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INVITED REVIEW

Sperm Biology

Sperm cryopreservation protocol for micro-TESE-retrieved sperm

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Azoospermia is characterized by the absence of sperm in the ejaculate and is categorized into obstructive azoospermia (OA) and nonobstructive azoospermia (NOA). For men with NOA, testicular sperm extraction (TESE) is the only method to obtain sperm for assisted reproductive technology (ART). Given the rarity of these sperm and the unpredictable success of subsequent retrieval attempts, cryopreservation of microdissection-TESE-obtained sperm is essential. Effective cryopreservation prevents the need for repeated surgical procedures and supports future ART attempts. After first delving into the physiological and molecular aspects of sperm cryopreservation, this review aims to examine the current methods and devices for preserving small numbers of sperm. It presents conventional freezing and vitrification techniques, evaluating their respective strengths and limitations in effectively preserving rare sperm, and compares the efficacy of using fresh versus cryopreserved testicular sperm.

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INTRODUCTION

Azoospermia affects approximately 1% of the male population.[1] It has a tremendous social and psychological impact, considering the significantly reduced probability of fathering a child by one's own genetic contribution.

In the context of male factor infertility, azoospermia refers to the absence of sperm in the ejaculate, including two main categories, obstructive azoospermia (OA) and nonobstructive azoospermia (NOA).[2] In the case of NOA, the absence of sperm is due to a problem in sperm production or sperm maturation within the testes, rather than a blockage that prevents sperm from being ejaculated. In such cases of NOA, currently, surgical retrieval through testicular sperm extraction (TESE) is the only option available to obtain sperm for use with assisted reproductive technology (ART).

Microdissection TESE (micro-TESE) is a specialized technique aimed at identifying specific regions within the testes where sperm production may occur despite the absence of sperm in the ejaculate. It is more precise in sperm retrieval, as the operating microscope effectively assists the surgeon in extracting the most probable site of finding the sperm, thus increasing the sperm retrieval rate. Further, minimum and precise extraction reduces testicular damage.

The sperm retrieval procedure demands skilled reproductive specialists and trained laboratory personnel to detect and isolate intact spermatozoa from the surgically retrieved seminiferous tubules. The probability of using TESE sperm for fertilization was predicted almost 30 years ago, offering hope to men with testicular failure.[3] Such rare sperm, most often retrieved after extensive surgical and laboratory searches, demands equally challenging efforts to cryopreserve for future use.

Micro-TESE sperm need to be cryopreserved as they are precious. Very often, they are retrieved in extremely low numbers. The probability of finding such rare sperm in the next micro-TESE attempt remains unpredictable. If micro-TESE is performed before the treatment cycle, in case no sperm are found during the attempt, the couple has the option to cancel the cycle without undergoing stimulation or to make the decision to use donor sperm. Retrieval of sperm with successful cryopreservation avoids repeating the procedure in case of failure to initiate the pregnancy. Frequently, the testicular sperm may lack motility. A report on the successful utilization of immotile but viable sperm emphasized the significance of cryopreservation as a crucial tool in the surgically retrieved sperm extraction procedure.[4]

In this review, the initial focus is on the physiological and molecular aspects of sperm cryopreservation, followed by an examination of the current preservation methods for low sperm numbers. Conventional freezing and vitrification techniques are presented with their respective strengths and limitations in preserving sperm effectively, and ART outcomes with cryopreserved versus fresh sperm are compared.

SPERM CRYOPRESERVATION: AN OVERVIEW

The history of sperm cryopreservation can be traced back nearly 75 years ago from 1949.[5] The astonishing fact is that they used the vitrification technique for sperm, which remained quiescent till the late 1990s. Even today, sperm vitrification is a field that needs optimization and is considered experimental according to the 6th edition of the WHO laboratory manual for the examination and processing of human semen.[6]

The sperm retrieval technique of micro-TESE was introduced in 1999[7] and has been reported as an advanced alternative for retrieving

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testicular sperm more efficiently than the conventional TESE (cTESE) method.[8] It is necessary to optimize an approach to effectively cryopreserve such "rare" sperm to be used for intracytoplasmic sperm injection (ICSI) in the future.

Routinely, the vapor freezing,[9] rapid method,[10] programmed freezing,[11] and modified vitrification[12] are used for sperm cryopreservation. Even after six decades, there has been limited advancement in the effectiveness of 45%–55% postthaw sperm survival rate.

The main reason for this viability loss is that cryopreservation can induce deleterious cellular changes, particularly to the delicate structure of sperm membranes,[13] leading to reduced sperm viability and postthaw fertilizing ability. Cryoprotective agents (CPAs), added to the sperm samples before cooling, are known to mitigate cryoinjury by protecting the sperm from ice-induced damage.[14]

However, the effect of various CPAs on the structure of sperm membranes remains a crucial area of research to optimize cryopreservation protocols and improve reproductive outcomes.

Pathophysiology of cell/sperm freezing

Sperm cryopreservation is a physical process that allows for preserving cell structure and functionality at very low temperatures for almost an indefinite time period.

Exposure of native cells to ultralow temperatures typically leads to cell death. As the cell content mainly consists of water (approximately 80%), freezing may provoke ice crystal formation intra- and extracellularly. Thus, water solidification has been implicated in the harmful biochemical and structural effects of freezing that jeopardize cell integrity postthaw.

The extent of cell damage is associated with the cooling speed. At slow rates, extracellular ice formation increases causing excessive cell dehydration. Contrastingly, at very rapid rates, intracellular water is solidified causing internal organelle damage.[15]

The use of cryoprotectant agents has been applied in view of mitigating the cryodamage caused on sperm cells. Permeating substances, such as glycerol, dimethyl sulfoxide (DMSO), glycol, ethylene and methanol, as well as non-permeating ones, such as polyethylene glycol (PEG), polynynilpyrrolidone (PVP), raffinose, sucrose and trehalose, are most commonly used.

Permeating CPAs, due to their lipophilic nature, are able to enter the cell, by crossing the cellular membrane. Their osmotic action induces water exit from the cell, thus minimizing intracellular ice formation and stabilizing the bilipid membrane layer. Nonpermeating CPAs consist of high-molecular-weight molecules that do not permeate the cell membrane, thus increasing the extracellular solute concentration, creating an osmotic gradient causing cell dehydration.

Vitrification achieves ultrarapid cooling of the cells without the requirement of osmotic balance (nonequilibrium) by the use of hyperosmolar media and very rapid cooling rates. The sample, therefore, rapidly passes from a liquid to a solid glassy state avoiding ice crystal formation in the intra- and extracellular milieu, while the water movement is more efficient, creating a less harmful effect than conventional freezing.[16]

Improper warming process of the intracellular environment may provoke serious damage. At low warming rates, *i.e*., under 1000°C per min, recrystallization occurs, inducing the formation of intra- and extracellular ice crystals. Contrastingly, when warming is performed at high rates, ice crystal formation is significantly decreased. Therefore, the warming temperature is critical for the protection of the plasma membrane structure and functionality, even if the cells seem apparently unaffected.[15]

Cryopreservation effects on sperm quality

The biochemical processes that take place during freeze-thawing/vitrification-warming induce molecular impairments associated with metabolic alterations, oxidative imbalance by increasing reactive oxygen species, and reducing the levels of antioxidant factors with associated DNA damage, epigenetic variation, and cell death.[15] Furthermore, alterations in proteins related to membrane function have various implications on sperm function, such as sperm motility, sperm viability, acrosome reaction, capacitation, metabolism, fertilizing ability, as well as cell death through apoptotic or other mechanisms.

The overall impact of sperm cryopreservation is further dependent on intra- and inter-individual sperm cryo-tolerance which varies among seasons, latitudes and ethnic backgrounds.[16–18]

The detrimental effects of sperm cryopreservation on sperm DNA fragmentation (SDF) have been extensively studied.[19] The main mechanism of post-thaw SDF has been associated with oxidative damage. In the attempt to counteract this cryo-induced oxidative stress, the supplementation of CPAs with antioxidant substances, such as vitamin E, hypotaurine, or other substances, has been suggested to prevent sperm cell damage in terms of DNA integrity and membrane function (**Figure 1**).[20,21]

The underlying causes of why a certain proportion of motile sperm becomes nonviable during the cryopreservation process remain elusive. Variation of this proportion of nonviability has also been observed among the samples and techniques applied.[22] Sperm positioned at the periphery of the freezing droplet at the time of freezing may be more prone to damage due to osmotic shock.[23]

In normal semen parameters, preserving sperm has an obvious advantage of quantity as sufficient sperm can be obtained for insemination even if 50%–60% of sperm become nonviable post thaw. However, in the case of very few sperm available, one needs a protocol that can retain the viability of almost all sperm cryopreserved. This is a daunting challenge considering the lack of information on variations in the structural configuration of sperm membranes and their response to the CPAs and cryopreservation protocols *per se*.

CRYOPRESERVATION OF "RARE" SPERM

Realizing the importance of preserving a low number of sperm obtained through challenging situations, researchers have attempted to establish various efficient methods described in the next sections.

Empty zona method

Cohen *et al*.[24] first proposed the concept of single-sperm cryopreservation in 1997, using the newly established micromanipulation technique. It was an unconventional, unique idea to use an empty zona, either from an animal or human, to store a single or a limited number of sperm, manually selected and deposited into the emptied zona pellucida.[24,25] Two groups adopted two methods for this technique. Levi-Setti *et al*.[26] filled the empty zona with CPA and injected sperm in it. Ye *et al*.[27] first inserted the sperm in an empty zona, and then exposed this structure to the CPA. Zona-containing sperm were then subjected to either vapor cooling or slow-freezing method. A few groups used empty zonae from humans,[24,28–30] whereas some used those from mice.[24,28,31]

The first live births using human and hamster empty zonae to cryopreserve epididymal and testicular spermatozoa were reported by Walmsley *et al*.[28] They used thawed sperm from oligozoospermic/azoospermic patients, cryopreserved in the empty zonae. Out of five embryo transfer cycles, three resulted in pregnancies, with two of them ending in the birth of two sets of twins. The efficacy of this technique was confirmed by Fusi *et al*.[32] in 2001 by reporting one

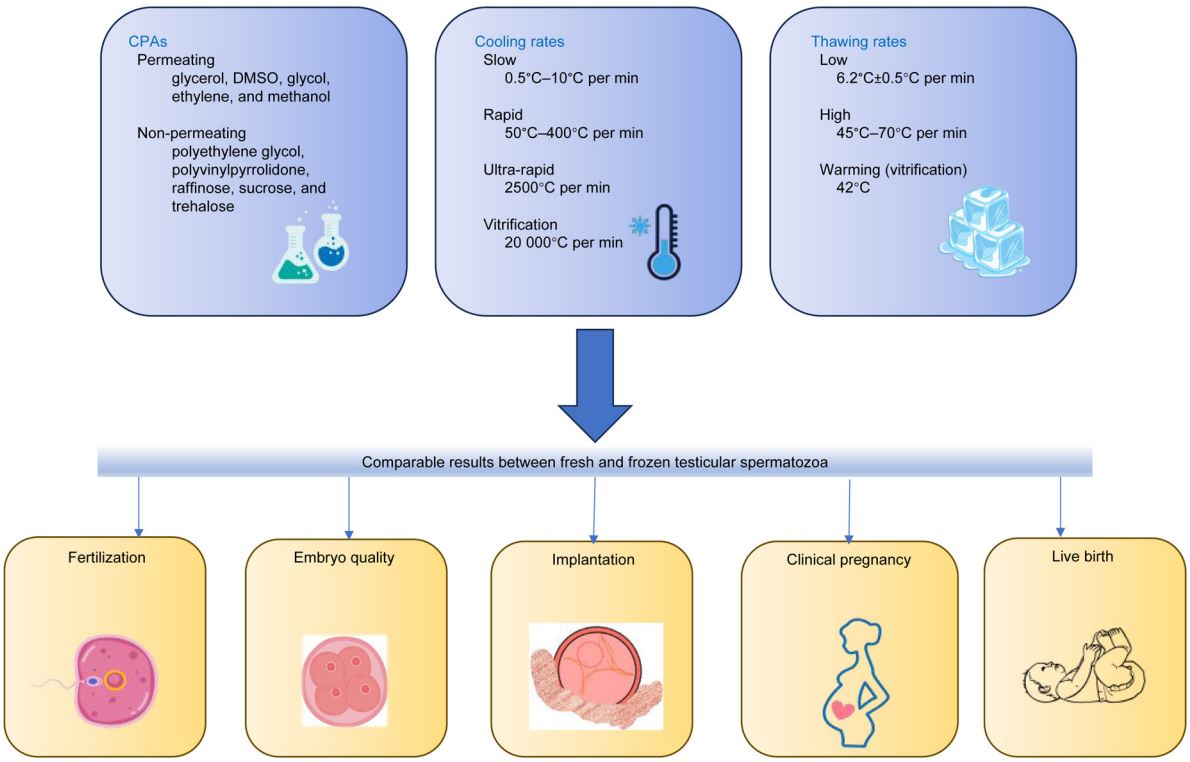

Figure 1: Graphic illustration of various factors affecting testicular sperm cryopreservation and their impact on major reproductive endpoints. CPA: cryoprotective agents; DMSO: dimethyl sulfoxide.

singleton pregnancy using empty human zona with epididymal sperm. Cohen *et al.*[24] postulated that low recovery (27 sperm lost or damaged of total 126 sperm) and fertilization rates (50%) were attributable to the induction of acrosome reaction or 1-day-old oocytes used for the experimentation, which might have a lower tolerance toward cryopreservation protocol and compromised membrane flexibility. So far, this method has not been applied with micro-TESE sperm.

The use of zona pellucida as a carrier for sperm cryopreservation presents several advantages and limitations. On the positive side, the zona provides a protective environment for the sperm, as it is nonpermeable and securely entraps the sperm. It also offers better visibility compared to a single sperm, making it easier to locate under a stereo microscope postthawing. Additionally, handling the zona is more convenient since it can be recovered without disturbing the preserved sperm inside. However, there are significant limitations to this technique. The use of rodent zonae for human sperm cryopreservation is restricted by regulations due to immunological concerns and the risk of microbiological cross-contamination. Access to human zonae is also limited, posing a challenge for routine implementation. Furthermore, recovering sperm can be difficult if it becomes embedded in the zona's porous inner surface, and a large injection hole can compromise motile sperm retention. Preparing the zona requires sophisticated instruments and skilled personnel, making the process tedious and time-consuming.

Microdroplet method

Considering the drawbacks of biological carriers, Gil-Salom *et al.*[33] and Rubio *et al.*[34] introduced the technique of microdroplets for the cryopreservation of rare sperm. Based on the simple principle of solidifying tiny droplets of 50–100 µl containing a mixture of sperm and CPA using dry ice or a cold steel plate, these tiny solid drops are thawed when required to retrieve sperm. Gil-Salom *et al.*[33] reported the successful application of this technique with testicular sperm recovery of 172 from 190 (90.5%) frozen-thawed testicular sperm, comparable to 50% in the zona method. The implantation, clinical pregnancy, and ongoing pregnancy rates with cryopreserved sperm were comparable with those for fresh testicular sperm (13.1% *vs* 12.2%, 27.8% *vs* 28.2%, and 21.8% *vs* 22.4%, respectively).

Attempts to use the microdroplet method to cryopreserve individually selected sperm were reported.[34–36] Bouamama *et al.*[35] used a similar setup with 100 testicular sperm and exposed the dish to liquid nitrogen (LN_2) vapors for 2 h before plunging it into LN_2. The survival was 100%, but motility dropped to 50%.[35] Sereni *et al.*[36] in their experiment with microdroplets used testicular sperm obtained with the testicular fine needle aspiration technique. Out of 431 retrieved sperm, only 15 (3.5%) sperm were motile. Postthaw retrieval rate was 100%, but motility was retained by only 11 sperm (2.6%). Astonishingly, out of 51 oocytes injected, only 9 were fertilized. Three embryo transfers resulted in one biochemical pregnancy.[36]

The technique of using polystyrene Petri dishes for sperm cryopreservation offers several advantages and limitations. Among its benefits, the method is simple and does not require any special equipment, making it quick to perform and ensuring a good recovery rate. However, there are notable drawbacks, including highly variable post-thaw motility and the risk of cross-contamination since the sperm is not encapsulated. Additionally, this method requires enormous storage space, rendering it impractical for large-scale use. Moreover, the polystyrene material of Petri dishes is unsuitable for long-term cryostorage, further limiting its practicality.

ICSI pipette in sperm cryostorage

Successful micromanipulation applications in ART proved beneficial in multiple ways. It revolutionized insemination outcomes with precision in

controlling the movement of selected sperm in the injecting pipette. Based on this ability, Gvakharia and Adamson[37] proposed cryopreservation of a few sperm inside the injecting pipette. The selected sperm picked up in a group of 5–50 were equilibrated within CPA droplets and either directly plunged into LN_2 or exposed to LN_2 vapors for 20 min before plunging into LN_2. They reported postthaw recovery and motility retention rates of 92% and 52%, respectively. Sohn *et al*.[38] applied the same technique to preserve single sperm obtained during TESE using slow cooling and ultrarapid cooling methods. They achieved recovery and motility rates of 90% and 29% for slow cooling method and 80% and 8% for ultrarapid cooling method, respectively.[38]

The use of a precise control method for sperm cryopreservation offers significant advantages and notable limitations. On the positive side, this method allows for precise control over the selection and movement of sperm, resulting in a very low probability of losing them during cryopreservation and a high recovery rate due to good visibility. However, the technique requires sophisticated equipment and skillful handling. The carrier used is highly fragile, increasing the likelihood of damage during handling, and its tiny nature makes efficient storage challenging. Additionally, the open system design makes it vulnerable to microbiological cross-contamination.

Volvox globator spheres method

Just *et al*.[39] proposed a rather unusual method to cryopreserve rare sperms. They used spheres formed by colonies of algae Volvox globator. The sphere was equilibrated with CP solution. A fixed number of 8 morphologically normal and motile sperm from severe oligozoospermic patients were taken into the injecting pipette. The algae sphere was held using a holding pipette, and gently, the sperm were released into the sphere, which was then loaded into the straw and vapor-cooled before storing in LN_2. After thawing, the sphere was released into the buffered medium and positioned with a holding pipette. Sperm were extracted using another injecting pipette. Just *et al*.[39] reported 100% recovery and motility retention of 50%.

The method for cryopreserving sperm using algae shows promise with good recovery and acceptable motility of cryopreserved sperm. However, it has several limitations. The method has not been reproduced by other research groups, and its complex methodology hinders routine implementation. There is also a risk of contamination from unknown genetic or biochemical factors of algae, and preparing and maintaining algae colonies contamination-free is tedious and time-consuming. Additionally, using algae-originated material for human purposes is forbidden in many countries, further restricting its application.

Alginate beads method

Herrler *et al*.[40] used alginate beads as carriers to preserve a low number of sperm. Alginate is a polysaccharide derived from brown seaweed. It forms a gel-like structure when conjugated with calcium compounds. Influencing factors such as concentration of alginic acid, size of drops, time of polymerization, and media were evaluated for optimum outcome. The final protocol was standardized as encapsulation by 7.3 mg ml^{-1} alginic acid forming 10-µl drops polymerized for 30 s and liquefied for 2.5 min in sodium citrate, maintaining a pH of 6. Briefly, after centrifugation of the semen, the pellet was mixed with CPA. Alginic acid in a concentration of 7.3 mg ml^{-1} was immediately added to it. Tiny drops of this mixture were exposed to calcium chloride solution to initiate encapsulation that formed tiny beads. These beads containing spermatozoa were cryopreserved using a slow-freezing program method. The beads were exposed to sodium citrate solution to dissolve them during thawing, and sperm were isolated, washed off the alginic acid, and used for ICSI.

The use of an inert capsule for sperm cryopreservation has the advantage of maintaining the sperm intact. However, this method has not been reproduced by other research groups and requires a complicated setup, making it highly time-consuming. Its routine application is limited by low efficiency, as the process involves preparing beads, applying polymerization, and maintaining an optimum pH to retain sperm viability. Additionally, alginic acid used in the process may negatively affect sperm motility due to its residual effects.

Agarose microspheres method

Intending to replace biological zona with a nonbiological carrier, Isaev *et al*.[41] used 2% agarose microspheres of about 100 µm diameter. About 1–10 sperm from ejaculates were deposited in these spheres, which were exposed to CPA for 5 min. Postequilibration, 5–10 microspheres were loaded in 0.25 ml plastic straws and cryopreserved using the vapor method. Isaev *et al*.[41] published the data of 318 motile sperm preserved using this technique in 67 spheres and in 19 straws. Upon thawing, two spheres that contained seven sperm each, were lost during the procedure. Out of the 311 remaining recovered sperm, 243 (78%) retained their motility.

The method presents a good alternative to biological carriers with an acceptable recovery and motility retention rate. However, it has several limitations, including the fact that it has not been reproduced by other groups and that there is no data on clinical pregnancy outcomes. In addition, the setup is complicated, potentially making it unsuitable for routine use.

Methods developed for sperm vitrification

The application of vitrification for cryopreserving testicular spermatozoa in the context of assisted reproduction seems more promising than conventional freezing in terms of motility recovery, DNA and membrane integrity preservation, and viability.

Vitrification became an indispensable part of ART procedures due to unparalleled efficacy as compared to the slow-freezing method. Even vitrification of embryos and oocytes resulted in better outcome as compared to sperm vitrification. Researchers are working on standardizing vitrification to optimize it for the fragile morphologic architecture of the sperm cell.

While the technique is still not established unequivocally for sperm preservation, a few groups tried using the vitrification method with various carriers for sperm with varying degrees of success.

Dry ice droplet method

Using the same principle of creating solidified microdroplets, Isachenko *et al*.[42] applied vitrification carriers with 40-µl droplets containing few sperm exposed to dry ice. However, they found 40% sperm with reduced motility as compared to control samples.[42]

Cryoloop and strip method

Schuster *et al*.[43] proposed using a cryoloop. They used a tiny loop with a diameter of about 1 mm at one end. A thin layer of sperm cryoprotectant was created on the surface of the loop. The spermatozoa to be cryopreserved were picked up using a micropipette under a stereoscope and loaded on this thin film. The loop was then exposed to liquid vapors for 15 min and plunged into LN_2 to vitrify the sperm.

Similar experiments to preserve a low number of sperm were carried out by Desai *et al*.[44] They reported cryopreservation of a low number of sperm, considering a minuscule volume of 0.1 µl to 0.01 µl of vitrification solution forming a thin film in the tiny circular

loop. They reported 73% retention of twitching motility. Further investigations of sperm function tests revealed that the sperm could undergo decondensation thus being capable of successful fertilization. They used 5 sperm on the cryoprotectant layer in the loop. Three sperm were recovered postwarming and 2 sperm could fertilize the oocytes. The effectiveness of this technique was confirmed by Isachenko *et al*.[42]

As it is a general trend to prefer a closed system over an open system to prevent possible cross-contamination through liquid nitrogen, Mangoli *et al*.[45] reported cryopreservation of a few number of sperm without any additional cryoprotectant using VitriMate® device. Hu *et al*.[46] recently confirmed the efficacy of the closed system with comparable results.

The technique offers the advantages of a simple setup, ease of handling, good recovery rates, and the ability to use a closed system to prevent cross-contamination for preserving a low number of sperm. However, there are limited studies to confirm the efficacy of the technique, and it is primarily used as an open system, which poses a risk of cross-contamination.

With regard to the preservation of low sperm numbers, Endo *et al*.[48] first reported the use of vitrification using a commercially available carrier Cryotop for single sperm preservation. Although effective in post-warming retrieval, the concern was that it involved an open system. The same group later reported using a closed system, "cell sleepers", and claimed 100% recovery and 72% viability retention. They used a vial-based system containing a tray on the inner side of the cap that holds the microdroplets (3.5 μl) with sperm. After loading a single sperm while monitoring under a stereoscope, the cap fitted in the vial creates a close system environment. The cell sleeper was held 4–5 cm above LN_2 vapor for 2.5 min before plunging into the LN_2.[48] Coetzee *et al*.[49] used cell sleepers to cryopreserve single testicular sperm on a droplet of 2 μl. A total of 304 sperm were cryopreserved, of which 265 were warmed. The recovery rate was 94%, with a viability retention rate of 56%.[49]

Mesh and pearl drop method

O'Neill *et al*.[56] published data on sperm vitrification using a novel device of "mesh and pearl drop". A specially developed funnel-shaped device was used, the bottom part of which is immersed in LN_2. The flat surface area has a mesh-like structure on which 20-μl drops containing an average of 5 sperm were placed. Once solidified, the drops were collected at the bottom tubular structure and stored in LN_2 (**Table 1** and **2**).[50]

COMPARISON OF THE EFFICACY OF FRESH *VS* CRYOPRESERVED TESTICULAR SPERMATOZOA IN MICRO-TESE-ICSI

According to the majority of available research data, the use of fresh versus frozen testicular spermatozoa seems to produce comparable results in terms of fertilization, embryo quality, implantation, clinical pregnancy, and live birth rates.[51–54] In a limited number of cases reported by Karacan *et al*.,[55] it was suggested that fresh testicular sperm from NOA patients might provide a slight, albeit not statistically significant, increase in delivery rates. According to the findings reported in three major meta-analyses, the use of fresh *vs* cryopreserved testicular sperm provided similar outcomes. Nicopoullos *et al*.[56] found comparable results in fertilization rates, as well as clinical and ongoing pregnancy rates in ICSI by use of fresh and frozen testicular sperm (in cases of both OA and NOA).The number of embryos transferred in these cases was not available.[56] Ohlander *et al*.[57] showed that when only NOA cases were analyzed, comparable fertilization and clinical pregnancy rates were obtained with fresh and frozen testicular sperm samples. In an analysis of more than 1000 samples, Yu *et al*.[52] reported a similar efficiency of fresh and frozen testicular sperm, in terms of fertilization, implantation and clinical pregnancy rates. The presence of motility in surgically retrieved sperm has also been evaluated in a limited number of studies as a contributing factor of success rates. The motility of testicular spermatozoa is expected to be low (usually nonprogressive, mostly immotile), as they have not yet matured, acquiring the ability of movement during their passage through the epididymis. In the particular case of frozen testicular spermatozoa, low motility is usually present due to the additional loss of motility encountered after cryopreservation, providing feebly motile or immotile viable spermatozoa for use in ICSI. Although lower fertilization rates occur by the use of poorly motile sperm, there are no meaningful differences in embryonic development or pregnancy rates between oocytes fertilized by motile and poorly motile sperm once fertilization is achieved.[58] In general, the use of feebly motile *vs* totally immotile cryopreserved testicular sperm produces better quality embryos, even if fertilization rates may be similar.[52,58]

Hence, in an effort to improve ICSI outcomes, techniques such as the addition of substances enhancing sperm motility, mainly pentoxifylline, have been applied to help distinguish live sperm in case of total immotility.

Alternative sperm selection strategies have also provided promising results in the attempt to select live sperm. These include the hypo-osmotic swelling test (HOST), which identifies sperm with viable and

Table 1: Cryopreservation chronicles: evolution of techniques for preserving limited sperms

Study	*Method/technique, sperm number*	*Sperm source*	*Viable sperm recovery (%), range*	*Fertilization rate (%)*	*Pregnancy (yes/no)*
Cohen *et al*.[24] 1997	Empty zona (124)	Ejaculate	65–100	50–53.2	No
Walmsley *et al*.[28] 1998	Empty zona (1056)	TESE, MESA	74	82	Yes
Gil-Salom *et al*.[33] 2000	Microdroplet (190)	TESE, MESA	90.5	63.2	Yes
Gvakharia and Adamson[37] 2001	ICSI micropipette (520)	Ejaculate, TESE	92	NA	No
Bouamama *et al*.[35] 2003	Culture dish (100)	Ejaculate	100	50	No
Desai *et al*.[44] 2004	Ultrarapid on cryoloop (24)	TESE, MESA	0–100	33.3–66.6	No
Just *et al*.[39] 2004	Volvox globator spheres (360)	Ejaculate	100	NA	No
Isachenko *et al*.[42] 2005	CP-free droplet (NA)	Ejaculate	100	NA	No
Herrler *et al*.[40] 2006	Alginate beads (NA)	Ejaculate, TESE, MESA	100	NA	No
Isaev *et al*.[41] 2007	Agarose microsphere (318)	Ejaculate	95.5	NA	No
Endo Yuji Y *et al*.[47] 2011	Vitrification (20)	Single sperm from ejaculate	98	30	No
Endo *et al*.[48] 2012	Cell sleeper (150)	Single sperm from ejaculate	100	NA	No
Mangoli *et al*.[45] 2016	VitriMate (close system), without cryoprotectant (750)	Ejaculate	78.63	84.5	No

NA: data not available; TESE: testicular sperm extraction; MESA: micro-epididymal sperm aspiration

Table 2: Summary of rare sperm post-cryopreservation retrieval methods

Study	*Method*	*Accessibility*	*Setup*	*Recovery*	*Biological material used (yes/no)*
Cohen *et al.*[24] 1997	Empty zona	Complex	Complex	Variable	Yes
Gil-Salom *et al.*[33] 2000	Microdroplet	Easy	Simple	Variable	No
Gvakharia Adamson.[37] 2001	ICSI pipette	Easy	Complex	Good	No
Just *et al.*[39] 2004	Volvox globator sphere	Complex	Complex	Good	Yes
Herrler *et al.*[40] 2006	Alginate beads	Complex	Complex	Low	No
Isaev *et al.*[41] 2007	Agarose microsphere	Complex	Complex	Good	No
Isachenko *et al.*[42] 2005	Dry-ice droplet	Easy	Simple	Variable	No
Schuster *et al.*[43] 2003	Cryoloop	Easy	Simple	Good	No
Mangoli *et al.*[45] 2016	Cryostrip	Easy	Simple	Good	No
O'Neill *et al.*[50] 2019	Mesh and pearl drop	Complex	Complex	Good	No

ICSI: intracytoplasmic sperm injection

functional membranes in the form of a coiled tail, as well as the laser-assisted immotile sperm selection (LAISS) technique, which induces a tail curl and coil using a laser shot.[59,60] Pentoxifylline is reported to be a better alternative than the HOST to select viable spermatozoa among the nonmotile TESE sperm population.[61]

CONCLUSION AND FUTURE ASPECTS

Cryopreservation of low numbers of surgically retrieved testicular spermatozoa should be considered whenever possible, as fresh and cryopreserved-thawed/warmed spermatozoa provide comparable results. Couples should also be informed about the possibilities offered and consider the asynchronous sperm retrieval during the time scheduling of the ICSI attempt. More studies are warranted to include higher numbers of subjects and further support the standardization and refinement of cryopreservation procedures applied in different fertility centers. The application of vitrification for cryopreserving low numbers of testicular spermatozoa in the context of assisted reproduction seems more promising than conventional freezing in terms of motility recovery, DNA and membrane integrity preservation, and viability.

Future considerations regarding the optimization of the efficient use of cryopreserved testicular spermatozoa in a micro-TESE-ICSI setting focus on ameliorating methodological protocols, including cooling and warming/thawing rates, CPA concentration and combinations, and osmotic equilibration conditions. The utilization of novel freezability markers derived from transcriptomics, proteomics, and bioinformatics, aiming to describe molecular alterations in spermatozoa before, during, and after cryopreservation, may enable the application of personalized protocols enhancing post-thaw recovery of viable and functional sperm.

SUMMARY

Cryopreservation of surgically extracted spermatozoa from the testis constitutes an integral facet of efficacious interventions for addressing male infertility, obviating the need for recurrent surgical procedures. Both biologic and nonbiologic carriers have been investigated in attempts to cryopreserve spermatozoa retrieved in limited quantities.

Surgically retrieved specimens frequently present with elevated levels of red blood cells, cellular debris, testicular cells, and a substantial number of nonviable or immotile sperm. The absence of easily implementable technologies capable of handling these cases represents a significant impediment to single-sperm freezing.

Over a decade has transpired since instances of pregnancies were documented, following the utilization of surgically retrieved human sperm cryopreserved through various methods. The lower-than-expected recovery and pregnancy rate underline the need to optimize the methods of rare sperm cryopreservation. The root cause for the poor outcome is multifaceted. Initial reasons include a scarcity of clinical data substantiating its effectiveness and the absence of a universally applicable device or vehicle. Each technique proposed possesses its own inherent limitations and drawbacks.

Innovation in cryopreservation technology custom-tailored for managing minute sperm quantities necessitates further exploration. Published studies focusing on cryopreservation of small numbers of testicular sperm in exceedingly compromised surgical specimens remain scarce. The current body of evidence is insufficient to advocate for the preference of one technology over another. Methodologically robust clinical trials incorporating appropriate sample sizes are imperative to evaluate the feasibility and efficacy of diverse cryopreservation techniques for low sperm count scenarios.

AUTHOR CONTRIBUTIONS

VM and EE contributed to the main writeup of the manuscript. CW was instrumental in designing the project outline, deciding the structure of the manuscript and critically reviewing the writeup. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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