Testicular biopsy for ICSI: technique and timing

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Surgical retrieval of testicular spermatozoa combined with intracytoplasmic sperm injection (ICSI) has become an established treatment for azoospermic patients. In obstructive azoospermia as well as in patients with hypospermatogenesis or incomplete spermatogenic arrest, sperm retrieval can be done either by open biopsy, or needle aspiration. The sperm retrieval rates are almost 100% by any technique and cryopreservation of excess spermatozoa is possible in all cases. The fertilization and pregnancy rates using cryopreserved spermatozoa are comparable to fresh spermatozoa. The timing of sperm retrieval in such cases does not cause any problem in busy IVF centers.

On the other hand, the overall sperm retrieval rates in non-obstructive azoospermia (NOA) range from 40 to 60%. Lower retrieval rates (18 - 25 %) are expected in patients with severe forms of NOA such as complete Sertoli cell only, Klinefelters syndrome and early complete spermatogenic arrest (1). Although some authors claim retrieval rates comparable to open biopsy using fine needle aspiration, most centers prefer open biopsy in NOA. It has been shown that microsurgical TESE offer better retrieval rates (±60%) with lower side effects (2). However, the procedure did not gain much popularity because it needs relatively expensive instruments, specialized training, and more operative time compared to standard biopsy. A compromised solution is to use surgical loupes during TESE and the stereomicroscope for dissection and selection of dilated tubules (3). However, at the present time, there are no studies comparing surgical loupes to microsurgery for sperm retrieval in NOA.

Failure to retrieve spermatozoa at the day of oocyte retrieval exposes the couple's to psychologically and financially stressful conditions. The cost and risks of oocyte retrieval can be avoided if sperm retrieval is planned several hours prior to oocyte retrieval. In Vitro culture of testicular tissue may allow scheduling the procedure longer time up to 72 hours prior to oocyte retrieval. Unfortunately, the results in patients with severe forms of NOA, in terms of survival of spermatozoa, fertilization and pregnancy rates, are not encouraging, as in patients with obstructive azoospermia (4). On the other hand, performing TESE after ovarian stimulation would eliminate the risks of ovarian stimulation and the psychological and financial impact of failed sperm retrieval. Several authors reported satisfying fertilization, implantation and pregnancy rates following ICSI using frozen-thawed testicular spermatozoa retrieved from patients with NOA. Therefore it was suggested to perform surgical sperm retrieval and cryopreservation prior to admitting the patients to an ICSI program (5).

In contrast to patients with obstructive azoospermia, where viable sperm can be easily retrieved from the frozen specimens, the impaired quality of the testicular tissue of NOA patients does not allow for cryopreservation and later use for ICSI in all cases. As has been demonstrated for ejaculated sperm, a significant decrease in motility and viability was observed after thawing of cryopreserved testicular sperm. This implies that cases with extremely low numbers of sperm retrieved can hardly be considered candidates for cryopreservation. The substantial risk of not finding sperm suitable for injection after thawing was about 20% in a non-selected group of patients with NOA (6). In order to overcome the risk that the frozen material is inadequate for injection upon thawing, some IVF centers define limits for...
testicular sperm quality suitable for freezing, and others only allocate patients for treatment on the basis of sufficient quality (motility) of a preliminary-thawed testicular sperm fraction. This means that the data presented in the literature about the successful sperm recovery after thawing and hence the fertilization and the pregnancy rates using cryopreserved spermatozoa may not represent all patients with NOA.

When no spermatozoa suitable for injection can be obtained after thawing cryopreserved testicular sperm in NOA patients, a repeat TESE procedure can be planned. Studies showing the sperm retrieval rates in repeated TESE are scarce. The largest available study showed that failure of sperm retrieval in the second TESE is ± 25% (7). This means that if TESE was performed prior to ICSI and sperm were found and cryopreserved there is still a small chance (±5 %) that ICSI might be cancelled or performed with immotile cryothawed spermatozoa. Moreover, only few studies took into consideration the testicular size, the technique of biopsy, and the degree of histopathological impairment. In patients with Klinefelter syndrome, although pregnancies could be achieved with cryopreserved spermatozoa, there was a high risk of cancellation or injecting oocytes with immotile sperm (8).

When very few sperm are available, Empty Zona pellucidae (ZP) can be used as a vehicle for the cryopreservation of sperm collected by testicular sperm extraction. This may avoid their loss after thawing. It has been shown that the ZP of different species such as mouse ZP can be used for human sperm storage (9). However, although the idea seems attractive, this technique did not gain much popularity.

In conclusion, until improved recovery procedures after cryopreservation of few spermatozoa are available, surgical sperm retrieval prior to ICSI should be individualized. All patients with NOA should be properly counseled about the pros and cons of performing TESE either prior or at the day of oocyte retrieval.

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Testicular sperm extraction and intracytoplasmic sperm injection (TESE/ICSI) became the standard protocol for treatment of
patients with non obstructive azoospermia and obstructive azoospermia, who are not amenable to surgical reconstruction. Many ICSI centers adapt their own protocol of sperm retrieval as regards the technique and time. Since there is no universal agreement; our opinion here is meant to reflect our own experience which we wish to be beneficial for all colleagues.

The Clinician counseling the azoospermic patient has a big dilemma as he can suggest many enthusiastic options coupled with some risks. In order to retrieve sperm, several techniques are already available in the form of needle and open biopsies. If he chooses the Testicular sperm extraction (TESE) option and retrieves fresh sample just after egg collection he carries the risk of finding no sperms after female ovarian stimulation and ovum pick up! He can go for fresh Testicular sperm extraction (TESE) sample before egg collection (4-8 hours) but again carrying the risk of finding no sperms after a pointless ovarian stimulation. He can schedule it 1-3 days before ovum pickup using the In vitro culture techniques of testicular sperm with the aim to increase the fertilization rate, pregnancy rate as well as the implantation rate but with possible deterioration of sperm vitality. Another option is to verify sperm presence before starting any female workup and to Freeze a diagnostic TESE sample for a later egg collection. Beside its obvious practicality in busy IVF lab, Freezing is also very convenient for the physicians and the patients being done electively. It will also circumvent the repetition of the invasive sperm retrieval technique. The availability of sperm cryopreservation offers additional advantages, negating the need for synchronization of sperm retrieval and ovulation, preventing pointless ovarian stimulation in the female partner with its hazards and expenses. However the risk of Survival of frozen sperm exists. So each option of these has significant advantages and drawbacks.

Actually I always ask myself two important questions when I see an azoospermic patient. The first one is the patient really azoospermic and or in need of sperm retrieval? After confirmation of the azoospermic status I have to plan and counsel the patient properly for the most appropriate technique and timing. To make a proper schedule I always remind myself with the following goals: I need to obtain the best quality of sperm possible, I have to retrieve an adequate number of sperm for both immediate use and for cryopreservation and I must minimize the possible damage to the reproductive tract so as not to jeopardize future attempts at sperm retrieval or surgical reconstruction.

The patient should have at least two previous analyses showing only azoospermia after centrifugation, moreover I need to confirm by a third semen analysis (double ejaculate) in our Andrology lab after a prolonged abstinence (2 weeks). Sometimes few motile sperms are found and can be detected and cryopreserved instantaneously saving the patient from an unnecessary invasive procedure. In case of no sperm detection, two smears are done from the pellet; one is subjected to nuclear-fast red-picroindigocarmine staining to exclude sperm presence (Amer et al., 2002), the other to May-grunwald-Giemsa (MGG) stain for detection of spermatogenic cells (Amer et al., 2001) for prognostic and diagnostic purposes.

Basically before discussing the optimum technique and timing of TESE, we should remember that the results of retrieval depend on the technique of biopsy as well as the technique of search for testicular sperm. Optimizing the technique of search for sperm can not be more emphasized and is far beyond the scope of this article.

Optimum technique for Biopsy?

Although the ideal method of sperm retrieval is debatable, the optimal method is that which is safe, efficient, and reliable in retrieving adequate amounts of sperm of optimal quality. Different methods for recovering epididymal or testicular spermatozoa have been described and each has its drawbacks and advantages. Percutaneous aspiration of the testis or epididymis may be the method of choice in cases of irreparable obstructive azoospermia. TESE in the form of open biopsy remains the most effective and reliable technique in retrieving sperm in men with non obstructive azoospermia (NOA). The microsurgical technique that have been applied to either identify areas of active spermatogenesis within the testis (Schlegel, 1999; Amer 2000, 2002 and 2007) or to aspirate sperm-containing fluid from the epididymis (microsurgical epididymal
sperm aspiration) is a valuable addition. NOA represents the most challenging group for sperm retrieval. Our experience for patients with NOA is now based on the microsurgical technique that we developed since 1998 (Amer et al. 2000) rather than the multiple random open biopsy that we previously applied (Amer et al. 1999). Men with NOA may have minute foci of spermatogenesis; this was observed in the early studies of quantitative analysis of spermatogenesis (Silber and Rodriguez, 1981). The role of the surgeon is to try to find these foci based on their appearance under magnification and this is the idea of using the surgical microscope. Instead, we can perform multiple large biopsies in a trial not to miss an active focus. But who guarantees? Ezeh et al (1998) found that focal areas of spermatogenesis coexisting with either maturation arrest or SCO pattern in 28% of their patients who underwent bilateral testicular biopsy. The tubule-to-tubule variability runs contrary to the orthodoxy that spermatogenesis in the testis is uniform and that a piece of testicular tissue as small as 10 mg is representative of the entire testis. While this may be the case in normal or oligozoospermic men, it is not the case in azoospermia due to primary gonadal failure. So the idea of taking multiple biopsies from different site is very appealing, however, multiple sampling may be hazardous. Testicular biopsy has the potential for causing permanent testicular damage especially if we remembered the fact that spermatogenesis is an intricate process of 74 days duration and it is highly sensitive to toxic effects and even minor alteration in temperature. Therefore, inflammatory changes in the testis following testicular surgery could adversely affect the spermatogenic process in those patients with marginal sperm production (Schlegel, 1999; Amer et al, 1999, 2000); again 29% of single open biopsy may result in intratesticular hematoma formation with associated inflammatory changes (Harrington et al., 1996). Schlegel and Su, 1997, evaluated 64 patients after TESE by physical examination, serial scrotal ultrasonography, histological analyses and evaluation of the successfullness of repeated sperm retrieval attempts. It was found that 82% of the evaluated patients had ultrasonic abnormalities in the testis suggesting resolving inflammation or hematoma at the biopsy site. By 6 months, these acute inflammatory changes typically resolved leaving linear scars or calcifications. Two patients had documented impaired testicular blood flow, with complete devascularization and atrophy of the testis in one patient after multiple biopsies. Repeated TESE procedures were more likely to retrieve spermatozoa if the second TESE procedure was performed after 6 months (80%), relative to those performed within 6 months (25%). The authors concluded that transient adverse physiological effects are common in the testis for up to 6 months after TESE. In addition permanent devascularization of the testis following TESE with multiple biopsies is high and they suggested using optical magnification to minimize the risk of this complication. In order to minimize tissue damage when taking multiple excision biopsies, small tissue samples may be taken using a microsurgical approach. We (Amer et al., 2000) compared the micro-dissection (on one side) with open, classic surgical biopsy (on the other side) in the same patient in 100 cases with NOA, the rate of recovery by micro-dissection TESE was significantly higher than by conventional method (47% vs. 30%). Furthermore, the risk of complications is far reduced by using the surgical microscope. Recently we were able to measure the diameter of Semineferous tubules (STs) during microdissection TESE using a micrometer fixed to the eyepiece of the operating microscope and introduced the new concept of a single tubule biopsy as a new objective microsurgical advancement for testicular sperm retrieval in patients with nonobstructive azoospermia. The STs were measured using the micrometer, and the tubule with the largest diameter was excised and freshly examined under an inverted microscope. If no spermatozoa were found, another sample was taken from the second most dilated tubule area and then at random until sperm were found or a maximum six samples were harvested. If no spermatozoa were detected, the contralateral testis was operated upon. The total sperm recovery rate was 105 out of 264 (39.8%). When ST measured \( \geq 300 \mu m \) the dissection and isolation of a single tubule enabled successfully retrieved spermatozoa in 16 out of 19 cases. When dilated tubules \( \geq 250 \mu m \) were observed in 48 out of 264 patients the
sperm recovery rate was 27 out of 48 (56.2%). When ST diameter was <300 μm, the sperm retrieval rate was 36.3% (89 out of 245). Unfortunately, this technique is not practical in cases of complete maturation arrest. Single tubule microsurgical sperm retrieval carries the advantage of being minimally invasive, reducing the time of search in the IVF lab, and maximizing the outcome of microsurgical TESE. Moreover, it is a very useful and objective tool for inexperienced surgeons during their practice of microsurgical TESE (Amer et al, 2007). As the microsurgical technique needs training and equipment which may not be available in every centre, some authors tried loop magnification with improved results. On the other some authors suggested testicular fine needle aspiration as an alternative method for sperm retrieval in men with non obstructive azoospermia (Lewin et al., 1999). In our experience, we should restrict FNA to the obstructive cases which in trained hands, a single puncture is always enough to retrieve enough sperm for ICSI and for cryopreservation. Multiple punctures, especially with large caliber needles, are associated with the risk of hematoma and this is highly expected in patients with NOA.

**Optimum timing for Biopsy?**

Whenever I see an azoospermic patient with clinical evidence of obstruction (Normal FSH, normal sized testicles and absent vas deferens or presence of epididymal nodule), or with NOA but with previous favorable histopathological diagnosis (i.e.: showing late spermatids), I usually propose the option of a fresh testicular biopsy to be programmed concurrently to wife stimulation. We found that it is better to schedule TESE 24h rather than 48h in NOA and 48 rather than 24 hours before ovum pickup in cases of OA. Of course, any superfluous testicular sperm is frozen for future trials. In-vitro culture of testicular sperm of NOA patients seems to impact positively through higher implantation rate, fertilization and early cleavage as well as through lower abnormal fertilization rates, this is reflected on higher number of embryos with adequate implantation potential to be frozen giving the possibility to a higher cumulative chances of conception. In-vitro cultured testicular sperm in obstructive azoospermic patients is also advantageous (48-hr rather than 24h) but in a different manner giving higher take home baby rate and lower abortion rate (Unpublished data).

Whenever I face an azoospermic patient with NOA with normal or Moderate size testicle, with previous histopathological diagnosis of unfavorable NOA (Fibrosis, hyalinization, Sertoli cell only, Early maturation arrest), I usually suggest the option of diagnostic TESE and freezing prior to wife stimulation to verify sperm presence provided that he accepts the minor risk of testicular sperm loss after freezing and thawing or the possibility of injection of the oocytes with immotile fresh testicular spermatozoa if sperm retrieval is done at the same day of egg collection and no motile sperm is found. The risk of Survival of frozen thaw sperm is not uniform in all centers (33%-90%). In our center only about 10% of our patients will need a second TESE the day of ovum pick up because of the sperm immobility after thawing. This usually happens when the total motile count of preserved sperm is less than 40. Although Pregnancy rate is variable from center to center with frozen thaw sperm, from our unpublished data, freezing seems to be an excellent way to improve pregnancy and implantation in non obstructive azoospermia (NOA) rather than using fresh testicular sperm. In obstructive azoospermia (OA) freezing provides us with higher fertilization rate and lower abortion rate than using fresh testicular sperm in ICSI cycles (unpublished data). Whenever repeated trials are decided, the use of frozen-thawed sperms is very suitable, economic and efficient rather than repetition of the invasive sperm retrieval technique.

The most difficult situation I frequently encounter is during counseling a patient with clinical evidence of severe gonadal failure (patients with small testes, especially those where the possibility of repeating biopsy seems improbable because of their testicular mass). I usually expect testicular sperm extraction to be very difficult and may even fail if after MGG stain only primary spermatocytes or no spermatogenic cells are detected in the ejaculate, especially in older men. In the contrary testicular sperm extraction is likely to be easier at younger ages, or when spermatids are detected in the ejaculate, and when testicular parenchyma is heterogeneous on microscopy.
However, it may take some time to extract and collect sufficient normal motile testicular sperm for injection of all available oocytes. As delayed injection is associated with low fertilization rate and poor embryo quality after ICSI, TESE is preferably scheduled for couples with severe gonadal failure on the same day 4-8 hours before ovum pick-up to minimize the risk of in vitro post maturity oocytes damage (Amer et al., 2002). Of course, the risk of finding no sperm is great and patients are counseled accordingly. The alternative considering a diagnostic biopsy and freezing carry the higher probability of testicular sperm loss after freezing and thawing or the possibility of injection of the oocytes with immotile fresh testicular spermatozoa if sperm retrieval is repeated at the same day of egg collection and no motile sperm is found.

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The introduction of testicular sperm recovery and the use of such sperm in intracytoplasmic sperm injection (ICSI) is a major milestone in the history of male infertility management. The knowledge about the heterogeneity of the testicular tissue allowed people with diagnosis like Sertoli cell syndrome or spermatogenic arrest to father a child when it was thought previously to be impossible.

Several sperm recovery techniques are described. The most commonly described is open surgical sperm retrieval (TESE). Although the procedure is relatively simple and safe, still it has some limitations. The inability of predicting a positive TESE outcome made many men undergo unsuccessful procedures and their spouses receive unnecessary stimulation. The lack of guidance to where the focal spermatogenesis is located and the fear of damaging the testis if too much tissue is removed is another limitation of the TESE procedure. Color flow Doppler mapping and retrieval of testicular tissues from areas with higher perfusion is reported to be associated with better sperm retrieval. However, the data available about this technique is still limited (1).

Percutaneous fine needle aspiration (FNA) is simpler and less invasive alternative way for sperm retrieval that needs no special training and possibly
has fewer complications and less patient perceived pain.

In cases with obstructive azoospermia FNA is usually successful in retrieving good number of viable sperm. However, FNA has significantly lower chances of sperm recovery in cases with functional azoospermia when compared with conventional TESE. This is due to the heterogeneity of the testicular structure in these cases and the limited amount of tissue obtained by FNA (2). This was reported in association with different dysfunctional testicular histology (Sertoli cell, hypospermatogenesis and spermatogenic arrest). In these patients TESE procedure to yielded more motile sperm allowing sperm freezing for utilization in subsequent ICSI attempts (3).

Microdissection TESE is another technique for testicular sperm recovery that has been described before and received more attention lately. It allows larger volume of the testicular tissue to be examined without damaging the testis. The main justification for this lengthy procedure is to avoid testicular vascular injury and to minimize the amount of tissue excised and consequently decrease the incidence of complications. However noting that the incidence of acute and chronic adverse post operative sequels in conventional TESE is low; these advantages have to be weighed cautiously against the increased surgical time and cost of the procedure. Probably of more interest are the reports of better sperm recovery using microdissection technique in cases of functional azoospermia compared with conventional TESE. Successful sperm recovery was reported even in cases where previous conventional TESE was negative (4). However the number of cases in these comparative studies is usually small and in some cases the difference is not statistically significant.

Conventional TESE is still the standard procedure for testicular sperm recovery. New techniques like microdissection, color Doppler mapping or combined techniques should be investigated further to improve the chance of sperm recovery.

Testicular sperm recovery procedure is usually scheduled on the same day of the oocyte collection. Retrieved sperm are used directly for ICSI. Surplus testicular sperm and tissue are frozen. In order to avoid unnecessary ovarian stimulation, decrease the cost or because of social reasons; TESE procedure can be done first independent from ovarian stimulation together with freezing of the retrieved sperm. Thawed testicular sperm can be used later when the female partner is stimulated. Although this approach seems appealing, it is not without concerns. First, freezing can be difficult in some cases when very few sperm are retrieved or when only immotile sperm can be harvested. Second, sperm freezing and thawing is usually associated with decline in motility. In some cases of functional azoospermia when only few twitching sperms are seen in the testicular tissue homogenate, post thawing recovery of motile sperm can be impossible. Motility is a sign of viability. The use of immotile sperm is associated with less favorable fertilization and pregnancy rate. Finally sperm freezing is associated with increased sperm DNA damage (5).

Retrieved testicular sperm can be cultured in different type of media for 24-72 hours prior to oocyte retrieval. This will help to decrease the work load and allows for better work organization in busy laboratories. In addition it enables physicians to withhold the HCG injections in negative cases and avoid unnecessary risk of ovarian hyperstimulation. Most of the studies on testicular sperm culture are done on cases with obstructive azoospermia where sperm motility increases during culture to reach the maximum within 24-72 hours. In cases with functional azoospermia the effect of culture is unpredictable. The increase in sperm motility is demonstrated in only 30% of the cases and in cases with totally immotile sperm, it does not develop any motility post incubation. Furthermore, immotile but viable sperm as demonstrated by supra vital stain loosen their viability during incubation (7). Thus culturing sperm in cases of functional azoospermia might end with no available sperm for ICSI. It is documented that short and long term sperm
incubation is associated with increased apoptosis associated DNA damage (8). This can increase pregnancy loss in spite of the good fertilization and pregnancy rate. There is no clear justification for sperm culture prior to ICSI. In cases of obstructive azoospermia motile sperms are readily available, while, in functional azoospermia the results are unpredictable.

Physicians have to cautious when advising sperm recovery procedure before the egg collection especially in cases of functional azoospermia, knowing that in 23% of the cases with positive first TESE, no sperm can be retrieved in the second attempt (9). Also second sperm retrieval procedure is preferably postponed for 3-12 month following the first one to allow complete healing of the testis and the resumption of testosterone level (10).

Open multi-site testicular biopsy TESE performed on the day of ICSI still the golden standard although other method of sperm recovery or scheduling might have advantages, the value of these alternative techniques are either needs further evaluation or is hampered by disadvantages.

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