# Cryopreservation of testicular and epididymal sperm: techniques and clinical outcomes of assisted conception

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The introduction of the technique of intracytoplasmic sperm injection to achieve fertilization, especially using surgically retrieved testicular or epididymal sperm from men with obstructive or non-obstructive azoospermia, has revolutionized the field of assisted reproduction. The techniques for the retrieval of spermatozoa vary from relatively simple percutaneous sperm aspiration to open excision (testicular biopsy) and the more invasive Micro-TESE. The probability of retrieving spermatozoa can be as high as 100% in men with obstructive azoospermia (congenital bilateral absence of the vas deferens, status post-vasectomy). However, in non-obstructive azoospermia, successful sperm retrieval has been reported in 10-100% of cases by various investigators. The surgical retrieval and cryopreservation of sperm, especially in men with non-obstructive azoospermia, to some extent ensures the availability of sperm at the time of intracytoplasmic sperm injection. In addition, this strategy can avoid unnecessary ovarian stimulation in those patients intending to undergo in vitro fertilization-intracytoplasmic sperm injection with freshly retrieved testicular sperm when an absolute absence of sperm in the testis is identified. Several different methods for the cryopreservation of testicular and epididymal sperm are available. The choice of the container or carrier may be an important consideration and should take into account the number or concentration of the sperm in the final preparation. When the number of sperm in a testicular biopsy sample is extremely low (e.g., 1-20 total sperm available), the use of an evacuated zona pellucida to store the cryopreserved sperm has been shown to be an effective approach.

**KEYWORDS:** Azoospermia; Testicular Sperm Retrieval; Epididymal Sperm Aspiration; Sperm Cryopreservation; Intracytoplasmic Sperm Injection.

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## INTRODUCTION

The introduction of intracytoplasmic sperm injection (ICSI), a technique for the fertilization of oocytes, has revolutionized the field of in vitro fertilization and assisted reproduction, especially in couples with male factor infertility. The report (1) of successful live births following the fertilization of metaphase II oocytes by the injection of a single sperm in couples for whom in vitro fertilization (IVF) and subzonal injection (SUZI) of sperm had previously failed represented a major milestone. Initially, these investigators used freshly ejaculated spermatozoa for ICSI, but soon thereafter, pregnancy and live births were reported using sperm retrieved from the testes (2) and epididymis (3). The technique of testicular sperm extraction was first introduced in 1993 (2,4) to retrieve sperm from patients with obstructive azoospermia (OA). Pregnancies resulting from the testicular sperm of obstructive azoospermic patients were followed by reports of successful outcomes using the testicular sperm from patients with non-obstructive azoospermia (NOA) (5-11). Nowadays, ICSI using frozen testicular (12) and epididymal (13) sperm has become an effective and standard approach to treating infertility secondary to obstructive and non-obstructive azoospermia.

The cryopreservation of mammalian sperm has been practiced for decades. In fact, ejaculated spermatozoa were the first successfully cryopreserved human cells (14). The use of cryopreserved ejaculated sperm in intrauterine insemination and IVF is a standard practice. The Food and Drug Administration enforces a quarantine period before sperm from anonymous donors can be used, thus requiring the cryopreservation and storage of donor sperm for at least six months. Generally, the large number of sperm (usually in the millions) in semen makes the cryopreservation of ejaculated sperm easy and feasible, as even after a loss in viability following the thawing of sperm, enough live and



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motile sperm are available for insemination or IVF. In comparison, the cryopreservation of surgically retrieved testicular sperm is more cumbersome and difficult because of the low number (total count) of sperm retrieved and their lack of motility. In addition, the testicular sperm preparations are almost always contaminated with a high proportion of cellular debris and blood cells. The basic principles and methods of sperm cryopreservation for testicular, epididymal, and ejaculated sperm are, however, similar and involve the use of glycerol as a cryoprotectant. The cryopreservation of testicular and epididymal sperm has now become a standard technique in the management of male factor infertility.

## REASONS FOR AND ADVANTAGES OF THE CRYOPRESERVATION OF TESTICULAR AND EPIDIDYMAL SPERM

The complete absence of sperm in the ejaculate following two-three days of abstinence on at least two occasions is the standard used to confirm the diagnosis of azoospermia. This diagnosis is the primary reason for attempting sperm retrieval from the testis for use in IVF-ICSI. Azoospermia can be caused by epididymal pathology or an obstruction in the reproductive tract at the post-testicular/epididymal locus in men who have otherwise normal spermatogenesis. Obstructive azoospermia (OA) also occurs in men with vasectomy or pathological blockage of the vas deferens. Mutations in the cystic fibrosis trans-membrane conductance regulator (*CFTR*) gene are a relatively frequent cause of the congenital bilateral absence of the vas deferens (CBAVD) leading to azoospermia.

In men with OA, the probability of acquiring sperm from testes is almost guaranteed, and the surgical retrieval of sperm can be scheduled at the convenience of the patient, urologist, and laboratory personnel. Thus, sperm can be cryopreserved in advance of the oocyte retrieval from the female partner. This flexibility not only avoids having to plan the testicular biopsy surgery to coincide with the egg retrieval but also avoids both partners having to undergo surgical procedures at approximately the same time. On rare occasions, men with an infertility diagnosis of ductal obstruction may present with a failure of spermatogenesis, and a fresh biopsy on the day of oocyte retrieval with no available sperm may cause undue psychological stress and a financial burden for the couple.

The cryopreservation of spermatozoa in multiple vials/aliquots confers the advantage of enabling multiple IVF-ICSI attempts without the male partner undergoing surgery for each attempt. Multiple testicular biopsies or the removal of an excessive amount of tissue can cause irreversible damage and in some cases may result in testicular atrophy (15). Patients who have had a vasectomy for personal reasons but wish to undergo vas reconstruction surgery to regain fertility are also candidates for the cryopreservation of testicular sperm. Testicular sperm can be retrieved and cryopreserved at the time of vas re-anastomosis. This strategy ensures the availability of sperm if the reconstruction fails (probability of 20-25%) (16) and avoids a repeat surgical sperm retrieval. Patients undergoing exploratory surgery to assess the cause of azoospermia should also be counseled to freeze testicular sperm at this time if possible, thus avoiding another surgical intervention.

With the advances in the field of cancer treatment, the survival rate for a variety of malignant diseases in recent years has significantly improved. A significant proportion of boys diagnosed with some form of childhood (pre-pubertal) cancer are cured and able to maintain a normal adult life. In male children (10-13 years of age) diagnosed with some form of malignancy, spermatogenesis may not be fully established, and ejaculated spermatozoa may not be available for banking. However, in such patients, isolated portions of the seminiferous tubules may contain enough sperm for cryopreservation and ICSI. Even in younger males in whom spermatogenesis has not yet been initiated, the cryopreservation of testicular tissue before starting chemo- or radiotherapy offers fertility potential in the future (17).

In men with non-obstructive azoospermia (NOA), obtaining enough sperm for cryopreservation may pose a challenge. Depending on the etiology and severity of the underlying condition, none to a fair number of testicular sperm can be retrieved. In 10-15% of patients diagnosed with NOA, the condition is attributed to micro-deletions in the long arm of the Y chromosome (AZF region). The micro-deletion of two genes, USP9Y and DDX3Y, in the AZFa region invariably results in the appearance of Sertoli cell-only syndrome and the complete absence of spermatogenesis in the seminiferous tubules. Depending on the extent and size of the deletions in the AZFb region, complete absence of spermatogenesis or focal hypo-spermatogenesis in isolated seminiferous tubules can occur. Deletions in the distal AZFc region produce a wide array of aberrations in spermatogenesis, often resulting in mild to severe hypo-spermatogenesis and leading to severe oligospermia or even ejaculatory azoospermia. Other causes of NOA may be attributed to cryptorchidism, spermatic cord torsion, testicular or inguinal surgery/infection/trauma, history of radiation and chemical exposure, endocrine disruption, or, in some cases, the use of medications associated with the impairment of sperm production. The probability of recovering sperm obviously depends on the extent and severity of each case.

In patients with NOA, it is difficult to predict a successful retrieval of testicular sperm, as no definitive markers of spermatogenesis have been described. Testicular measurements (e.g., size, volume, and plasma FSH concentrations) do not accurately predict the success of testicular biopsy in obtaining enough sperm for ICSI. The treatment of the female partner of NOA patients with gonadotropins in anticipation of oocyte retrieval and ICSI may be unnecessary in up to 50% of patients, as it is possible that no sperm may be available following the testicular biopsy. An exploratory testicular biopsy surgery with ''possible testicular sperm freeze'' is a valid option and should be offered to the couple. If there is evidence of rare sporadic hypo-spermatogenesis in the seminiferous tubules, the few spermatozoa that are retrieved may be frozen at this time. If it is doubtful whether enough spermatozoa will be available for ICSI post-thaw, the couple may be advised to undergo a fresh biopsy on the day before or on the morning of oocyte retrieval. Patients with NOA often have decreased testicular volumes, and multiple biopsies with the excision of excessive testicular parenchyma carry the risk of irreversible damage and atrophy. Couples hesitant to undergo a repeat biopsy may be offered donor sperm as a back-up in case the number of mature oocytes exceeds the number of available viable testicular sperm for ICSI. This strategy offers the patient and the healthcare provider the advantage of knowing if there

are sperm available and if alternate arrangements may be needed. Because the number of sperm retrieved for cryopreservation in NOA patients may be extremely low, the loss of a few sperm during the freeze/thaw cycle can be significant and is a very real disadvantage.

## SURGICAL TECHNIQUES FOR SPERM RETRIEVAL

In patients with documented OA, sperm can be surgically retrieved from the testis or epididymis. In comparison, men with NOA are restricted to testicular surgery for the retrieval of male gametes. A variety of surgical approaches, ranging from percutaneous aspiration to open biopsy, have been successfully employed for sperm retrieval. Although enough sperm for IVF-ICSI in a fresh cycle may be retrieved by a particular surgical technique (such as needle aspiration), if the intent is to cryopreserve multiple aliquots for future use, techniques that yield higher numbers of sperm, such as open biopsy and micro-TESE, may be more suitable options.

### Retrieval of testicular sperm

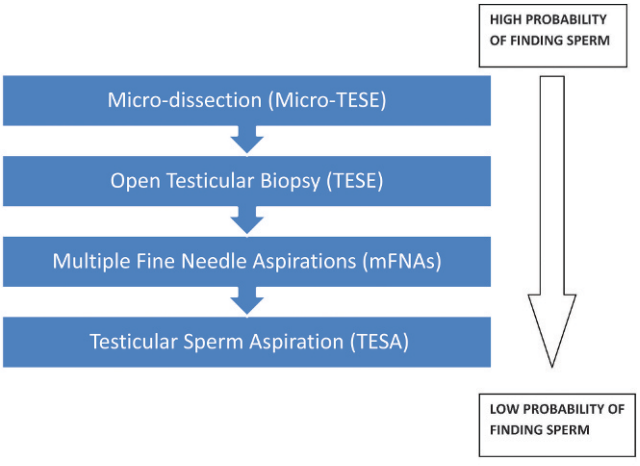
**Open testicular biopsy.** This conventional method of surgical sperm extraction generally offers the best chance of retrieving spermatozoa, irrespective of the etiology of azoospermia. Open biopsy also allows the excision of a larger tissue mass, allowing access to a greater number of sperm available for freezing. The major drawback of open biopsy, from the point of view of the patient, is the size of the wound and the healing time compared with other forms of aspiration, namely needle aspiration. In men with NOA, open testicular biopsy is more effective than testicular sperm aspiration (TESA) or multiple fine needle aspirations (FNAs). Patients with focal spermatogenesis or hypospermatogenesis are also best served with open biopsy or, in some cases, with micro-TESE (or micro-dissection TESE). Micro-TESE is a more invasive surgical procedure involving a thorough examination under an operating microscope of the bifurcated gonad to locate and excise the seminiferous tubules exhibiting active spermatogenesis. The sperm retrieval rate by micro-TESE has been reported as superior to that of conventional TESE (18-20), especially in patients with focal and sporadic spermatogenesis. The excision of isolated seminiferous tubules with active spermatogenesis in micro-dissection TESE results in the removal of significantly less tissue (average of 9.4 mg) compared with a standard biopsy (720 mg) (18). The advantages of micro-TESE in addition to the superior sperm retrieval rate include the avoidance of complications such as hematoma, fibrosis, and impaired androgen production (18).

**Testicular sperm aspiration (TESA).** Testicular sperm aspiration or percutaneous testicular sperm aspiration is a technique originally described as a diagnostic procedure used to evaluate spermatogenesis in azoospermic patients (21). In this technique, a 19- or 21-gauge needle attached to a 20-ml syringe is used to percutaneously puncture the testis, and negative pressure is applied to aspirate the tubules. Fragments of the seminiferous tubules are analyzed for the presence of sperm. Since the development of ICSI, in which a single viable sperm per mature oocyte is needed for fertilization, the availability of a few spermatozoa has been enough to offer a chance of pregnancy. In men with OA, testicular sperm aspiration invariably generates enough sperm for IVF-ICSI. In general, the number of sperm obtained by open biopsy (TESE) is significantly higher than that obtained by needle aspiration. A variation of TESA, testicular fine needle aspiration (TEFNA, FNA), involves the aspiration of tubules from multiple sites in the gonad. Multiple testicular fine needle aspirations have been attempted in men with NOA with varying success. In men with NOA, the success of sperm retrieval by FNA depends on the etiology and severity of the underlying cause of the azoospermia. In a prospective study comparing the efficacy of multiple needle biopsies with open testicular excision, spermatozoa were retrieved in only 14% of the patients with non-obstructive azoospermia by multiple needle aspirations and in 63% of patients following open excision biopsy (22). The probability of retrieving testicular sperm in NOA by various surgical techniques is presented schematically in Figure 1.

### Retrieval of epididymal sperm

**Percutaneous epididymal sperm aspiration (PESA).** PESA involves the insertion of a 23-gauge butterfly needle through the scrotal skin into the epididymis. Negative suction pressure is applied using a 10-ml syringe containing a small volume of culture medium. The epididymal fluid mixed with culture medium is then examined for the presence of motile sperm. The procedure may involve multiple punctures at different locations in the epididymis until live sperm are found. PESA may be a good option for patients with ductal obstruction distal to the epididymis.

**Microsurgical epididymal sperm aspiration (MESA).** The technique of MESA involves the surgical exposure of the epididymis and the aspiration of the effluent from the epididymal tubules under optical magnification. It is the technique of choice for some surgeons to surgically retrieve sperm because of the high concentration and quality of spermatozoa obtained compared with that of testicular sperm, especially in patients with irreparable epididymal obstruction. Epididymal spermatozoa are mature and progressively motile, and epididymal aspirates are much cleaner and devoid of the cellular debris that is seen in testicular sperm preparations. The motility of epididymal sperm makes sperm selection during ICSI easier without the introduction of additional steps (such as treatment with




Micro-dissection (micro-TESE) > Open testicular biopsy (TESE) > Multiple fine needle aspirations > Single testicular sperm aspiration (TESA)

Figure 1 - Probability of retrieving sperm in NOA by various surgical procedures (high to low).



motility-enhancing agents) that may be needed to identify viable gametes if testicular sperm are used. In a study comparing the two techniques of sperm retrieval from the epididymis (PESA and MESA), sperm were successfully retrieved by PESA in 61% of patients with obstructive azoospermia, compared with a 93% sperm retrieval rate by MESA (23).

## CRYOPRESERVATION OF TESTICULAR SPERM

### I. Materials and Equipment

Sterile culture dishes
Polystyrene conical tubes
Sterile glass pipettes
Syringes (50 ml)
Syringes (3 ml) with 21-gauge needles
Syringe filter (Nalgene, 0.22 μm)
Microscope glass slides
Coverslips
Gloves
Sterile pair of curved iris scissors
Sterile pair of forceps
Bench-top clinical centrifuge
Microscope
Refrigerator
Weighing chemical balance
Cryo vials
Aluminum canes
Plastic cryosleeves
Liquid nitrogen dewar
Liquid nitrogen
Personal protective gear for handling liquid nitrogen

### II. Reagents

Quinn's Sperm Washing Medium (modified human tubal fluid with human serum albumin, 5 mg/ml; SAGE IVF Inc., Trumbull, CT, USA).

Sperm Freezing Medium (20% TEST Yolk buffer, 12% glycerol with gentamycin sulfate; Irvine Scientific, Santa Ana, CA, USA).

Tissue culture-grade water (SAGE IVF Inc., Trumbull, CT, USA).

RBC Lysis Buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, 2 mM EDTA; pH 7.2).

### Preparation of RBC lysis buffer

| | |
|---|---|
| Ammonium chloride ($NH_4Cl$) | 0.829 g |
| Potassium bicarbonate ($NaHCO_3$) | 0.100 g |
| Ethylenediaminetetraacetic acid (EDTA) | 0.074 g |
| Tissue culture-grade water | 100 ml |

Dissolve and adjust the pH to 7.2. Sterilize the buffer using a 0.2-μm syringe filter and store in the refrigerator at 4℃ for up to four weeks.

### Procedure

(The procedure can be performed at room temperature)

1. Rinse the tissue in sperm-washing medium to remove the blood and transfer it to a sterile petri dish.
2. Using a pair of sterile curved iris scissors, mince the tissue well. Keep the tissue moist with the sperm-washing medium during mincing. Add 1.0 ml of the sperm-washing medium to the finely minced tissue. Tilt the dish, aspirate the medium, and transfer the suspension to a conical tube (A) while leaving the larger tissue fragments in the dish.
3. Add 2.0 ml of the sperm-washing medium to the petri dish. Using a 3-cc syringe attached to a 21-gauge needle, gently aspirate the tissue suspension repeatedly. The repeated aspiration through the syringe needle dissociates the seminiferous tubules and releases the sperm from the tubular lumen.
4. Transfer the suspension to the same conical tube (A) used in step 2 and let it stand at room temperature for 5-10 minutes. The large tissue fragments tend to settle at the bottom. Transfer the supernatant to a new conical tube (B).
5. Add 1-2 ml of the sperm-washing medium to the tissue pellet and mix well with a Pasteur pipet.
6. Allow the tissue fragments to settle for 5 minutes and transfer the supernatant to the conical tube (B).
7. Centrifuge the conical tube (B) at 400 g for 10 minutes.
8. Gently aspirate and discard the supernatant, leaving the pellet at the bottom.
9. Add 3-4 ml of RBC Lysis Buffer to the pellet and mix with a Pasteur pipet.
10. Centrifuge at 400 *g* for 5 minutes.
11. Gently aspirate the supernatant and discard it without disturbing the pellet.
12. Re-suspend the pellet in 3.0 ml of the sperm-washing medium and centrifuge the conical tube at 400 g for 10 minutes to wash off the RBC Lysis Buffer.
13. Discard the supernatant and resuspend the pellet in 0.5-1.0 ml of the sperm-washing medium.
14. Add an equal volume of TEST-Yolk sperm cryopreservation medium to the sperm suspension and aliquot the mixture into 3-4 labeled cryovials.
15. Cool the cryovials to 4℃ (in the refrigerator) for 30-45 minutes.
16. Expose the vials to the liquid nitrogen vapor phase (8-10 inches above the liquid nitrogen level) for an hour.
17. Plunge the frozen vials into liquid nitrogen for storage.

## CRYOPRESERVATION OF EPIDIDYMAL SPERM

1. Once the presence of sperm is documented by microscopic examination of the epididymal aspirate, the fluid is transferred to a conical tube.
2. Add 1-2 ml of the sperm-washing medium to the conical tube containing the epididymal fluid. Mix gently.
3. Centrifuge the conical tube at 400 g for 10 minutes.
4. Discard the supernatant. If the pellet appears to be contaminated with erythrocytes, blood cells may be removed by washing the pellet with RBC Lysis Buffer as follows.
5. Re-suspend the pellet in 2 ml of RBC Lysis Buffer. Mix gently.
6. Centrifuge the conical tube at 400 g for 5 minutes.
7. Gently aspirate the supernatant and discard without disturbing the sperm pellet.
8. Wash the pellet with 1-2 ml of the sperm-washing medium by centrifuging the tube at 400 g for 10 minutes.
9. Re-suspend the pellet in 1.0 ml of the sperm-washing medium.

10. Add an equal volume (1.0 ml) of TEST-Yolk sperm cryopreservation medium to the epididymal sperm suspension.
11. Aliquot the mixture into 3-4 labeled cryovials for cryostorage.
12. Place the cryovials in a 4℃ refrigerator for 30-45 minutes and then freeze the cryovials in liquid nitrogen vapor for 1 hour.
13. Plunge the vapor-frozen vials into liquid nitrogen for storage.

## PROCEDURE FOR THAWING THE TESTICULAR AND EPIDIDYMAL SPERM

Note: Use proper safety precautions and personal protective gear while handling liquid nitrogen.

1. Before thawing, locate, identify, and confirm the patient information on the vial.
2. Take the vial out of the liquid nitrogen and thaw at room temperature for 10 minutes.
3. Transfer the contents of the vial into a conical tube. Add 1-2 ml of the sperm-washing medium slowly, drop by drop, to the thawed suspension. Mix the contents gently.
4. Centrifuge the tube at 400 g for 10 minutes.
5. Gently aspirate the supernatant and discard it without disturbing the sperm pellet.
6. Re-suspend the pellet in 1-2 ml of the sperm-washing medium and centrifuge the tube again at 400 g for 10 minutes.
7. Gently remove the supernatant. Re-suspend the sperm pellet in 50-100 µl of the sperm-washing medium.
8. The sperm suspension is ready for ICSI.

## METHODS FOR THE CRYOPRESERVATION OF SINGLE (OR FEW) SPERM

The procedure for the cryopreservation of testicular and epididymal sperm as described in detail in the preceding section is a standard technique when several hundred to thousands or a million sperm are available for preservation. For some patients with NOA, the retrieval of only a few (less than 100, and sometimes as few as 10 or less) sperm poses a technical challenge for cryopreservation and, later, at the time of warming for the successful retrieval of these few gametes for ICSI. Several investigators have attempted to cryopreserve extremely low number of sperm (and sometimes a single sperm) in small volumes using various carriers (Table 1). The two methods that have resulted in successful pregnancies and live births involve the use of empty zona (human, mouse, or hamster) and cryoloops. These methods are briefly summarized below.

### Cryopreservation of a single human sperm using a zona pellucida

Cohen and colleagues (24) were the first to report this unique method of the cryopreservation of a single spermatozoon inside empty zona obtained from mouse, hamster, or human oocytes. The oocytes were treated with hyaluronidase to remove the cumulus and corona cells. The denuded oocyte was held by a holding pipette, and two small holes were drilled into the zona (by mechanical breach, acid tyrodes solutions, or a laser). The ooplasm was aspirated by suction, leaving the zona empty of its contents. The testicular suspension was carefully examined under the microscope for the presence of spermatozoa, and once a twitching/moving sperm was found, it was transferred to a PVP (10%) droplet. This procedure was repeated until a few sperm had been retrieved. The next step involved the insertion of one or more (up to 15) sperm into empty zona using an ICSI needle. Injected zonae containing sperm were placed into an 8% glycerol solution in phosphate-buffered saline (PBS) containing human serum albumin (3%). The zonae were then frozen individually in sterile straws (0.25 ml). Each zona was placed in a column of cryopreservation medium sandwiched between two air bubbles. The straws were heat-sealed and exposed to liquid nitrogen vapor for two hours, followed by storage in liquid nitrogen.

The spermatozoa were recovered by thawing the straw in a water bath (30℃) for 30 seconds. One end of the straw was cut with a pair of sterile scissors, and the content of the

**Table 1** - Carriers used for sperm cryopreservation in microquantities.

| Carrier | Advantages | Disadvantages | Comments | References |
|---|---|---|---|---|
| Empty zona (mouse, hamster, or human) | Easy to handle the zona envelope; good sperm recovery and survival | Human or non-human biological material; Labor-intensive; Requires micromanipulation and in-house evacuation and preparation of the zona envelope | Successful pregnancy | 24,25,45,46 |
| Cryoloop | Commercially available | Requires a micromanipulator to load sperm onto the loop and is somewhat labor-intensive | Successful pregnancy | 27,28,47 |
| Mini straws or open-pulled straws | Easy and simple technique | Not feasible for an extremely low volume/number of sperm | Can be used to freeze severely oligospermic samples | 48 |
| ICSI pipette | Commercially available; found in every IVF lab | Fragile glass pipette; difficult to store and handle in liquid nitrogen | No reported pregnancies | 49 |
| Microdroplets | Easy and relatively simple technique | Difficult to handle and store in liquid nitrogen; Culture dishes are fragile when stored in liquid nitrogen; Variable recovery rates | Has yielded successful pregnancies; has not received widespread acceptance | 50-52 |
| Volvox globator algae | Inexpensive; plentifully available | Non-human biological material; labor-intensive advance preparation of algae spheres | Not suitable for human clinical use | 53 |
| Alginate beads or agarose microspheres | Inert polymers used as carriers | Very labor-intensive technique | No report of clinical pregnancies | 54,55 |
| Cryotop | Commercially available, easy to load and handle | - | Sperm recovery, survival similar to that obtained using the empty zona method | 56 |



**Table 2** - Outcomes using testicular sperm in patients with obstructive azoospermia.

| Testicular Sperm | Fertilization (%) | Embryonic Cleavage (%) | Implantation (%) | Clinical Pregnancies (%) | Ongoing/Live births (%) | References |
|---|---|---|---|---|---|---|
| Fresh | 52 | 99 | - | 0 | 0 | 12 |
| Frozen-thawed | 51 | 96 | - | 6 | 6 | |
| Fresh | 58 | 98 | 33 | 33 | - | 57 |
| Frozen-thawed | 64 | 95 | 14 | 32 | - | |
| Fresh | 64 | 99 | - | 30 | 20 | 58 |
| Fresh | 62 | 93 | - | 25 | 20 | |
| Fresh | - | - | - | - | 23 | 59 |
| Frozen-thawed | - | - | - | - | - | |
| Fresh | 50 | - | 18 | 26 | 22 | 60 |
| Frozen-thawed | 49 | - | 14 | 29 | 19 | |
| Fresh | 72 | - | 33 | 69 | 44 | 31 |
| Frozen-thawed | 68 | - | 17 | 42 | 25 | |
| Fresh | 52 | 106* | 14 | 24 | 15 | 29 |
| Frozen-thawed | - | - | - | - | - | |

*>100% because embryos that did not show normal signs of fertilization (2 pronuclei) but that did show cleavage are included.

straw was expelled into a sterile dish. The zona was recovered and washed several times in HEPES-buffered medium. The zona containing the sperm was then transferred to a droplet of PVP (12%). Using a holding pipette, the zona envelope was positioned to permit the penetration of the ICSI needle through the slit (used earlier to evacuate the ooplasm). The ICSI needle was inserted into the zona, and the sperm was aspirated gently and released into the PVP droplet. The sperm was immobilized and injected into the metaphase II oocyte, held in a drop of HEPES-buffered medium. This technique of the cryopreservation of individual spermatozoa inside an empty zona, although quite labor-intensive and time-consuming, offers an opportunity to retrieve and store sperm in extreme cases of male factor infertility. Successful pregnancies and live births have been reported with this method (25).

### Cryopreservation of sperm using the cryoloop method

The use of a cryoloop as a carrier to contain the frozen embryos was initially proposed by Lane and colleagues (26). The premise of holding the embryo on a thin film of cryoprotectant solution on a small (0.5-0.7 mm) loop has the advantage of enabling the handling of a very small volume (2 µl) with excellent recovery rates. The use of cryoloops was thereafter extended to freeze a very small number of sperm (27). The method of cryopreservation on cryoloops was refined to enable the freezing of individual sperm by loading the sperm onto the cryoloop by a micromanipulation technique (28). The procedure of single sperm cryopreservation involves holding the cryoloop on a magnetic wand. The magnetic wand is attached to the micromanipulator on an inverted microscope and is lowered to visualize the loop. The spermatozoa are isolated using a micropipette (e.g., ICSI pipet) and concentrated onto a microdrop of HEPES-buffered medium, supplemented with 1% human serum albumin. Just before loading the sperm onto the cryoloop, the individual sperm is transferred to another microdrop of a 1:1 mixture of HEPES medium and sperm cryopreservation medium (TEST-Yolk buffer containing 12% glycerol; Irvine scientific, Santa Ana, CA). Using a micropipette, sperm are then loaded onto the film in the loop. The cryoloop attached to the cap is screwed onto the cryovial, and the vial is exposed to liquid nitrogen vapor for up to 30 minutes before being plunged into liquid nitrogen for storage.

Sperm frozen on the cryoloop are thawed at room temperature using a dissecting binocular microscope. The cryoloop attached to the cap is unscrewed under liquid nitrogen and is swiftly shifted under the binoculars over a sterile culture dish. The loop is held so that it touches the surface of the dish, and it is cut with a scalpel from the metal shaft. Immediately, 2 µl of HEPES-buffered medium supplemented with albumin is laid on top of the loop, and the droplet is overlaid with mineral oil. Spermatozoa may be individually moved to a fresh drop of medium, washed, and then transferred to PVP before ICSI.

The cryoloop method for the cryopreservation of a single spermatozoon is also labor-intensive and time-consuming and requires extensive training and experience. The cryopreservation of a single sperm, although it is feasible and has had documented success, is rarely practiced, and alternative options, such as fresh biopsy with donor sperm back-up, are more prevalent.

## OUTCOME

### Obstructive azoospermia

The use of testicular and epididymal sperm in the treatment of male factor infertility secondary to OA has now become a standard approach. The probability of retrieving sperm in men with OA is close to 100%. In men with OA, the use of fresh testicular sperm for ICSI invariably offers fertilization, pregnancy, and live birth rates that are comparable to those derived from the use of ejaculated sperm in age-matched controls. The use of frozen testicular sperm from OA patients for ICSI offers fertilization, implantation, and clinical pregnancy rates that are equivalent to those derived from the use of freshly retrieved testicular sperm (Table 2). Fertilization and clinical pregnancy rates are also similar between spermatozoa of epididymal or testicular origin (29). MESA is the preferred method of sperm recovery if the obstruction is determined to be at a location distal to the epididymis. The use of either fresh or frozen epididymal sperm offers comparable fertilization, embryonic development, implantation, and clinical pregnancy rates (30). Fertilization and pregnancy rates across different sperm retrieval methods and obstruction etiologies are also comparable (23). The number of

high-quality embryos obtained using frozen testicular sperm is similar to that obtained using fresh testicular sperm (31). Despite the high pregnancy rates achieved using fresh and frozen surgically retrieved sperm, there is some concern that the use of testicular sperm results in higher miscarriage rates compared with the use of ejaculated sperm (31-33). The rate of aneuploidy in testicular and epididymal spermatozoa in OA patients is similar to that observed in sperm from normal men (34), suggesting that the higher incidence of early miscarriages cannot be attributed to the paternal genome. Testicular sperm are generally immature but undergo maturation during their passage through the epididymis. Immature testicular sperm are easily recognizable by the presence of large cytoplasmic droplets attached to their middle pieces and necks. It has been suggested that reactive oxygen species in cytoplasmic droplets may cause irreversible DNA damage (35) in immature testicular sperm, resulting in a higher miscarriage rate (31).

## Non-obstructive azoospermia

The probability of sperm retrieval in NOA is dependent on two factors, namely the etiology of NOA and the surgical approach. In general, microdissection TESE or open biopsy is more successful than needle aspiration. In approximately 13% of men diagnosed with NOA, the failure of spermatogenesis may be attributed to Y chromosome microdeletions. The probability of retrieving spermatozoa in men who have microdeletions in the AZFa and/or AZFb regions is close to zero, whereas men with AZFc microdeletions have an approximately 70% chance of having enough sperm available for ICSI (36). Although the reports detailing the rates of testicular sperm retrieval in men with NOA range from 14-87% (Table 3), a conservative estimate of an overall 50-60% chance of successful sperm retrieval is appropriate. In general, the total number of sperm retrieved in NOA is significantly less than that obtained in OA. The cryopreservation of sperm in general results in a decrease in post-thaw motility and vitality. This loss of viability can be of great concern in NOA because of the low number of sperm available; however, unique approaches to the freezing of single/individual sperm now offer excellent post-thaw recoveries.

It is well documented that spermatozoa in men with oligoasthenoteratozoospermia exhibit an increased incidence of chromosomal abnormalities (37-40). Interestingly, a comparison of aneuploidy frequency between embryos derived from testicular sperm from men with OA and from

**Table 3 -** Rate of testicular sperm retrieval in men with non-obstructive azoospermia.

| Sperm Retrieval Technique | Rate of Sperm Retrieval | Comment | References |
|---|---|---|---|
| TESE | 13/15 (87%) | | 5 |
| Micro-TESE | 17/27 (63%) | | 9 |
| TESE | 15/25 (60%) | | |
| TESE | 14/18 (78%) | | 61 |
| TESE | 22/35 (63%) | Compared TESE and Multiple FNAs | 22 |
| Multiple FNAs | 5/35 (14%) | | |
| TESE | 33/55 (60%) | | 43 |
| TESE | 43/64 (67%) | | 62 |
| TESE | 15/42 (36%) | | 63 |
| TESA | 22/86 (26%) | | 64 |
| TESE | 5/48 (10%) | 48 patients with failed TESA underwent TESE | |
| TESE | 10/22 (45%) | Compared TESE and Micro-TESE | 18 |
| TESE | 10/17 (59%) | | 65 |
| TESE | 18/31 (58%) | | 66 |
| Multiple TESE | 13/37 (35%) | Compared multiple TESE and Micro TESE | 67 |
| Micro-TESE | 24/56 (43%) | | 67 |
| FNA | 35/51 (69%) | 2 patients who had no sperm retrieved during the first FNA attempt had a positive sperm retrieval during the second FNA attempt | 68 |
| TESE | 5/12 (42%) | Post-chemotherapy azoospermia | 69 |
| TESE | 23/30 (77%) | | 31 |
| TESE | 261/628 (42%) | First attempt | 70 |
| If no sperm retrieved, patients had multiple repeat biopsies | 77/103 (74%) | Second attempt | |
| | 28/34 (82%) | Third attempt | |
| | 11/11 (100%) | Fourth attempt | |
| | 5/6 (83%) | Fifth Attempt | |
| | 2/2 (100%) | Sixth attempt | |
| | 384/784 (49%) | Overall | |
| Micro-TESE | 57% | Compared TESE and Micro-TESE | 71 |
| TESE | 32% | | |
| TESE/Micro-TESE | 65/138 (47%) | | 72 |
| TESE | 87/258 (34%) | Compared TESE and Micro-TESE | 73 |
| Micro-TESE | 16/77 (21%) | | |
| TESE+Micro-TESE | 131/258 (51%) | | |
| Micro-TESE | 37/65 (57%) | Compared TESE and Micro-TESE | 20 |
| TESE | 26/68 (38%) | | 20 |
| Micro-TESE | 27/73 (37%) | Post-chemotherapy azoospermia | 74 |



**Table 4** - Outcome using testicular sperm in patients with non-obstructive azoospermia.

| Testicular Sperm | Fertilization (%) | Embryonic Cleavage (%) | Implantation (%) | Clinical Pregnancies (%) | Ongoing pregnancies/ Live births (%) | References |
|---|---|---|---|---|---|---|
| Fresh | 48 | 78 | 19 | - | 20 | 5 |
| Frozen-Thawed | - | - | - | - | - | |
| Fresh | 39 | 68 | 25 | 60 | | 9 |
| Frozen-Thawed | | | | | | |
| Fresh | 47 | 93 | 9 | 26 | 22 | 61 |
| Frozen-thawed | 44 | 89 | 11 | 27 | 9 | |
| Fresh | 66 | - | 13 | 30 | - | 62 |
| Frozen-Thawed | 58 | - | 18 | 50 | - | |
| Fresh | 54 | 97 | 13 | 27 | 20 | 43 |
| Frozen-thawed | 51 | 97 | 9 | 22 | 13 | |
| Fresh | 52 | 75 | 13 | 33 | - | 57 |
| Frozen-Thawed | 56 | 94 | 24 | 67 | - | |
| Fresh | 58 | 93 | - | 32 | - | 66* |
| Frozen-thawed | 55 | 93 | - | 29 | - | |
| Fresh | 67 | 93 | - | 29 | 18 | 58 |
| Frozen-Thawed | - | - | - | - | - | |
| Fresh | 68 | 85 | 7 | 13 | 13 | 70 |
| Frozen-Thawed | - | - | - | - | - | |
| Fresh | - | - | - | - | - | 44 |
| Frozen-thawed | 58 | 93 | 11.3 | 25 | 22 | |
| Fresh | 52-60 | - | - | 40-52 | 31-39 | 74 |
| Frozen-Thawed | - | - | - | - | - | |
| Fresh | 64 | - | - | 8 | 7 | 75 |
| Frozen-thawed | - | - | - | - | - | |

*Data represent a combination of OA (10 patients) and NOA (18 patients).

those with NOA revealed no difference between the two groups (41), suggesting the chromosomal normalcy of the testicular sperm. However, a study comparing surgically retrieved versus ejaculated sperm showed a significantly higher incidence of chromosomal abnormalities in surgically retrieved sperm from men with OA and NOA compared with normospermic ejaculated sperm (42). Despite differences in the study designs that make any comparison of outcome results between OA and NOA difficult, it appears that if sperm are retrieved and viable gametes are available for ICSI, the fertilization, implantation, and pregnancy rates resulting from the use of fresh sperm from men with NOA (Table 4) are in line with those resulting from the use of sperm from men with OA (Table 3). Therefore, the cryopreservation of sperm does not affect the fertilization and pregnancy outcomes.

## CONCLUSION

Successful pregnancies resulting from the use of surgically retrieved spermatozoa for ICSI (2) were first reported in 1993. Various surgical techniques, ranging from less invasive percutaneous aspiration to highly invasive open testicular biopsy and micro-dissection TESE, are employed. In OA, the probability of obtaining sperm from the testis is almost guaranteed. In men with non-obstructive azoospermia, freshly retrieved sperm offers the best chance of pregnancy; however, in 30-50% of NOA patients, it is still possible that no sperm may be retrieved (43). In patients with frozen sperm, sometimes no viable sperm may be available at the time of ICSI. The incidence of complete non-viability or the inability to find any injectable sperm post-thaw in NOA patients is reported to be approximately 20% (44). The availability of viable sperm, regardless of the source (testis or epididymis), at the time of ICSI largely dictates the outcome of fertilization. Frozen (testicular or epididymal) sperm are as effective as freshly retrieved sperm. In NOA patients with severe hypospermatogenesis or focal spermatogenesis, where very few sperm are retrieved and the cryopreservation of individual (few) sperm using specialized methods (such as the empty zona or cryoloop method) is not feasible, a fresh biopsy should be offered. The injection of sperm using non-motile spermatozoa results in a significantly lower fertilization and live birth rate, emphasizing the importance of the motility/viability of sperm (43). Currently, there are no definitive parameters, besides surgical testicular exploration, that are reliable in predicting the presence of sperm in men with NOA.

## REFERENCES

1. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;340(8810):17-8, http://dx.doi.org/10.1016/0140-6736(92)92425-F.
2. Schoysman R, Vanderzwalmen P, Nijs M, Segal-Bertin G, van de Casseye M. Successful fertilization by testicular spermatozoa in an in-vitro fertilization programme. Hum Reprod. 1993;8(8):1339-40.
3. Tournaye H, Devroey P, Liu J, Nagy Z, Lissens W, Van Steirteghem A. Microsurgical epididymal sperm aspiration and intracytoplasmic sperm injection: a new effective approach to infertility as a result of congenital bilateral absence of the vas deferens. Fertil Steril. 1994;61(6):1045-51.
4. Craft I, Bennett V, Nicholson N. Fertilizing Ability of Testicular Spermatozoa. Lancet. 1993;342(8875):864, http://dx.doi.org/10.1016/0140-6736(93)92722-6.
5. Devroey P, Liu J, Nagy Z, Goossens A, Tournaye H, Camus M, et al. Pregnancies after Testicular Sperm Extraction and Intracytoplasmic Sperm Injection in Nonobstructive Azoospermia. Hum Reprod. 1995;10(6):1457-60, http://dx.doi.org/10.1093/HUMREP/10.6.1457.
6. Silber SJ, Nagy Z, Liu J, Tournaye H, Lissens W, Ferec C, et al. The Use of Epididymal and Testicular Spermatozoa for Intracytoplasmic Sperm Injection - the Genetic-Implications for Male-Infertility. Hum Reprod. 1995;10(8):2031-43.
7. Silber SJ, Vansteirteghem AC, Devroey P. Sertoli-Cell Only Revisited. Human Reproduction. 1995;10(5):1031-2.
8. Silber SJ, Van Steirteghem AC, Liu J, Nagy Z, Tournaye H, Devroey P. High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. Hum Reprod. 1995;10(1):148-52, http://dx.doi.org/10.1093/humrep/10.1.148.

9. Silber SJ, van Steirteghem A, Nagy Z, Liu J, Tournaye H, Devroey P. Normal pregnancies resulting from testicular sperm extraction and intracytoplasmic sperm injection for azoospermia due to maturation arrest. Fertil Steril. 1996;66(1):110-7.
10. Gil-Salom M, Remohi J, Minguez Y, Rubio C, Pellicer A. Pregnancy in an azoospermic patient with markedly elevated serum follicle-stimulating hormone levels. Fertil Steril. 1995;64(6):1218-20.
11. Mansour RT, Aboulghar MA, Serour GI, Fahmi I, Ramzy AM, Amin Y. Intracytoplasmic sperm injection using microsurgically retrieved epididymal and testicular sperm. Fertil Steril. 1996;65(3):566-72.
12. Gil-Salom M, Romero J, Minguez Y, Rubio C, De los Santos MJ, Remohi J, et al. Pregnancies after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Hum Reprod. 1996;11(6):1309-13, http://dx.doi.org/10.1093/oxfordjournals.humrep.a019377.
13. Nagy Z, Liu J, Cecile J, Silber S, Devroey P, Vansteirteghem A. Using Ejaculated, Fresh, and Frozen-Thawed Epididymal and Testicular Spermatozoa Gives Rise to Comparable Results after Intracytoplasmic Sperm Injection. Fertil Steril. 1995;63(4):808-15.
14. Smith AU, Polge C. Survival of Spermatozoa at Low Temperatures. Nature. 1950;166(4225):668-9, http://dx.doi.org/10.1038/166668a0.
15. Schlegel PN, Su LM. Physiological consequences of testicular sperm extraction. Hum Reprod. 1997;12(8):1688-92, http://dx.doi.org/10.1093/humrep/12.8.1688.
16. Kolettis PN, Sabanegh ES, D'Amico A M, Box L, Sebesta M, Burns JR. Outcomes for vasectomy reversal performed after obstructive intervals of at least 10 years. Urology. 2002;60(5):885-8, http://dx.doi.org/10.1016/S0090-4295(02)01888-5.
17. Keros V, Hultenby K, Borgstrom B, Fridstrom M, Jahnukainen K, Hovatta O. Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. Hum Reprod. 2007;22(5):1384-95, http://dx.doi.org/10.1093/humrep/del508.
18. Schlegel PN. Testicular sperm extraction: microdissection improves sperm yield with minimal tissue excision. Hum Reprod. 1999;14(1):131-5, http://dx.doi.org/10.1093/humrep/14.1.131.
19. Okada H, Dobashi M, Yamazaki T, Hara I, Fujisawa M, Arakawa S, et al. Conventional versus microdissection testicular sperm extraction for nonobstructive azoospermia. J Urol. 2002;168(3):1063-7.
20. Ghalayini IF, Al-Ghazo MA, Hani OB, Al-Azab R, Bani-Hani I, Zayed F, et al. Clinical comparison of conventional testicular sperm extraction and microdissection techniques for non-obstructive azoospermia. J Clin Med Res. 2011;3(3):124-31.
21. Persson PS, Ahren C, Obrant KO. Aspiration biopsy smear of testis in azoospermia. Cytological versus histological examination. Scand J Urol Nephrol. 1971;5(1):22-6, http://dx.doi.org/10.3109/00365597109133568.
22. Ezeh UI, Moore HD, Cooke ID. A prospective study of multiple needle biopsies versus a single open biopsy for testicular sperm extraction in men with non-obstructive azoospermia. Hum Reprod. 1998;13(11):3075-80, http://dx.doi.org/10.1093/humrep/13.11.3075.
23. Lin YM, Hsu CC, Kuo TC, Lin JS, Wang ST, Huang KE. Percutaneous epididymal sperm aspiration versus microsurgical epididymal sperm aspiration for irreparable obstructive azoospermia—experience with 100 cases. J Formos Med Assoc. 2000;99(6):459-65.
24. Cohen J, Garrisi GJ, Congedo-Ferrara TA, Kieck KA, Schimmel TW, Scott RT. Cryopreservation of single human spermatozoa. Hum Reprod. 1997;12(5):994-1001, http://dx.doi.org/10.1093/humrep/12.5.994.
25. Walmsley R, Cohen J, Ferrara-Congedo T, Reing A, Garrisi J. The first births and ongoing pregnancies associated with sperm cryopreservation within evacuated egg zonae. Hum Reprod. 1998;13 Suppl 4:61-70, http://dx.doi.org/10.1093/humrep/13.suppl_4.61.
26. Lane M, Bavister BD, Lyons EA, Forest KT. Containerless vitrification of mammalian oocytes and embryos. Nat Biotechnol. 1999;17(12):1234-6, http://dx.doi.org/10.1038/70795.
27. Schuster TG, Keller LM, Dunn RL, Ohl DA, Smith GD. Ultra-rapid freezing of very low numbers of sperm using cryoloops. Hum Reprod. 2003;18(4):788-95, http://dx.doi.org/10.1093/humrep/deg162.
28. Desai NN, Blackmon H, Goldfarb J. Single sperm cryopreservation on cryoloops: an alternative to hamster zona for freezing individual spermatozoa. Reprod Biomed Online. 2004;9(1):47-53, http://dx.doi.org/10.1016/S1472-6483(10)62109-8.
29. Buffat C, Patrat C, Merlet F, Guibert J, Epelboin S, Thiounn N, et al. ICSI outcomes in obstructive azoospermia: influence of the origin of surgically retrieved spermatozoa and the cause of obstruction. Hum Reprod. 2006;21(4):1018-24.
30. Tournaye H, Merdad T, Silber S, Joris H, Verheyen G, Devroey P, et al. No differences in outcome after intracytoplasmic sperm injection with fresh or with frozen-thawed epididymal spermatozoa. Hum Reprod. 1999;14(1):90-5, http://dx.doi.org/10.1093/humrep/14.1.90.
31. Wu B, Wong D, Lu S, Dickstein S, Silva M, Gelety TJ. Optimal use of fresh and frozen-thawed testicular sperm for intracytoplasmic sperm injection in azoospermic patients. J Assist Reprod Genet. 2005;22(11-12):389-94, http://dx.doi.org/10.1007/s10815-005-7481-y.
32. Pasqualotto FF, Rossi-Ferragut LM, Rocha CC, Iaconelli A, Jr., Borges E, Jr. Outcome of in vitro fertilization and intracytoplasmic injection of epididymal and testicular sperm obtained from patients with obstructive and nonobstructive azoospermia. J Urol. 2002;167(4):1753-6.
33. Anderson AR, Wiemer KE, Weikert ML, Kyslinger ML. Fertilization, embryonic development and pregnancy losses with intracytoplasmic sperm injection for surgically-retrieved spermatozoa. Reprod Biomed Online. 2002;5(2):142-7, http://dx.doi.org/10.1016/S1472-6483(10)61616-1.
34. Palermo GD, Colombero LT, Hariprashad JJ, Schlegel PN, Rosenwaks Z. Chromosome analysis of epididymal and testicular sperm in azoospermic patients undergoing ICSI. Hum Reprod. 2002;17(3):570-5, http://dx.doi.org/10.1093/humrep/17.3.570.
35. Said TM, Agarwal A, Sharma RK, Thomas AJ, Jr., Sikka SC. Impact of sperm morphology on DNA damage caused by oxidative stress induced by beta-nicotinamide adenine dinucleotide phosphate. Fertil Steril. 2005;83(1):95-103, http://dx.doi.org/10.1016/j.fertnstert.2004.06.056.
36. Oates RD, Silber S, Brown LG, Page DC. Clinical characterization of 42 oligospermic or azoospermic men with microdeletion of the AZFc region of the Y chromosome, and of 18 children conceived via ICSI. Hum Reprod. 2002;17(11):2813-24, http://dx.doi.org/10.1093/humrep/17.11.2813.
37. Moosani N, Pattinson HA, Carter MD, Cox DM, Rademaker AW, Martin RH. Chromosomal analysis of sperm from men with idiopathic infertility using sperm karyotyping and fluorescence in situ hybridization. Fertil Steril. 1995;64(4):811-7.
38. Bernardini L, Gianaroli L, Fortini D, Conte N, Magli C, Cavani S, et al. Frequency of hyper-, hypohaploidy and diploidy in ejaculate, epididymal and testicular germ cells of infertile patients. Hum Reprod. 2000;15(10):2165-72, http://dx.doi.org/10.1093/humrep/15.10.2165.
39. Colombero LT, Hariprashad JJ, Tsai MC, Rosenwaks Z, Palermo GD. Incidence of sperm aneuploidy in relation to semen characteristics and assisted reproductive outcome. Fertil Steril. 1999;72(1):90-6, http://dx.doi.org/10.1016/S0015-0282(99)00158-2.
40. Pang MG, Hoegerman SF, Cuticchia AJ, Moon SY, Doncel GF, Acosta AA, et al. Detection of aneuploidy for chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X and Y by fluorescence in-situ hybridization in spermatozoa from nine patients with oligoasthenoteratozoospermia undergoing intracytoplasmic sperm injection. Hum Reprod. 1999;14(5):1266-73, http://dx.doi.org/10.1093/humrep/14.5.1266.
41. Platteau P, Staessen C, Michiels A, Tournaye H, Van Steirteghem A, Liebaers I, et al. Comparison of the aneuploidy frequency in embryos derived from testicular sperm extraction in obstructive and non-obstructive azoospermic men. Hum Reprod. 2004;19(7):1570-4, http://dx.doi.org/10.1093/humrep/deh306.
42. Rodrigo L, Rubio C, Mateu E, Simon C, Remohi J, Pellicer A, et al. Analysis of chromosomal abnormalities in testicular and epididymal spermatozoa from azoospermic ICSI patients by fluorescence in-situ hybridization. Hum Reprod. 2004;19(1):118-23, http://dx.doi.org/10.1093/humrep/deh012.
43. Ben-Yosef D, Yogev L, Hauser R, Yavetz H, Azem F, Yovel I, et al. Testicular sperm retrieval and cryopreservation prior to initiating ovarian stimulation as the first line approach in patients with non-obstructive azoospermia. Hum Reprod. 1999;14(7):1794-801, http://dx.doi.org/10.1093/humrep/14.7.1794.
44. Verheyen G, Vernaeve V, Van Landuyt L, Tournaye H, Devroey P, Van Steirteghem A. Should diagnostic testicular sperm retrieval followed by cryopreservation for later ICSI be the procedure of choice for all patients with non-obstructive azoospermia? Hum Reprod. 2004;19(12):2822-30, http://dx.doi.org/10.1093/humrep/deh490.
45. Fusi F, Calzi F, Rabellotti E, Papaleo E, Gonfiantini C, Bonzi V, et al. Fertilizing capability of frozen-thawed spermatozoa, recovered from microsurgical epididymal sperm aspiration and cryopreserved in oocyte-free human zona pellucida. Hum Reprod. 2001;16:117-8.
46. Hsieh YY, Tsai HD, Chang CC, Lo HY. Cryopreservation of human spermatozoa within human or mouse empty zona pellucidae. Fertil Steril. 2000;73(4):694-8, http://dx.doi.org/10.1016/S0015-0282(99)00612-3.
47. Desai N, Glavan D, Goldfarb J. A convenient technique for cryopreservation of micro quantities of sperm. Annual meeting program supplement. Fertil Steril. 1998;70:S197-8
48. Isachenko V, Isachenko E, Montag M, Zaeva V, Krivokharchenko I, Nawroth F, et al. Clean technique for cryoprotectant-free vitrification of human spermatozoa. Reprod Biomed Online. 2005;10(3):350-4, http://dx.doi.org/10.1016/S1472-6483(10)61795-6.
49. Gvakharia M, Adamson GD. A method of successful cryopreservation of small numbers of human spermatozoa. Fertil Steril. 2001;76(3):S101, http://dx.doi.org/10.1016/S0015-0282(01)02297-X.
50. Gil-Salom M, Romero J, Rubio C, Ruiz A, Remohi J, Pellicer A. Intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Mol Cell Endocrinol. 2000;169(1-2):15-9, http://dx.doi.org/10.1016/S0303-7207(00)00345-2.
51. Quintans CJ, Donaldson MJ, Asprea I, Geller M, Rocha MG, Pasqualini RS. Pregnancy after ICSI with Spermatozoa Cryopreserved with a Novel Technique Useful for the Cryostorage of Very Small Numbers of Sperm

Cells. Fertility and sterility. 2000;74(3):S50, http://dx.doi.org/10.1016/S0015-0282(00)00853-0.
52. Sereni E, Bonu MA, Fava L, Sciajno R, Serrao L, Preti S, et al. Freezing spermatozoa obtained by testicular fine needle aspiration: a new technique. Reprod Biomed Online. 2008;16(1):89-95, http://dx.doi.org/10.1016/S1472-6483(10)60560-3.
53. Just A, Gruber I, Wober M, Lahodny J, Obruca A, Strohmer H. Novel method for the cryopreservation of testicular sperm and ejaculated spermatozoa from patients with severe oligospermia: a pilot study. Fertil Steril. 2004;82(2):445-7, http://dx.doi.org/10.1016/j.fertnstert.2003.12.050.
54. Herrler A, Eisner S, Bach V, Weissenborn U, Beier HM. Cryopreservation of spermatozoa in alginic acid capsules. Fertil Steril. 2006;85(1):208-13, http://dx.doi.org/10.1016/j.fertnstert.2005.06.049.
55. Isaev DA, Zaletov SY, Zaeva VV, Zakharova EE, Shafei RA, Krivokharchenko IS. Artificial microcontainers for cryopreservation of solitary spermatozoa. Hum Reprod. 2007;22:I154-I5.
56. Endo Y, Fujii Y, Shintani K, Seo M, Motoyama H, Funahashi H. Single Spermatozoon Freezing Using Cryotop. J Mamm Ova Res. 2011;28(1):47-52, http://dx.doi.org/10.1274/jmor.28.47.
57. Habermann H, Seo R, Cieslak J, Niederberger C, Prins GS, Ross L. In vitro fertilization outcomes after intracytoplasmic sperm injection with fresh or frozen-thawed testicular spermatozoa. Fertil Steril. 2000;73(5):955-60, http://dx.doi.org/10.1016/S0015-0282(00)00416-7.
58. Windt ML, Coetzee K, Kruger TF, Menkveld R, van der Merwe JP. Intracytoplasmic sperm injection with testicular spermatozoa in men with azoospermia. J Assist Reprod Genet. 2002;19(2):53-9, http://dx.doi.org/10.1023/A:1014487412975.
59. Osmanagaoglu K, Vernaeve V, Kolibianakis E, Tournaye H, Camus M, Van Steirteghem A, et al. Cumulative delivery rates after ICSI treatment cycles with freshly retrieved testicular sperm: a 7-year follow-up study. Hum Reprod. 2003;18(9):1836-40, http://dx.doi.org/10.1093/humrep/deg346.
60. Nicopoullos JD, Gilling-Smith C, Almeida PA, Ramsay JW. The results of 154 ICSI cycles using surgically retrieved sperm from azoospermic men. Hum Reprod. 2004;19(3):579-85, http://dx.doi.org/10.1093/humrep/deh092.
61. Friedler S, Raziel A, Soffer Y, Strassburger D, Komarovsky D, Ron-el R. Intracytoplasmic injection of fresh and cryopreserved testicular spermatozoa in patients with nonobstructive azoospermia—a comparative study. Fertil Steril. 1997;68(5):892-7, http://dx.doi.org/10.1016/S0015-0282(97)00358-0.
62. Gianaroli L, Magli MC, Selman HA, Colpi G, Belgrano E, Trombetta C, et al. Diagnostic testicular biopsy and cryopreservation of testicular tissue as an alternative to repeated surgical openings in the treatment of azoospermic men. Hum Reprod. 1999;14(4):1034-8, http://dx.doi.org/10.1093/humrep/14.4.1034.
63. Prins GS, Dolgina R, Studney P, Kaplan B, Ross L, Niederberger C. Quality of cryopreserved testicular sperm in patients with obstructive and nonobstructive azoospermia. The J Urol. 1999;161(5):1504-8.
64. Westlander G, Hamberger L, Hanson C, Lundin K, Nilsson L, Soderlund B, et al. Diagnostic epididymal and testicular sperm recovery and genetic aspects in azoospermic men. Hum Reprod. 1999;14(1):118-22, http://dx.doi.org/10.1093/humrep/14.1.118.
65. Ballesca JL, Balasch J, Calafell JM, Alvarez R, Fabregues F, de Osaba MJ, et al. Serum inhibin B determination is predictive of successful testicular sperm extraction in men with non-obstructive azoospermia. Hum Reprod. 2000;15(8):1734-8, http://dx.doi.org/10.1093/humrep/15.8.1734.
66. Fukunaga N, Haigo K, Kyono K, Araki Y. Efficiency of using frozen-thawed testicular sperm for multiple intracytoplasmic sperm injections. J Assist Reprod Gen. 2001;18(12):634-7, http://dx.doi.org/10.1023/A:1013107132110.
67. Tsujimura A, Matsumiya K, Miyagawa Y, Tohda A, Miura H, Nishimura K, et al. Conventional multiple or microdissection testicular sperm extraction: a comparative study. Hum Reprod. 2002;11:2924-9, http://dx.doi.org/10.1093/humrep/17.11.2924.
68. Fasouliotis SJ, Safran A, Porat-Katz A, Simon A, Laufer N, Lewin A. A high predictive value of the first testicular fine needle aspiration in patients with non-obstructive azoospermia for sperm recovery at the subsequent attempt. Hum Reprod. 2002;17(1):139-42, http://dx.doi.org/10.1093/humrep/17.1.139.
69. Meseguer M, Garrido N, Remohi J, Pellicer A, Simon C, Martinez-Jabaloyas JM, et al. Testicular sperm extraction (TESE) and ICSI in patients with permanent azoospermia after chemotherapy. Hum Reprod. 2003;18(6):1281-5, http://dx.doi.org/10.1093/humrep/deg260.
70. Vernaeve V, Verheyen G, Goossens A, Van Steirteghem A, Devroey P, Tournaye H. How successful is repeat testicular sperm extraction in patients with azoospermia? Hum Reprod. 2006;21(6):1551-4, http://dx.doi.org/10.1093/humrep/del012.
71. Ramasamy R, Yagan N, Schlegel PN. Structural and functional changes to the testis after conventional versus microdissection testicular sperm extraction. Urology. 2005;65(6):1190-4, http://dx.doi.org/10.1016/j.urology.2004.12.059.
72. Colpi GM, Colpi EM, Piediferro G, Giacchetta D, Gazzano G, Castiglioni FM, et al. Microsurgical TESE versus conventional TESE for ICSI in non-obstructive azoospermia: a randomized controlled study. Reprod Biomed Online. 2009;18(3):315-9, http://dx.doi.org/10.1016/S1472-6483(10)60087-9.
73. Turunc T, Gul U, Haydardedeoglu B, Bal N, Kuzgunbay B, Peskircioglu L, et al. Conventional testicular sperm extraction combined with the microdissection technique in nonobstructive azoospermic patients: a prospective comparative study. Fertil Steril. 2010;94(6):2157-60, http://dx.doi.org/10.1016/j.fertnstert.2010.01.008.
74. Hsiao W, Stahl PJ, Osterberg EC, Nejat E, Palermo GD, Rosenwaks Z, et al. Successful Treatment of Postchemotherapy Azoospermia With Microsurgical Testicular Sperm Extraction: The Weill Cornell Experience. J Clin Oncol. 2011;29(12):1607-11, http://dx.doi.org/10.1200/JCO.2010.33.7808.
75. Cavallini G, Magli MC, Crippa A, Resta S, Vitali G, Ferraretti AP, et al. The number of spermatozoa collected with testicular sperm extraction is a novel predictor of intracytoplasmic sperm injection outcome in non-obstructive azoospermic patients. Asian J Androl. 2011;13(2):312-6.