and allows the non-magnetic cells to pass [Miltenyi et al. 1990; von Schonfeldt et al. 1999].

Said et al. [2005] evaluated the utility of MACS to obtain high-quality sperm after cryopreservation. Post-thaw motility was higher in the sample that had undergone MACS before cryopreservation than in the sample that had undergone MACS after cryopreservation. The cryosurvival rate was higher in the sperm sample selected by MACS before cryopreservation than the sample where it was done afterwards and in a sample where no selection was performed [Said et al. 2005]. The sperm sample separated by MACS before cryopreservation also contained a larger number of sperm with an intact mitochondrial membrane. The mitochondrial survival rate (MSI), which is the ratio of the percentage of sperm with intact mitochondrial membrane integrity (MMP) after cryopreservation to that in the neat sample, increased by 36.1% ± 18.9% when MACS was used before cryopreservation [Grunewald et al. 2006]. Hence, sperm selection using MACS before cryopreservation can improve motility and cryosurvival of a sperm sample.

Zeta potential

Zeta potential is the negative electric potential between the sperm membrane surface and outside medium. This potential difference occurs mainly due to the negative charge of the epididymal proteins that are present on the sperm cell membrane [Ishijima et al. 1991]. Zeta potential is lower in sperm with DNA damage, and this property can be used to select sperm with intact DNA [Chan et al. 2006]. With this method, negatively charged sperm are immobilized on a container wall that is positively charged. Even though progressive motility declines after zeta selection on a cryopreserved sample, the selected sperm are more likely to have an intact acrosome and normal morphology. In one study, the markers of apoptosis and sperm necrosis were significantly reduced in a zeta selected sample [Kam et al. 2007]. However, the recovery rate was only 8 to 10% of the original concentration used for cryopreservation. An adequate sperm concentration could not be obtained when sperm selection using zeta potential was used before cryopreservation [Kam et al. 2007]. Because the recovery rate is low after zeta selection, it should not be used prior to cryopreservation.

Electrophoresis

Electrophoresis may also be used to separate out negatively charged sperm. A recovery rate of 27% was reported after electrophoresis in cryopreserved samples [Ainsworth et al. 2007] (Table 1). However, the process is labor intensive and is not yet a popular method for selection before cryopreservation. A poor recovery rate is another disadvantage of the newer techniques, but this should not be an issue if ICSI is used for gamete transfer.

Considerations

Even though a large number of advanced sperm separation techniques are available, only a few studies have attempted to integrate the selection techniques with cryopreservation protocols. There is a paucity of literature on the feasibility of integrating newer advanced methods of sperm separation into the cryopreservation protocol. This stems from the fact that many of the newer studies have a low sperm recovery rate. From the available literature, we conclude that density gradient centrifugation combined with MACS before cryopreservation may produce a sample with the highest sperm quality when compared to the other methods. But whether the addition of a new technique into the cryopreservation protocol will affect outcomes in terms of fertilization and pregnancy rates has yet to be studied since ICSI needs only one functional sperm for successful fertilization. Hence, more studies are needed to evaluate the benefits and feasibility using the newer techniques of sperm selection in cryopreservation protocols.

Newer methods of cryopreservation

Vitrification

Vitrification is the process of cooling cells at an extremely high rate (by directly plunging samples into liquid nitrogen) to produce a glass-like solidification, which prevents ice crystal formation [Kuleshova and Lopata 2002]. The history of vitrification dates back to the 1930s, but scientists at that time failed to achieve the high cooling rates necessary for this procedure [Luyet 1937]. Vitrification has now become a common technique in the preservation of oocytes and
embryos. However, developing methods of vitrification for spermatozoa was challenging due to their unique properties. For example, spermatozoa are osmotically fragile, and the use of a high concentration of permeable cryoprotectants is cytotoxic and may even cause genetic damage in sperm cells [Fraga et al. 1991; Gilmore et al. 1997]. Therefore, initial attempts to perform vitrification used either no cryoprotectant or very low levels [Nawroth et al. 2002]. They compensated for the lack of cryoprotectant by using high cooling rates (they directly plunged samples into LN2 (~720,000 K/minute) and increasing the surface area for heat exchange (using an extremely small sample volume) [Nawroth et al. 2002].

Vitrification is quite simple compared to conventional slow freezing. A semen sample is processed using the swim-up method and is then loaded into an appropriate container-like cryoloop or onto straws. These are rapidly cooled by directly plunging into LN2 (−196 °C), then placing in direct contact with the cold surface of a cryochamber (−180 °C), or using precooled aluminum blocks with long-term storage in LN2 [Nawroth et al. 2002; Satirapod et al. 2012; Vutyavanich et al. 2010]. The cryoprotectant-free vitrification system yielded sperm with similar viability, morphology, acrosome reacted cells, and a slightly higher motility compared to conventional programmable slow cooling with cryoprotectant [Nawroth et al. 2002].

One of the challenges in developing vitrification techniques for sperm preservation centers was its inability to preserve a large volume of sperm. Sperm are stored during vitrification using various instruments such as cryoloop droplets, open pulled straws, and open straws, and these methods do not differ in terms of motility and viability in post-thaw samples. But open pulled straws were preferred among these four methods cryoloops, droplets, open pulled straw and open straw due to the minimal risk of contamination from LN2 [Isachenko et al. 2005]. Additionally, it is difficult to quantify the amount of sample drawn into an open pulled straw, so to standardize the amount of sample drawn for storage, capillaries of standard diameter may also be used [Isachenko et al. 2012].

Vutyavanich et al. [2010] were able to vitrify samples of up to 0.25 mL in straws using pre-cooled aluminum blocks. When a low concentration of cryoprotectant medium with trehalose was used as the vitrification medium, post-thaw sperm parameters were better than those of the slow freezing method. This method also prevented the straws from breaking when they were directly immersed in the LN2 [Vutyavanich et al. 2010]. Samples of up to 0.5 mL were vitrified in a single straw with increased capacitation potential [Isachenko et al. 2011].

Recent studies evaluating sperm parameters (motility, mitochondrial membrane potential, and DNA integrity) in vitrified samples showed no significant difference when they were stored at −86 °C compared to −196 °C. Because the samples were stored only for 60 days, further evaluation is needed to determine whether storing vitrified samples long term at −86 °C is effective. This method has the potential to drastically decrease the cost of sperm cryopreservation and dependence on LN2. It is also safer than conventional cryopreservation because it decreases potential hazards associated with the handling of LN2 [Sánchez et al. 2012a].

On one hand, vitrification has many advantages over conventional cryopreservation. It does not require costly programmable freezers and takes much less time to perform, approximately 10 minutes compared to 1 hour for slow freezing [Vutyavanich et al. 2010]. Because vitrification is performed after swim-up, it is free of seminal plasma, which may harbor pathogens [Isachenko et al. 2012]. Also, conventional slow cooling requires post-thaw processing to separate sperm from the permeable cryoprotectant, which not only adds to the cost of the procedure but requires the sensitive spermatozoa to be mechanically manipulated [Isachenko et al. 2012]. Vitrification, on the other hand, does not require any post-thaw processing. In cases where repeated cryopreservation is done on the same sample, vitrification resulted in better post-thaw sperm parameters than standard slow programmable freezing [Vutyavanich et al. 2012]. This study was performed using high-quality semen samples, so further studies are needed using patients with low-quality sperm [Vutyavanich et al. 2012]. Case reports of live birth using vitrified spermatozoa and ICSI or IUI suggests it may be widely used in the near future [Sánchez et al. 2012b].

**Lyophilization**

Lyophilization or freeze drying is an innovative technique for preserving sperm. It essentially involves removing all moisture or water molecules in a semen sample, which is commonly done in the preservation of food and pharmaceuticals. Lyophilization involves cooling the sample below the triple point of water and then reducing pressure. Triple point is the temperature at which the solid, liquid, and gaseous state of a substance co-exists. Below the triple point temperature, a substance changes directly from a solid to a gaseous state when pressure is reduced. Hence, cooling below triple point and reducing pressure causes solid water, ice, to sublime directly into a vapor state.

Unfortunately, freeze drying is highly damaging to sperm membranes and hence, the outcome of lyophilization is sperm with zero motility and viability [Gianaroli et al. 2012]. But the overall integrity of the genetic material is preserved and there is less DNA damage with this method than with cryopreservation in LN2 [Gianaroli et al. 2012]. Animal studies show that mouse spermatozoa can be stored up to 1.5 years without significant DNA damage and can be used for fertilization [Ward et al. 2003]. Because the spermatozoa are dead, ICSI is the only method by which fertilization can be achieved. One case report discussed the successful use of ICSI for fertilization using non-viable sperm [Stecher et al. 2011], which suggests that freeze drying could be used as a method of sperm storage.

The greatest advantage of freeze drying is that a lyophilized sample can be stored at 4 °C and transported at room temperature. Moreover, lyophilization inactivates viruses [Unger et al. 2009]. Currently, its use is restricted due to the need for oocyte activation and the absence of human studies.

**Short-term storage methods**

Spermatozoa are usually cryopreserved before *in vitro* fertilization to ensure their availability during oocyte retrieval.
As stated earlier, the process of cryopreservation causes DNA damage and reduces sperm quality. Even though cryopreservation in LN₂ as a technique for long term storage of spermatozoa is undisputable at this stage, it is expensive, labor intensive, and potentially dangerous to handle. However, it may be possible to store sperm for short periods of time with acceptable post-thaw sperm parameters using electrolyte free media (EFM) [Riel et al. 2011]. One study reported that sperm motility and viability were comparable and chromatin integrity higher in sperm samples that were stored in EFM for one week compared to cryopreservation sperm [Riel et al. 2011]. In fact, sperm stored in EFM took four weeks to achieve a level of DNA fragmentation equivalent to that of cryopreserved sperm. Even though EFM-stored sperm have fertilized in animal studies [Riel et al. 2011], definitive human studies are lacking. Moreover, ICSI is the only method by which fertilization was achieved since the quality of sperm was low. Thus, storage in EFM has the potential to replace cryopreservation for short-term storage of sperm, but further human studies and more refined techniques are necessary.

**Storage methods for small samples**

Patients with azoospermia have no sperm in their ejaculate, leading to infertility. Patients with obstructive and non-obstructive azoospermia can have their sperm extracted from the testis or epididymis directly using various techniques. Open testicular biopsy, which is the most invasive method, involves cutting the testicle open to harvest sperm. Micro-TESE uses a microscope during the surgical procedure to locate and excise the tubules that show spermatogenesis. Testicular sperm aspiration (TESA) involves the use of a needle to aspirate the tubules; the aspirate is then analyzed for the presence of sperm. The process is named multiple testicular fine needle aspiration when multiple sites of the testis are aspirated. An adequate amount of sperm needed for IVF is usually obtained with the use of TESA in patients with obstructive azoospermia. But more invasive techniques like micro-TESE or open biopsy are usually needed in patients with non-obstructive azoospermia (NOA). Micro-TESE has the highest sperm retrieval rate compared to open testicular biopsy, multiple fine needle aspiration, and TESA in that order in patients with NOA [Gangrade 2013]. Epididymal sperm are more mature and therefore more motile than testicular sperm and are available for extraction in patients with obstructive azoospermia. Epididymal sperm can be harvested either by microsurgical techniques such as microsurgical epididymal sperm aspiration (MESA) or by percutaneous epididymal sperm aspiration (PESA). PESA is effective in patients who have obstructive azoospermia at a site distal to the epididymis. Sperm retrieval is better, as expected, with MESA than with PESA in patients with obstructive azoospermia but overall fertilization and pregnancy rates are similar [Lin et al. 2000].

In patients with obstructive azoospermia, there is no significant difference in fertilization and clinical pregnancy rates with the use of testicular and epididymal sperm [Buffat et al. 2006]. Additionally, cryopreserved and fresh epididymal sperm provide similar outcomes in terms of fertilization and clinical pregnancy rates [Tournaye et al. 1999] The preferred method of sperm retrieval in obstructive azoospermia is multiple needle aspiration because the probability of success is very high. Because there is a higher incidence of miscarriage due to possible interference with normal embryonal development, mature epididymal sperm should be used rather than the less mature testicular sperm if available [Buffat et al. 2006].

In patients with non-obstructive azoospermia, sperm retrieval by micro dissection TESE or open biopsy is preferred, and the success rate is estimated to range from 50 to 60% [Gangrade 2013]. The cause for non-obstructive azoospermia also plays a role in determining the success of sperm retrieval. Patients with unfavorable micro deletions in AZFa, AZFb regions have a very poor sperm retrieval rate of almost zero [Oates et al. 2002]. However, fertilization rates depend on whether a single viable sperm is available for ICSI rather than the source of the sperm or even the use of cryopreservation. But it is indeed influenced by the cause of azoospermia, i.e. whether the patient is having OA/NOA [Gangrade 2013; Verheyen et al. 2004].

Sperm cryopreservation after surgical retrieval decreases the need for repeated surgeries in cases of a failed ICSI attempt. It also ensures that sperm are available on the day of an IVF procedure. Repeated surgery not only increases the cost but also causes inflammation to the testis, leading to testicular devascularization and fibrosis [Schlegel and Su 1997]. Also, it avoids the need for the couple to both undergo a surgical procedure on the same day [Oates et al. 1997].

Fertilization and pregnancy rates were similar between fresh and frozen thawed sperm in patients with OA and NOA [Karacan et al. 2013; Nicopoullos et al. 2004]. Thus, it is useful to cryopreserve surgically retrieved sperm samples in patients with OA/NOA as the outcomes are only affected by the type of azoospermia and not by the cryopreservation process itself [Schwarzer et al. 2003].

Conventional freezing of testicular sperm yields a sperm recovery rate of only 1% [Borini et al. 2000]. Hence, there is a need for special techniques for storing a small number of sperm. Moreover, sperm recovery for ICSI can be difficult if the sample is diluted in a large volume of cryoprotectant as is done in conventional freezing techniques [Podsiadly et al. 1996].

Different carriers have been used in the cryopreservation of micro quantities of spermatozoa. In general, the carriers can be classified as biological and non-biological. Biological carriers include empty zona and Volovox globator algae. Non-biological carriers include straws, mini straws, copper loop cryoloop, calcium alginate beads, ICSI pipette microdroplets, agarose gel microspheres [AbdelHafez et al. 2009], and slopey cells [Endo et al. 2012].

**Storage of sperm in empty zona pellucida**

A small number of sperm can be effectively stored in empty zona pellucida. A human or animal oocyte is stripped of its cytoplasmic contents using micromanipulation techniques, and selected spermatozoa are then inserted into the zona pellucida using an ICSI pipette [Cohen et al. 1997]. The holes needed to remove the cytoplasmic contents can be made using
from the DNA of the host cell may thus get transferred into the oocyte during ICSI. Large holes have been closed using oil, but this may decrease post-thaw sperm recovery [Levi-Setti et al. 2003; Montag et al. 1999; Spadafora 1998]. In comparison, small holes might cause DNA entrapment causing DNA of the host cell to adhere to the sperm and get transferred. After the sperm are inserted into the zona, they are frozen either using conventional slow freezing or programmable freezing. Test yolk-glycerol cryoprotectant is used for cryopreservation, and empty zona may be pre-filled with the cryoprotectant before sperm insertion [Levi-Setti et al. 2003].

Cryopreservation in an empty zona significantly improves post-thaw sperm recovery and maintains motility and sperm chromatin and DNA integrity [Ye et al. 2009], making it an excellent method for storing small sperm samples. Various studies have reported high post-thaw sperm recovery (59 to 92%) and fertilization rates (23 to 65%) [Borini et al. 2000; Cohen et al. 1997; Levi-Setti et al. 2003; Montag et al. 1999; Walmsley et al. 1998]. The first successful pregnancy using an empty zona was reported by Walmsley et al. [1998].

Sperm binding to ZP3 (Zona pellucida sperm-binding protein 3) and the induction of acrosome reaction are believed to decrease recovery and fertilization rates with the use of a human zona [Cohen et al. 1997]. Also, the routine use of this technique is limited by the availability of human zona. Moreover, the use of animal zona is restricted by federal regulations to prevent human gametes from interacting with animal products.

Volvox globator spheres
Volvox globator spheres are sphere-shaped colonies formed by volvox globator algae. Just et al. [2004] showed that a small number of sperm can be cryopreserved in these colonies. Selected sperm were introduced into the spheres in cryoprotectants using an ICSI pipette and were then frozen using a conventional sperm freezing protocol. The post-thaw recovery rate was 100% and the motility rate was approximately 60%. With this method, however, human gametes are exposed to algae cells, which is restricted by new FDA and European tissue directive regulations [AbdelHafez et al. 2009; Just et al. 2004].

Cryoloop
A cryoloop consists of a nylon loop that can trap a small sample volume using capillary action. Cryoloops were initially used to store embryos [Lane et al. 1999] and later to cryopreserve a small volume of sperm samples by vitrification [Nawroth et al. 2002]. Storage of a small number of sperm using cryoloop was studied by Schuster et al. [2003] using ultra rapid and conventional slow freezing protocols. Sperm function after storage in cryoloops was evaluated by Desai et al. [2004] who reported sperm head decondensation and pronuclear formation when it was introduced into a human oocyte. Up to 73% of individually cryopreserved sperm were motile. Epididymal and testicular sperm cryopreserved using cryoloops were able to fertilize oocytes; the recovery rate was 72%.

The advantages of using a cryoloop to store a small number of sperm include ease of retrieval and the lack of exposure to animal products. Because nylon is an inert substance, it is safe, and the small surface area of contact between the sample and the nylon thread in cryoloops helps prevent sperm from adhering to the side of the container, minimizing sample loss [Schuster et al. 2003]. The major drawback is that it is an open system and is thus susceptible to contamination during storage inLN2.

Straws and ICSI pipette
Small quantities of sperm can also be stored in open straws, open pulled straws, and mini straws. An open straw system can be converted into a closed system by placing the straw inside another 90-mm straw. Open pulled straws are preferred for vitrification because they prevent contamination from LN2 [Isachenko et al. 2005].

Ministraws were used as early as 1998 by Desai et al. [2004] to preserve a small quantity of sperm. A small sample with CPA was loaded, and the ends were sealed. Multiple straws were stacked into cryovials and frozen in LN2 after exposure to LN2 vapor [Desai et al. 2004]. One disadvantage to using straws is that sperm may be lost if they adhere to the walls of the straw. Also, it is difficult to store individual sperm in them.

Sperm storage using ICSI pipettes was also evaluated. Post-thaw recovery and motility rates of 90 and 52%, respectively, were reported. Selected sperm from severe oligospermic samples stored in an ICSI pipette following conventional slow freezing lead to a recovery rate of 90% and a viability rate of 29% [AbdelHafez et al. 2009]. Even though ICSI pipettes are useful for storing a small number of sperm, they commonly break and are an open system, which increases the risk of cross contamination.

A newer improved version of storage called High Security Straws (HSV) was developed by Desai et al. [2012]. They reported a successful clinical pregnancy using this method. This system is closed and does not require any interaction between human sperm and animal products [Desai et al. 2012].

Cryopreservation of sperm in microdroplets
Small quantities of sperm mixed with CPA can be cooled rapidly on dry ice or in cooled stainless steel form microdroplets. These droplets can then be introduced into LN2 for storage. In one study, this method of storing sperm produced a clinical pregnancy, and implantation rates were similar to those of fresh testicular samples [Gil-Salom et al. 2000].

Microencapsulation of sperm in alginate beads and freezing sperm in agarose microspheres are other techniques for sperm preservation. However, these are not popular due to lack of adequate data for clinical use [Herrler et al. 2006].

Utility of cryoadditives
The addition of antioxidants during cryopreservation can also be used to improve post-thaw sperm motility, viability, and
DNA damage. Supplementation of the cryopreservation medium with 50 μM quercetin resulted in a significant improvement in post-thaw motility, viability, and DNA integrity [Zribi et al. 2012]. The addition of catalase resulted in a significant increase in the percent of progressive motility and viability, along with a significant decrease in DNA damage [Moubasher et al. 2013]. However, the addition of one mM reduced glutathione to the freezing and thawing extenders was of limited use in terms of increasing the percentage of motile sperm but not in viability or lipid peroxidation [Gadea et al. 2011]. Similarly, other reports also suggest varying degrees of improvement in sperm quality after processing. These depend on the freezing medium and the antioxidant used [Branco et al. 2010; Taylor et al. 2009; Thomson et al., 2009] while some were beneficial, others did not result in reducing ROS production or lipid peroxidation levels [Khodayari et al. 2014; Sariozkan et al. 2009]. Thus the use of antioxidants during freezing and the novelty of antioxidant proteins needs further investigation.

Antifreeze proteins (AFP) have the ability to depress the freezing point, modify the ice-crystal formation, prevent recrystallization, and interact with plasma membrane at low temperatures [Tomczak et al. 2002; Venkatesh and Dayananda 2008]. These proteins have been demonstrated in fish living in polar environments that have the ability to resist freezing temperatures. Improved sperm quality has been reported after cryopreservation with AFPs [Prathalingam et al. 2006; Younis et al. 1998]. AFPs especially AFPIII seem to interact with unsaturated fatty acids in fish (S. aurata) and stabilize the plasma membrane organization during cryopreservation and thereby improve sperm quality after thawing [Beirao et al. 2012]. However the beneficial effects of AFP on human spermatozoa have not been demonstrated.

**Future of cryopreservation**

As above, sperm cryopreservation using conventional techniques causes a significant decline in motility, viability, chromatin integrity, and mitochondrial potential. Conventional techniques are popular and have been used for decades even though they are cumbersome, time consuming, and expensive. Storage in LN2 also creates a safety hazard for the operator, and open systems increase the risk of cross contamination of samples. With this background, scientists are working on finding novel ways to store sperm and select high quality samples to improve sperm parameters and pregnancy outcomes. Table 2 compares the post-thaw parameters of different sperm storage techniques to those of conventional slow freezing. Clearly, many of the techniques, especially vitrification, have post-thaw parameters that are similar to those of conventional freezing. Live births have been reported after vitrification after IUI, which suggests that it can be used as an alternative to conventional freezing [Sanchez et al. 2012a]. Vitrification is easy and less time consuming because it requires minimal sperm processing. Moreover, it does not require the use of expensive programmable freezers. The ability to store vitrified samples at −86 °C would significantly decrease the need for LN2 [Sánchez et al. 2012b]. Recent improvements in the volume of a sample that can be vitrified in a single container have made it a good alternative to conventional freezing [Isachenko et al. 2011]. The development of closed systems for vitrification using sleeper cells has decreased the risk of cross contamination [Endo et al. 2012]. Thus, vitrification has the potential to become the preferred technique used for sperm cryopreservation in the near future. More research is still needed with larger sample sizes to assess fertilization and pregnancy outcomes using vitrified sperm.

Lyophilization is another technique that has the potential to develop into a popular method. Even though sperm that undergo lyophilization are technically dead, the DNA damage in lyophilized sperm is less than that seen in conventional freezing. The ability to store the samples at 4 °C is an attractive advantage [Gianaroli et al. 2012]. Techniques are now available that allow fertilization with dead sperm [Stecher et al., 2011]. More human studies are needed to demonstrate the effectiveness of this technique.

Many of the assisted reproductive techniques require only short-term storage sperm. For example, cryopreservation of sperm is used to ensure that sperm are available during the time of oocyte retrieval. Short-term storage media may be employed in such situations, which is more cost effective. The limited number of studies available suggests that it is possible to store sperm samples without a loss of sperm DNA integrity for up to four weeks. Table 3 illustrates the advantages and disadvantages of the main storage methods compared to conventional cryopreservation.
<table>
<thead>
<tr>
<th>Sperm Storage Technique</th>
<th>Special Remarks</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Literature</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>2. Faster</td>
<td>2. Cryoloops are open systems. Hence increased risk of disease transmission</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Less osmotic damage to spermatozoa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lyophilization</td>
<td></td>
<td>1. Does not require LN₂ for storage – Cheap, easy to maintain, improved safety</td>
<td>1. More studies needed on long term storage</td>
<td>[Sánchez et al. 2012b].</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Transportation of sample in room temperature</td>
<td>1. Sperm are dead and hence ICSI and oocyte activation mandatory</td>
<td></td>
</tr>
<tr>
<td>LN₂ vapor storage</td>
<td></td>
<td>1. No direct contact with the LN₂. Hence decreased risk of sample contamination</td>
<td>1. Storage still requires LN₂</td>
<td>[Lim et al. 2010]</td>
</tr>
<tr>
<td>Storage using cryoloop</td>
<td>For storing small number of spermatozoa</td>
<td>1. No need for preparing the carrier since its commercially available</td>
<td>1. Open system – risk of cross contamination</td>
<td>[Desai et al. 2004; Schuster et al. 2003].</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Sperm loss due to adherence to the vessel wall is avoided</td>
<td>2. Requires micromanipulator to load the sample</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Nylon is inert and no exposure to animal products</td>
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</table>

LN₂: liquid nitrogen
Storing a small number of sperm surgically recovered from the testis and epididymis is usually done using either empty zona or cryoloops. These are the most popular methods even though many other carriers for sperm storage in microquantities have been described in the literature. The cryoloop is preferred since it is easy to use. Human zona are difficult to obtain, and expertise is needed to remove the cytoplasmic contents with micromanipulation tools and are subject to a series of regulations. However, as stated previously, cryoloops are an open system. Newer methods of storing sperm in high security straws provide an alternative to cryoloop since it is a closed system. Vitrification using sleeper cells is another technique that can be used to preserve a small number of sperm [Endo et al. 2012]. The advantage here is that it becomes a closed system. Currently, there are no randomized controlled studies comparing the effectiveness of different methods of storage for a small number of sperm [AbdelHafez et al. 2009].

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Declaration of interest
The authors report no declaration of interest.

Author contributions
Conceived the idea, supervised the study, and edited the article for submission; RS; Collection of literature and preparation of the manuscript: AK, JG; Reviewing and editing of the manuscript: AA.

References
Comparison of newer sperm freezing methods


