Laboratory processing and intracytoplasmic sperm injection using epididymal and testicular spermatozoa: what can be done to improve outcomes?

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There are two main reasons why sperm may be absent from semen. Obstructive azoospermia is the result of a blockage in the male reproductive tract; in this case, sperm are produced in the testicle but are trapped in the epididymis. Non-obstructive azoospermia is the result of severely impaired or non-existent sperm production. There are three different sperm-harvesting procedures that obstructive azoospermic males can undergo, namely MESA (microsurgical epididymal sperm aspiration), PESA (percutaneous epididymal sperm aspiration), and TESA (testicular sperm aspiration). These three procedures are performed by fine-gauge needle aspiration of epididymal fluid that is examined by an embryologist. Additionally, one technique, called TESE (testicular sperm extraction), is offered for males with non-obstructive azoospermia. In this procedure, a urologist extracts a piece of tissue from the testis. Then, an embryologist minces the tissue and uses a microscope to locate sperm. Finding sperm in the testicular tissue can be a laborious 2- to 3-hour process depending on the degree of sperm production and the etiology of testicular failure. Sperm are freed from within the seminiferous tubules and then dissected from the surrounding testicular tissue. It is specifically these situations that require advanced reproductive techniques, such as ICSI, to establish a pregnancy. This review describes eight different lab processing techniques that an embryologist can use to harvest sperm. Additionally, sperm cryopreservation, which allows patients to undergo multiple ICSI cycles without the need for additional surgeries, will also be discussed.

■ INTRODUCTION

Azoospermia is defined as an absence of spermatozoa in the ejaculate (1), and it is classified as either obstructive or non-obstructive. Obstructive azoospermia is the result of a blockage in the male reproductive tract; therefore, sperm production in the testicle is normal, but the sperm are trapped inside the epididymis. The absence of sperm in the ejaculate can be due to an abnormality in the epididymis, vas deferens, or ejaculatory ducts. The obstruction can be caused by many factors, such as infection, surgery (vasectomies), or an absent vas deferens. Non-obstructive azoospermia is the result of severely impaired or non-existent sperm production. Approximately 10% of male factor infertility has been attributed to complete azoospermia, which presents either in an obstructive or a non-obstructive form (2).

There are four different harvesting techniques that can be used to obtain sperm for in vitro fertilization (IVF). Each procedure has advantages and disadvantages and is described below. Sperm-harvesting techniques used to obtain sperm from men with obstructive azoospermia are described in the following paragraphs.

MESA (microsurgical epididymal sperm aspiration): Using an intraoperative stereomicroscope, a cut is made in a single tubule of the epididymis. The contents therein, which should contain sperm, are then aspirated with a fine-gauge needle. An embryologist examines the sample for the presence of motile sperm. If no motile spermatozoa are found at the first site, the maneuver is repeated. Typically, only a few microliters of epididymal fluid are retrieved because sperm are highly concentrated in the epididymal fluid (approximately 1×10^6 sperm/μl). A MESA approach should provide more than adequate numbers of sperm for immediate use, as well as for cryopreservation. As reported by Dr. Shlegel and colleagues, who used MESA and ICSI in

a group of men with obstructive azoospermia, clinical pregnancies were detected by a fetal heartbeat in 75% (57/76) of attempts, and healthy deliveries occurred in 64% (49/76) of attempts (3).

PESA (percutaneous epididymal sperm aspiration): This simple technique can be performed under local anesthesia or mild sedation. Unlike MESA, where the surgeon is able to visualize the exposed epididymal tubules, PESA is a blind procedure. A fine needle is used to puncture the epididymis, and sperm are aspirated and given to an embryologist to examine. Again, if no motile spermatozoa are found at the first site, the surgeon will repeatedly draw samples for an embryologist to examine.

TESA (testicular sperm aspiration): For obstructive azoospermia patients in whom sperm cannot be found in the epididymis, it is always possible to find sperm in the testis. TESA may be performed as a primary harvesting technique if there is an absent epididymis or severe epididymal fibrosis. This blind procedure is usually performed under local anesthesia or mild sedation. A wide-bore needle is pushed into the testis through the skin, suction is applied, and the contents of the needle are flushed into a petri dish containing culture media. Then, an embryologist examines the aspirate for motile or immotile spermatozoa with a stereomicroscope. Using fine needles, sperm are released from within the seminiferous tubules, where sperm are produced, and they are then dissected from the surrounding tissue. Next, the embryologist determines whether there is a sufficient number of sperm for treatment or cryopreservation for future use. If not, several more needle biopsies will be attempted by the surgeon in each testis to obtain a sufficient amount.

TESE (testicular sperm extraction): This technique is reserved for men with non-obstructive azoospermia. This procedure is also routinely carried out under mild sedation or local anesthesia. The surgeon exposes a small area of the testis by making an incision in the scrotum. The seminiferous tubules are forced out through the incision by gently squeezing the testis, the tubules are excised and the biopsy sample is placed in a petri dish and given to an embryologist to examine. An approximately 500-g biopsy sample is rinsed in culture media to remove red blood cells and divided into small pieces, using fine needles, under a stereomicroscope to check for sperm (4). If no sperm are found, the surgeon will continue taking biopsies from different areas of the testis, and the embryologist will continue to examine the samples.

Sperm can usually be easily obtained from infertile men with obstructive azoospermia for intracytoplasmic injection (ICSI); however, individuals who exhibit non-obstructive azoospermia have historically been difficult to treat (4). Examining biopsies for sperm under a microscope can be very time-consuming and difficult. Finding sperm in the testicular tissue can be a laborious 2- to 3-hour process depending on the degree of sperm production and the etiology of testicular failure, especially in men with partial testicular failure. For men with non-obstructive azoospermia, some IVF clinics ask that a TESE be performed a day before the egg retrieval process because they believe culturing the testicular tissue in an incubator overnight will help sperm to acquire motility. When sperm are not found after surgery, the couple must decide whether they want to cancel the egg retrieval, cancel the fertility cycle, or proceed with donor sperm. Alternatively, oocyte collection can be

performed even if no sperm are found in the TESE process and there is no donor sperm back-up. In this case, eggs can be cryopreserved to enable the couple to make future fertility decisions. Importantly, this new option is available because vitrification has significantly improved oocyte survival rates. For men who must undergo sperm-harvesting procedures to recover testicular or epididymal sperm, it is now possible to cryopreserve the excess sperm for future cycles if needed, thus eliminating the need for repeat surgery. However, some clinics still prefer to use freshly retrieved epididymal or testicular sperm instead of frozen-thawed samples.

■ INTRACYTOPLASMIC SPERM INJECTION (ICSI)

Two major breakthroughs have recently occurred in the area of male infertility (4). The first is the development of the intracytoplasmic sperm injection (ICSI) technique for the treatment of male factor infertility due to severely abnormal semen quality. The second is the extension of ICSI to azoospermic males and the demonstration that spermatozoa derived from either the epididymis or the testis are capable of producing normal fertilization and pregnancy (4). ICSI is now the primary technique used to treat male-factor infertility. ICSI has achieved consistent fertilization and high pregnancy rates in couples with suboptimal spermatozoa (5). Although the fertilization rates obtained with surgically retrieved spermatozoa were satisfactory, they were significantly lower than those achieved with fresh ejaculates (6). A concern associated with the use of suboptimal spermatozoa for ICSI is the potential for transmitting the genetic abnormalities responsible for male infertility to the offspring (4). Despite this concern, ICSI is the customary therapeutic approach, although genetic screening and patient counseling are still imperative for these patients.

In IVF clinics, surgically retrieved sperm samples are most often prepared for use before a patient progresses through an ICSI cycle. This is a safe course of action in cases of obstructive azoospermia, but in cases of non-obstructive azoospermia, it is beneficial to undergo the surgery before the cycle because the female has already been stimulated with medication. At that time, a couple would have to decide whether to proceed with donor sperm or cryopreserve the oocytes for use at a later time. Epididymal aspirates are relatively easy to prepare in the lab, either for ICSI or for cryopreservation. Typically, an embryologist washes the sample with culture media to dilute any epididymal fluid or blood contamination. The most common issues are a high level of red blood cells, an absence of observed sperm, poor sperm motility, and slow progression (5).

■ DIFFERENT METHODS FOR PROCESSING EPIDIDYMAL AND TESTICULAR SPERM SAMPLES

Shredding method

The preparation of testicular samples is more difficult if the sperm are contained within the seminiferous tubules. Many of the sperm found are immature, with a large cytoplasmic drop attached to the head, and some sperm are motile; however, most are still viable for ICSI. It is advantageous to perform the sperm retrieval procedure at least 24 hours in advance of the egg retrieval process, as

motility improves with time in culture (7). Biopsies will always require incubation before motility is observed. In some cases, it was found that the sample needed to be cultured for at least 24 hours at 37℃.

A common procedure associated with the shredding method is the excision of testicular tissue by a surgeon and its subsequent examination by an embryologist. The tissue can be finely minced and teased apart with fine needles or glass slides in HEPES-buffered media. Then, the product is placed in a 5-ml Falcon tube and centrifuged for 5 minutes at 1,800 g. The supernatant is removed with a Pasteur pipette, and the pellet is resuspended in 0.2 ml of culture media. The number of spermatozoa in the droplet can be very low, as well as poor motility when visualized microscopically. An embryologist must retrieve sperm from the field of debris, red blood cells, and Sertoli cells for ICSI. Then, the sperm suspension can be incubated at 37℃ until the time of ICSI (8).

Squeezing method

Seminiferous tubules are teased apart and rinsed to remove blood contamination, and they are then placed into a petri dish with fresh culture media. Tubules are then cut into short lengths (1-2 cm) with fine needles. A long, thin Pasteur pipette is pulled over a flame and then bent (ideally at an angle of 45°). A second pipette (without a bend) should be heated and used to pick up the tubule contents. By holding one end of a cut tubule with the point of a needle, the bent pipette can be run along the length of the tubule while simultaneously pushing down against the base of the petri dish. This procedure squeezes the entire contents of the tubule into the medium. The contents can now be picked up with the second pulled pipette and placed in a test tube filled with clean sperm media or placed on a slide to look for sperm (9).

Cell strainer

A method for processing large biopsy samples that has the benefit of removing unwanted debris consists of the use of a cell strainer. First, the biopsy sample is teased apart and sliced with fine needles. These slices are rinsed in a series of petri dishes containing sperm preparation media to remove any blood contamination. The tubules are then placed in a cell strainer (Becton Dickinson & Company, Franklin Lakes, New Jersey, USA). Heat-treating the end of a clean, sterile Pasteur pipette produces a sphere-shaped tip approximately 5 mm in diameter. This pipette is used as a pestle to grind and break up the seminiferous tubules against a mesh strainer (9).

Tissue grinder method

Another method for processing large tissue samples is to use a mini-tissue grinder. The tissue is teased apart, sliced with fine needles and placed in a test tube with fresh culture media and a glass pestle. With the glass pestle, the sample is ground at the bottom of a glass tube. Then, the sample is concentrated via centrifugation. After centrifugation, the pellet is resuspended with fresh culture media and placed on a Petri dish in 10-µl drops to look for sperm.

Erythrocyte lysing buffer method

Sperm recovery from epididymal fluid is usually rapid and efficient, whereas the recovery of sperm cells from testicular biopsy specimens is more difficult, especially in cases of non-obstructive azoospermia. The main difficulty is due to the abundance of red blood cells in shredded testicular biopsy specimens. The presence of a very high concentration of erythrocytes and the very infrequent occurrence of sperm cells sometimes result in a much longer examination (10). Therefore, one option for testicular biopsy specimens is suspension in Hepes-buffered medium. This can then be centrifuged for 5 minutes at 300 g. Treatment of the testicular sperm pellet with erythrocyte lysing buffer is performed by resuspending the testicular sperm pellet in 2 to 4 ml of erythrocyte lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 2 mM ethylenediaminetetracetic acid; pH 7.2) for 10 minutes at room temperature. Then, 5 to 10 ml of Hepes-buffered medium supplemented with protein is added to the suspension in the same tube and then centrifuged for 10 minutes at 500 g. The pellet is then resuspended with 1.5 ml of culture media with protein. This resuspended pellet can be transferred to an Eppendorf tube and centrifuged again at 500 g for 5 minutes. After centrifugation, the pellet is resuspended in 50 µl of culture media supplemented with protein. This pellet is then placed in an injection dish that contains 20-30 droplets containing 5 µl of Hepes-buffered medium; the central droplet is composed of 10% PVP solution. Subsequently, the embryologist can search for spermatozoa in the sample that will be used for ICSI. This method is beneficial when there is a large amount of red blood cell contamination in the sample. This technique has been reported and described in more detail by Nagy and colleagues (10). One of the main difficulties in finding spermatozoa in a testicular sample is the abundance of erythrocytes (10).

The hypoosmotic swelling test (HOS test)

The hypoosmotic swelling test (HOS) was developed by Jeyandran et al. (1984) (11) to evaluate the functional integrity of the sperm membrane. Viable sperm with normal membrane function will exhibit tail swelling and curving due to the influx of water when exposed to hypoosmotic conditions. The use of this test for distinguishing viable from non-viable spermatozoa for ICSI was first proposed by Desmet et al. in 1994 (12). It has been shown that the fertilization rate after ICSI of randomly selected, immotile spermatozoa is usually very low, especially with ejaculated spermatozoa (13,14). There is a reduced success rate of fertilization for azoospermic males due to the injection of non-viable spermatozoa, which cannot be distinguished from viable spermatozoa with no motility. Application of the hypo-osmotic swelling test, however, seems to be a promising method of identifying live spermatozoa for ICSI (14). Casper et al. (1996) (15) conducted a study that showed a higher fertilization rate after injecting spermatozoa selected by the HOS test than after injecting randomly selected spermatozoa.

Another study using the HOS test has also been reported. In that study, immotile spermatozoa were suspended in a microdroplet consisting of 50% culture medium and 50% Millipore-grade water. After a maximum of 10 seconds, the viable spermatozoa, whose tails were curved and swollen, were selected and transferred to another microdroplet of Hepes-buffered medium where they were washed three times to osmotically re-equilibrate them before transferring them to a PVP drop. The group found that the modified HOS test resulted in improved fertilization and pregnancy

rates compared with the regular HOS test and non-use of the HOS test (16).

Pentoxyfylline method

Pentoxyfylline is a phosphodiesterase inhibitor of the methylxantine group. It inhibits the breakdown of cyclic adenosine monophosphate (cAMP), a molecule known to play a role in sperm motility (17). It was found that adding pentoxyfylline to a testicular sperm sample caused sperm to be motile. This procedure is performed by adding pentoxyfylline to the sperm suspension at a 1:1 ratio so that the final concentration of pentoxyfylline in the sample is 0.5 mg/ml. The sample is incubated at 37℃ for 20 minutes. Next, the sample is observed for the identification and isolation of motile sperm. Some studies have shown that pentoxyfylline is toxic and should not be used (18). It has been suggested that a lower concentration of pentoxyfylline with shorter exposure times may be beneficial (17). One study found that this was a better and quicker identification and selection method for vital sperm for use with ICSI. This procedure also resulted in better fertilization rates and less time consuming for staff involved in TESA/TESE (17).

Collagenase method

In a study by Crabbe et al. in 1997 (19), the enzymatic treatment of testicular biopsies with collagenase type IV was applied in clinical ICSI cases where no spermatozoa had been found after the sample was minced using two fine needles. This procedure was performed to determine whether spermatozoa can be recovered from the residual tissue pieces. This method is performed by shredding the testicular biopsy sample with fine needles. Microscopic examination of the wet preparation is carried out at 400x magnification under an inverted microscope. Biopsy factions are further minced with two fine forceps in the Petri dish until tissue pieces of ~1 mm^3 or free tubule pieces of a few millimeters in length are obtained (19). Spermatozoa are directly recovered from the pellet after centrifuging the supernatant of the shredded tissue at 300 g for 5 minutes. Then, erythrocyte-lysing buffer is used to increase the probability of visualizing any spermatozoa or elongated spermatids (19). The residual pieces are placed in 1 ml of pre-warmed HEPES medium supplemented with protein, 1.6 mM CACL$_2$, 25 μL/ml DNase, and 1000 IU/ml collagenase Type IV. The tissue samples are then placed in an incubator at 37℃ for 1 hour to allow digestion to occur. To facilitate complete enzymatic digestion, the samples are shaken every 10-15 minutes during the incubation period. The digested tissue solution is gently centrifuged for 5 minutes at 50 g to remove any residual pieces or debris not dissolved by the enzymes. The remaining cell suspension (supernatant) containing loose cells is then washed twice with culture media and centrifuged for 5 minutes at 1000 g. The supernatant is removed, and the pellet is resuspended once more with 50-100 μl. A 5-μl drop of the suspension is examined on a glass slide with a coverslip and placed under an upright phase contrast microscope. When spermatozoa are found in one of the suspensions, multiple small droplets are added under oil in a Petri dish to further examine and retrieve spermatozoa for ICSI.

This study concluded that the fertilization rate obtained using the samples treated with collagenase type IV was comparable to that obtained using the samples retrieved via the shredding method. This method can be considered successful in reducing sperm recovery failure and may increase the chance of selecting the highest quality spermatozoa in patients with non-obstructive azoospermia (19).

■ CRYOPRESERVATION OF TESTICULAR AND EPIDIDYMAL SAMPLES

Successful sperm cryopreservation allows patients to have multiple ICSI cycles without the need for additional sperm harvesting procedures. Because ICSI enables severely impaired viable sperm to fertilize an oocyte, cryopreserved sperm can achieve rates of fertilization and pregnancy similar to those achieved using fresh sperm (4). Studies have also found that the cryopreservation of testicular sperm is more problematic than the cryopreservation of epididymal sperm because testicular tissue yields a much lower number of spermatozoa with limited motility. Nevertheless, reports have indicated that it is possible to cryopreserve testicular biopsy tissue samples and subsequently extract spermatozoa after thawing, with at least isolated pregnancies being achieved (8).

Although the use of cryopreserved testicular sperm for ICSI has several advantages, the data concerning the outcomes of IVF-ICSI procedures using frozen-thawed testicular sperm are still controversial. Some investigators claim that they have demonstrated, in obstructive and non-obstructive azoospermic men, that cryopreserved sperm can function as well as fresh sperm (20). Additionally, some labs have shown that the use of frozen-thawed testicular sperm for ICSI results in higher abortion rates and lower live birth rates compared with fresh testicular sperm (20).

Without cryopreservation, testicular tissue and testicular sperm can only be used for one ICSI cycle. Lacking cryopreserved sperm, each cycle of ICSI in these couples requires repeated surgeries. Repeated testicular surgeries can cause permanent testicular damage, irreversible atrophy, deterioration of spermatogenic development, and even loss of endocrine function (2). The cryopreservation of samples allows for multiple ICSI cycles and minimizes the number of invasive testicular surgeries.

Surgically recovered sperm from PESA can be cryopreserved in a manner similar to the cryopreservation of fresh ejaculates, although the post-thaw survival rates are usually very poor. Due to extreme variability in post-thaw survival rates, it is important to freeze a small aliquot for a post-thaw survival test. This test requires thawing of the sample the day before the egg retrieval. In cases of virtually zero post-thaw survival, having results from the test sample allows time for the patient to consider what precautions to take if no viable sperm are identified on the day of their partner's egg retrieval. Because there is never an absolute guarantee that cryopreserved sperm will thaw with appropriate viability, counseling patients regarding the use of donor sperm as a back-up should be considered, as well as options of repeat surgery or oocyte cryopreservation.

Haberman described the following cryopreservation technique (20). The biopsy specimen is placed in a petri dish with HEPES buffer supplemented with protein at 37℃. Under a dissecting microscope, the seminiferous tubules are teased apart using fine needles, and the contents are squeezed into the surrounding media. The tubules are transferred to a 15-ml conical tube containing 1 ml of fresh

media. The cell suspension is then transferred to a separate centrifuge tube. Both tubes are incubated at 37°C for 15-30 minutes, and the supernatant (containing tubules) is combined within the cell suspension in the second tube. The suspension is centrifuged at 500 *g* for 5 minutes, and the pellet is resuspended in 1 ml of fresh media with protein. A cell count is performed, and the suspension is diluted or concentrated to 0.5-1.0 million sperm/ml. Before freezing, an aliquot is removed to assess sperm quality. At the time of cryopreservation, multiple aliquots of sperm are frozen whenever possible along with a test cryovial. The cell suspension is slowly mixed in a 1:1 ratio with test yolk buffer and 12% glycerol so that the final concentration of glycerol is 6%. The samples are slowly cooled at a rate of -0.5°C/min to 4°C and then packaged in 1-ml cryovials. The vials are frozen at a rate of -10°C/min to -90°C and are stored in liquid nitrogen at -196°C (20). A study was conducted using this protocol for 29 patients with obstructive and non-obstructive azoospermia undergoing testicular sperm extraction for a total of 46 IVF-ICSI cycles (12 fresh, 34 frozen). No statistically significant differences were noted between the fresh and frozen-thawed testicular spermatozoa (20).

Cohen et al. 1997 (2) described a method where cryopreservation and recovery of spermatozoa can be performed even in patients who have fewer than 100 spermatozoa present in the final testicular tissue homogenate. A porous capsule, such as an emptied zona pellucida, is used as a vessel to contain individual spermatozoa. Empty zona pellucida is prepared by microscopic dissection; specifically, small incisions are made in the zona using a microsuction device. The emptied zona pellucida is then maintained in Hepes-buffered media supplemented with protein. A sperm suspension in 10% PVP is then injected into the zona, placed in an 8% glycerol solution and cryopreserved using a standard freeze protocol with plastic straws. The use of an empty zona pellucida is very advantageous for samples with very low numbers of spermatozoa and also reduces the loss of motility associated with post-thaw dilution and sperm washing, which is observed when thawing frozen donor sperm (2).

In cases where freezing intact testicular tissue is required, a method adapted from Allan and Cotman (1997) is used (21). Testicular tubules are rinsed in HEPES-buffered medium to eliminate or reduce red blood cell contamination. The tubules are sectioned with a scalpel blade into pieces of approximately 2-3 mm and placed into a 35-mm dish that contains HEPES-buffered medium and cryoprotectant medium at a 1:1 v/v ratio at room temperature. Then, after 30 minutes, the samples are loaded into as many cryostraws as required. The 30-minute room-temperature equilibration period is ongoing during the loading process. The straws are sealed and labeled with the patient's name. At the end of the 30-minute room-temperature equilibration period, the straws are loaded into the goblets and cryocanes and placed horizontally in the freezer for 30 minutes. At the end of the 30-minute cooling period, the specimens are hung over liquid nitrogen, well below the frost line of the tank, for 30 minutes. Then, the specimens are plunged into liquid nitrogen for continued storage. It is recommended to cryopreserve a test straw and then thaw it to determine the success of the cryopreservation procedure. To thaw these samples, the straws must be taken out of the liquid nitrogen tank and placed in a room-temperature water bath for 10

minutes. The contents are then expelled into room-temperature HEPES-buffered medium, rinsed with fresh HEPES-buffered medium and allowed to stand for 10 minutes before the sample is processed. The tissue is placed in a sterile Petri dish with a small volume of HEPES-buffered medium, sufficient to cover the tissue, and this sample is then homogenized with fine needles. Using a pulled pipette, a small volume of the homogenate is moved to the ICSI dish, and motile sperm are captured using an assisted hatching pipette. These sperm are then placed in a drop of PVP. Note that overnight incubation of the cryopreserved, thawed, and processed homogenate may increase the effective yield of the preparation (22).

■ FUTURE DIRECTIONS

Further studies and data will help to identify and define how to more effectively extract sperm from men with obstructive and non-obstructive azoospermia. A key issue identified by Silber et al. (2003) is that there is a high incidence of mosaicism in embryos derived from TESE. Sperm retrieved from men with non-obstructive azoospermia may have a higher rate of compromised or immature centrosome structures, which may lead to mosaicism of the embryo. Their results showed a significant difference in mosaicism rates between embryos from ICSI cycles using ejaculated sperm and embryos from ICSI cycles using TESE for non-obstructive azoospermia (23).

Another key issue is the increased DNA fragmentation observed in testicular sperm samples from patients with azoospermia, due either to spermatogenic failure or duct obstruction. Several studies have shown that male fertility can be affected by DNA damage found in sperm (24,25). One study yielded two interesting conclusions resulting from the measurement of DNA fragmentation using the sperm chromatin dispersion (SCD) test (25). The first conclusion was that patients exhibiting low implantation rates and low embryo quality may benefit from this test, which determines the levels of DNA fragmentation. Second, the observation of nucleolus asynchrony in a zygote from a sperm sample with a high DNA fragmentation rate could indicate the possibility of an upcoming low-quality blastocyst cohort (25). In addition, sperm DNA fragmentation appears to be related to the ability of sperm to fertilize the oocyte (25,26). The use of fragmented sperm is still a matter of concern because of the long-term consequences on the development and behavior of the offspring. Still, studies have shown that oocytes may partially repair fragmented DNA, depending on the level of DNA fragmentation, producing blastocysts that are able to implant and produce live offspring (23).

In addition, it has been shown that testicular cultures should not be cultured for more than 48 hours to increase motility. Aged spermatozoa may be more susceptible to the damaging effects of free oxygen radicals. Moreover, an increase in structural chromosomal abnormalities in in vitro-stored spermatozoa has been reported (27).

The efficacy and safety associated with the retrieval of incompletely developed spermatozoa (i.e., spermatids) from men with non-obstructive azoospermia will also need to be clarified over time. Anecdotal pregnancies and deliveries from these clinical scenarios have been reported (28). However, spermatids usually cannot be extracted from the testis if TESE does not yield fully developed spermatozoa. However, other new therapies, including spermatogonial

transplants, have been reported in animal models. Because many men with non-obstructive azoospermia may have significant genetic abnormalities, caution must be exercised before these fertility treatments can be accepted as standard therapies (28).

In conclusion, the use of ICSI combined with sperm-harvesting techniques has transformed fertility treatment for azoospermic males. In both obstructive and non-obstructive azoospermia, the sperm retrieval technique itself seems to have no impact on the success rates of ICSI. Laboratory techniques that recover sperm from surgical specimens should be carried out with great care and caution. It is important to not jeopardize the potential of the sperm to fertilize the oocyte. The chances of retrieving and recovering spermatozoa and of achieving a live birth by ICSI are increased in couples in which the male partner has obstructive rather than non-obstructive azoospermia (5). Each laboratory technique described in this paper has advantages and disadvantages; the embryologist must determine which method yields the best chance of extracting viable sperm for ICSI and enabling cryopreservation of a patient's sperm for future IVF cycles.

■ AUTHOR CONTRIBUTIONS

Popal W wrote the paper and Nagy ZP was responsible for proofreading the manuscript.

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