Evaluation & Treatment of Men with Non-obstructive Azoospermia

Azoospermia due to low sperm production (non-obstructive azoospermia) affects approximately 1% of the male population and 10% of men who seek fertility evaluation. Testis biopsy reveals that these men have Sertoli cell-only pattern, maturation arrest, or hypospermatogenesis. Until recently, it was assumed that men with non-obstructive azoospermia were untreatable. Indeed, these patients were often referred to as being "sterile" or having "testicular failure." The only way these couples could have children was to use donor spermatozoa or to adopt. Several observations have changed our approach to this condition. First, we have observed that direct evaluation of testis biopsy specimens often demonstrates sperm in men with non-obstructive azoospermia, despite severe defects in spermatogenesis (Jow et al., 1993). This observation has been interpreted to mean that a low level of sperm production may be present in the testes of men with azoospermia, but the sperm do not survive epididymal transit and ejaculation.

In addition, it was previously thought that sperm must traverse the male reproductive tract before acquiring the ability to normally fertilize an egg. The success of treatment of men with obstructive azoospermia using sperm extracted from the epididymis or testis has changed this view. Although testicular sperm have dramatically lower motility than those that have transited the male reproductive tract, these sperm can be used for intracytoplasmic sperm injection (ICSI) during in vitro fertilization (IVF). Such observations led investigators to perform testicular sperm extraction (TESE) with ICSI for men with non-obstructive azoospermia. Low pregnancy rates of 20 to 21% per attempt have been reported. The following is our approach to evaluation and attempted sperm retrieval for men with non-obstructive azoospermia.

Patient Evaluation

To determine if a semen sample is truly azoospermic, centrifugation of the semen sample with meticulous microscopic examination of the pellet is necessary. Although this might seem obvious, Ron-El et al. (1997) reported that sperm was found on extended sperm analysis of a centrifuged semen specimen in up to 35% of men who were thought to have non-obstructive azoospermia. In addition, we have found that up to 10-20% of men who have inadequate sperm on preoperative semen analysis will actually have sperm usable for ICSI on the day of oocyte retrieval. Therefore, *we always repeat a semen analysis on the day of planned sperm retrieval for men with non-obstructive azoospermia.*

For all patients with azoospermia, a complete history and physical examination is necessary to identify potentially correctable causes of male factor infertility. Typically, the man with non-obstructive azoospermia will have small testes (< 15 cc) with a flat epididymis. Some men may have a history of cryptorchidism. Hormonal evaluation of a man with non-obstructive azoospermia (NOA) will typically demonstrate an elevated serum FSH, with normal or nearly normal testosterone and estradiol levels. Prior to further intervention, we will usually treat any correctable abnormalities that are found on evaluation of a man with NOA, including surgical repair of large varicoceles, correction of hormonal abnormalities, and avoidance of gonadal toxins for at least three

months prior to attempted TESE. Orchiopexy and varicocele repair are usually considered if female age is less than 38, because with advanced female age the window of opportunity to achieve pregnancy is limited and these procedures benefit only a small percentage of men and require six months of recovery prior to attempted sperm retrieval.

With advanced female age (>38) the window of opportunity to achieve a pregnancy is more limited, making orchiopexy and varicocele repair less worthwhile because they benefit only a small percent of men and require six months of recovery prior to attempted TESE.

Scrotal ultrasound

The ASRM/AUA Practice Guidelines do not recommend routine application of scrotal ultrasound for evaluation of the infertile male, although ESHRE guidelines do support routine ultrasound examination. Scrotal ultrasound should be strongly considered for patients with a history of cryptorchidism or prior germ cell tumors, and for men who have any unexplained abnormalities on testicular examination. In addition, it should be considered if the patient's body habitus limits physical examination of the scrotum or if the findings on physical examination are equivocal. Routine use of a scrotal ultrasound to screen for subclinical varicoceles is not recommended, as repair of very small varicoceles has not been shown to be effective in improving semen parameters or fertility. Ultrasound may also be of value in following patients after testicular biopsy, since intratesticular hematomas or scar tissue formation commonly occur and affect the timing for subsequent sperm retrieval procedures. We particularly appreciate the value of scrotal ultrasound for men who have had prior surgery to evaluate for intratesticular abnormalities including inflammation, hematoma, detection of postoperative scar and other, unexpected findings. This may be particularly helpful for patients travelling from a distance to Cornell for treatment, where initial physical examination is not done at our center.

Genetic Abnormalities and Testing

Genetic abnormalities include both chromosomal abnormalities, detectable with routine karyotype testing, and Y chromosome microdeletions, so called "AZF defects." Other rare genetic causes of male infertility are nicely reviewed in Mak and Jarvi: *J Urology* 156:1245-57, 1996. For men with severe male factor infertility, including sperm concentrations less than 10×10^6/cc and non-obstructive azoospermia, karyotype evaluation and Y chromosome microdeletion analysis is recommended before treatment with assisted reproduction.

Evaluation of a sequential series of 170 men with non-obstructive azoospermia who were candidates for TESE at Weill Cornell revealed that 17% of these men had definable genetic defects (Y chromosome microdeletions or karyotype abnormalities; Rucker et al., 1998.) We have found that the knowledge of having a genetic defect leads many men to pursue options other than TESE-ICSI and that, regardless of treatment choice, the majority of men find it reassuring to know the cause of their infertility. For men with AZFa or AZFb defects, the prognosis for sperm retrieval is important to consider before proceeding with IVF and attempts at sperm retrieval.

Karyotype evaluation

The most common karyotypic abnormality in men with severe male factor infertility is Klinefelter syndrome, affecting up to 7-13% of azoospermic men. Almost all men with the "pure, classic form" (47,XXY) of Klinefelter will be azoospermic, whereas limited sperm production is commonly found in men with a mosaic pattern of Klinefelter syndrome. It was previously felt that only spermatogonia with a 46,XY complement could produce spermatozoa (Martini et al., *Hum Reprod* 11:1638, 1996); however, other observations suggest that a significant proportion of 24,XY spermatozoa are present in the testes of men with Klinefelter (Cozzi et al., *Hum Genet* 93:32, 1994). General teaching has suggested that men with Klinefelter syndrome can be readily identified by their typical physical appearance of tall stature, gynecomastia and small, firm testes. Again, however, clinical observations contradict this statement. I have detected a number of men in my practice who were normally masculinized, between 5'6" and 5'10" in height, but had non-mosaic Klinefelter syndrome. Observations reported by Oates et al. (*J Urol* 155:476A, abstract 660; 1996) also confirm that some men with chromosomal abnormalities will have an otherwise normal phenotypic appearance except for their infertility. Most men with Klinefelter syndrome have sperm retrievable with testicular sperm extraction (TESE) and can have children with ICSI. We have attempted treatment of over 15 men with this condition and eight children have been born to these couples. Other karyotypic abnormalities identified include Robertsonian translocations, chromosomal inversions and other sex chromosome abnormalities.

Y chromosome (AZF) microdeletions

Specific regions of the long arm (Yq) of the Y chromosome are commonly deleted in men with non-obstructive azoospermia or severe oligospermia. Vogt et al. have suggested that three relatively discrete regions of Yq, AZFa, AZFb, and AZFc, are deleted in severely infertile men and that the deleted region determines the chance of sperm production. Reijo et al. have shown similar deletions in men with different levels of sperm production, suggesting that other genetic or environmental factors may affect the phenotype of sperm production in men with Y chromosome microdeletions. Subsequent studies have shown that regions AZFb and AZFc overlap, with a common deletion occurring that involves both segments of AZFb and AZFc (AZFb/c deletion.) Since most men have a specific region of the Y chromosome deleted and we have attempted treatment of a large proportion of these men, we can provide significant clinical information on the significance of a Y chromosome microdeletion on sperm retrieval results.

As noted above, Y chromosome deletions affecting fertility usually involve deletion of one or more of the entire AZFa, AZFb, or AZFc regions. Deletions involving *b1/*b3 and *gr/gr* regions have also been described. These areas are within or adjacent to the AZFc region. The limited deletions of *b1/b3* and *gr/gr* appear to have no prognostic significance and little effect on sperm production. An additional region of the Y chromosome referred to as AZFd has been described, however, AZFd is within AZFc, again, has no prognostic significance, is not associated with impaired sperm production, and therefore such deletions are clinically irrelevant. **The specific region that is missing on the Y chromosome may provide prognostic significance.** Approximately two-thirds of men with deletions involving only AZFc, have sperm present in the ejaculate. So, if we know nothing else about an infertile man except that he has an AZFc deletion, then this is a very encouraging prognostic sign – most of the men will have enough sperm in the ejaculate to proceed with ICSI using ejaculated sperm. In azoospermic men with AZFc deletions,

sperm production is commonly present within the testicle, and TESE is as successful as for other men with non-obstructive azoospermia. At Weill Cornell, sperm was found by TESE in 70% of men with AZFc deletions and azoospermia..

For men with deletions involving the AZFb region, the chance of having sperm in the ejaculate or finding sperm with TESE is severely decreased. Sperm was found in zero out of 23 men we evaluated with deletions involving AZFb who had a biopsy or sperm retrieval attempted with TESE (Hopps et al., 2003). TESE should not be routinely recommended for men with complete deletions of the AZFb region of the Y chromosome because the chance of sperm retrieval is extremely low.

Deletions involving the entire AZFa region are relatively rare, but are typically associated with a Sertoli cell-only pattern on diagnostic biopsy (Kamp et al., 2001). The need to discriminate between partial and complete deletions of an AZF region is reflected in the observation that at least one patient with a deletion of part of the AZFa region had germ cells on testis biopsy, however, to-date **no sperm has been retrieved from men with complete deletions of AZFa or AZFb**. Because the documented number of cases in the literature is limited, absolute predictive statements are not possible to make at this time. However, the prognosis is clearly different and dramatically worse for men with complete AZFa and AZFb deletions than for other patients with non-obstructive azoospermia.

Because Y chromosome abnormalities, including deletions, will be passed on to any male child who is produced after assisted reproduction, these men must have genetic counseling prior to treatment. Since men with these genetic defects have rarely or never fathered children naturally, it is uncertain whether any medical conditions will be present in the offspring with Y chromosome microdeletions, except for infertility. This knowledge makes genetic counseling difficult. On the other hand, common sense suggests that because the fathers are otherwise healthy and normal, the presence of a Y chromosome microdeletion does not pose a high risk for major congenital defects in potential offspring.

<u>Hormonal therapy</u>

Hormonal therapy has never been demonstrated in randomized controlled trials to increase sperm production or quality in men with normal hormone levels. Therefore, *I use hormone therapy only in men with demonstrated hormone abnormalities.* Hormonal evaluation of a man with non-obstructive azoospermia (NOA) will typically demonstrate an elevated serum FSH, with normal or nearly normal testosterone levels**.** However, many men with NOA have an abnormal testosterone (ng/dL) to estradiol(pg/mL) ratio (T/E_2 ratio), which is correctable with treatment using aromatase inhibitors like testolactone or anastrozole (Pavlovich et al., 2001). Of note, testolactone is no longer clinically available through most areas of the world. Whereas normal fertile men will have a T/E_2 ratio of 16 ± 3; men with NOA have a ratio of 7, and men with Klinefelter syndrome have a ratio of 4. The inbalance is correctable with aromatase inhibition, or delivery of exogenous testosterone, suggesting that increased aromatase activity comes from the testis, where relative hyperplasia of Leydig cells within the testicle may be the cause of this phenomenon. Correction of abnormal T/E_2 ratios in men with severe oligospermia can effect dramatic improvements in sperm concentration and motility. We now routinely evaluate testosterone and estradiol levels for men with non-

obstructive azoospermia or severe oligospermia. Men with low testosterone and a low T/E_2 ratio are routinely treated with anastrazole, one milligram per day (Raman & Schlegel, 2002.) Letrozole 2.5 mg/day may be equally effective in increasing endogenous testosterone production, but it is such an effective aromatase inhibitor that it may cause greater side effects (decreased libido; Schlegel, F&S, 2012).

The response of patients to hormonal therapy may predict the chance of sperm retrieval as well. In our experience FSH, LH, and testicular volume had no predictive value for sperm recovery, at least in the subset of men with KS Many men with Klinefelters will have low baseline testosterone levels that do not predict the chance of sperm retrieval. However, the preoperative testosterone serum level after three months of medical treatment is predictive of success of sperm retrieval by micro-TESE in men with Klinefelters. Men with low baseline testosterone who responded to medical therapy with a resultant testosterone of greater than 250 ng/dl had a 77% chance of sperm retrieval versus 55% for men that did not respond to therapy initiated to enhance testosterone production. Medical therapy in those who responded appeared to identify a subgroup of men with sperm retrieval rates similar to that in men with baseline normal testosterone. The benefit, if any, for patients who responded to medical therapy appeared to occur within one-two months of treatment to optimize their testosterone levels. Of note, the baseline FSH level in Klinefelter patients did not affect the chance of sperm retrieval (Ramasamy et al., 2009).

Varicocele repair

The role of the varicocele in male infertility continues to be controversial. As early as 1987, the World Health Organization stated that varicoceles are associated with decreased male fertility. Numerous uncontrolled studies have suggested increased sperm count, motility and morphology after varicocele repair. However, few randomized controlled studies have documented a beneficial effect of varicocele repair on male fertility. A recent meta-analysis published in Lancet reported little beneficial effect of varicocele repair on male fertility (Evers & Collins, 2003). Unfortunately, this review included studies in which patients had normal semen parameters or were treated for "subclinical varicoceles," a practice that is not supported by even the most ardent supporters of varicocele repair.

Of more relevant interest is the increasing practice of varicocele repair for men with non-obstructive azoospermia. Studies have reported a return of sperm to the ejaculate in 20-43% of men with non-obstructive azoospermia followed for up to 24 months. Unfortunately, when you have a patient with non-obstructive azoospermia, a repeat semen analysis finds sperm even if no intervention occurred. Similarly, rare sperm in the ejaculate can be lab errors – it is only important when enough motile sperm for ICSI are in the ejaculate to avoid surgery to extract sperm. When we reviewed a series of patients with varicoceles and non-obstructive azoospermia who were evaluated and treated at Weill Cornell, we looked at the critical outcome – does varicocele repair improve sperm production enough so that you don't have to do sperm retrieval? Of 31 men who underwent varicocele repair for documented non-obstructive azoospermia, 20% (7/31) had sperm on at least one postoperative semen analysis. However, only 9.6% (3/31) of men after varicocele repair had adequate motile sperm in the ejaculate for ICSI (without TESE). A history of prior varicocele repair did not affect the results of TESE, for men with non-

obstructive azoospermia and varicoceles. Retrospective analysis of patients with clinical varicoceles identified before TESE shows that the rate of sperm retrieval was identical in those who had their varicoceles repaired before TESE as compared with those who did not have them repaired before TESE, 60% (41/68) and 60% (42/70) respectively (Schlegel & Kaufman, 2004.) Although some other studies have suggested that sperm retrieval rates can increase with varicocele repair, this observation has not been confirmed with prospective randomized trials. Based on these data, varicocele repair is of limited value for men with non-obstructive azoospermia and varicoceles. In my opinion, surgical repair is probably most helpful for men who are younger (or at least their spouse is younger, so she has a longer period of time to conceive) and the man has large varicoceles, and possibly for those with testicular atrophy associated with a varicocele.

Diagnostic biopsies in men with presumed non-obstructive azoospermia

The diagnosis of NOA can only be definitively made on testicular biopsy. Testis biopsy can also rule out the unlikely possibility of testicular intratubular germ cell neoplasia (carcinoma-in-situ) that is more common in men with unexplained unilateral testicular atrophy, history of contralateral germ cell tumor (testicular cancer) or with a history of cryptorchidism.

All men with non-obstructive azoospermia have impaired sperm production and will have normal testis biopsies, usually with a predominant pattern showing a Sertoli cell-only pattern. **The most advanced spermatogenic pattern, as opposed to the predominant pattern, appears to affect the results of sperm retrieval.** For men who had at least one area of hypospermatogenesis present on diagnostic testis biopsy, spermatozoa were retrieved in 81% (57/73) of attempts, whereas for men with maturation arrest as the most advanced pattern, spermatozoa were retrieved in only 44% (27/62) of attempts. If the entire diagnostic biopsy had a Sertoli cell-only pattern, our most recent data found that sperm could be retrieved for 41% (98/257) of TESE attempts. Although no finding absolutely determined sperm retrieval or negated the possibility of successful TESE, the findings of diagnostic biopsy were helpful in evaluating the chance of success with TESE (Ramasamy et al., 2005). In addition to the role of diagnostic biopsy in identifying rare cases of intratubular germ cell neoplasia (carcinoma-in-situ) and confirming the diagnosis of non-obstructive azoospermia, diagnostic biopsy helps to predict the chance that a TESE procedure will obtain sperm. However, since no finding on biopsy absolutely precludes the chance of sperm retrieval, we do not routinely perform a biopsy – it just doesn't tell you whether sperm are present in the testicle because there are so many different sections of the testicle, and a biopsy samples very little of that tissue. Microdissection TESE (microTESE) is necessary to search though the multiple areas of the testicle to find sperm.

When do you perform a diagnostic testis biopsy before TESE for ICSI?

Testicular biopsy provides limited benefit in determining the prognosis for sperm retrieval, and it will not provide definitive proof of whether sperm will be found with a more intensive evaluation of the testis (TESE or microdissection TESE). *Therefore, we prefer not to perform a diagnostic testis biopsy prior to TESE-ICSI for non-obstructive azoospermia.* A diagnostic biopsy should be performed if the etiology of azoospermia is not clear, if the risk of carcinoma-in-situ is

high (rare), or if the results of biopsy will affect the couple's choice to undergo TESE-ICSI. The reasons to determine the etiology are multiple:

1. Some men with obstructive azoospermia prefer reconstruction to sperm retrieval-ICSI.
2. The genetic abnormalities associated with obstructive and non-obstructive azoospermia are different, and appropriate genetic testing cannot be done without knowing which defects to search for.
3. There is a risk of not finding sperm in men with non-obstructive azoospermia. These couples should be counseled regarding the potential use of donor spermatozoa if sperm are not identified in the man. It is, likewise, inappropriate to discuss the use of donor sperm with men who have normal sperm production and a 100% chance of sperm retrieval.
4. The procedure of sperm retrieval is different in obstructive & non-obstructive cases.

If a biopsy is performed, testicular tissue should be frozen for possible subsequent use. I perform diagnostic testis biopsies if a couple will not proceed to TESE-ICSI with only 35-40% chance of sperm retrieval.

What if a previous biopsy/TESE attempt did not find sperm?

We recently retrospectively examined the chance of sperm retrieval with microdissection TESE after prior random biopsies had failed to find sperm in an azoospermic man. **The chance of sperm retrieval with microdissection TESE was no different for men who had had no prior biopsy, or if one or two random biopsies per testis failed to find.** If 3 or more biopsies were done per testis and no sperm were found, then the chance of successful microdissection TESE decreased to only 22%. In no case, even with multiple random biopsies, did a prior "non-microdissection" approach to biopsy preclude the chance of success with microdissection TESE. Even if Sertoli cell-only was found on 4 or more biopsies per testis, the chance of finding sperm elsewhere with microdissection TESE. This observation supports the effectiveness of this technique to find rare areas of sperm production within the failing testis (Ramasamy & Schlegel, J Urol 177:1447, 2007).

Considerations before treatment

Sperm retrieval by testicular sperm extraction (TESE) can be performed prior to or coincident with an IVF cycle for the female partner. *We prefer to perform microTESE procedures on the day prior to oocyte (egg) retrieval during a programmed IVF cycle to maximize the potential to retrieve viable spermatozoa for use with ICSI and avoid the need to freeze sperm.* Therefore, the female partner must be evaluated by the IVF center and ovarian stimulation initiated before the TESE procedure.

Sperm retrieval is occasionally done on the same day as egg retrieval at our center when logistically necessary or there is a high chance of finding sperm in the ejaculate on the morning of planned sperm retrieval. In a retrospective analysis, we compared pregnancy outcomes for couples where the man had NOA and sperm retrieval was done on the day of oocyte retrieval or the day before. For 149 cases involving "same-day" retrievals, the clinical pregnancy rate was 43%,

whereas the pregnancy rate was 45% in 265 cases where sperm were retrieved on the day before oocyte retrieval (with sperm incubated overnight in sperm-wash medium.)

Because men with NOA have marginal sperm production, TESE procedures should be delayed for at least 6 months after any intervention such as a prior biopsy or TESE procedure, or other inguinal/scrotal surgery (Schlegel & Su, 1997). I commonly wait for 1 or 2 years to allow complete healing if multiple biopsies were previously done, an extensive microdissection procedure was previously required, or extensive scar within the testis is noted on scrotal ultrasound or after clinically detectable postoperative bleeding/infection. Because the testicular blood supply penetrates the tunica albuginea and then disperses in a series of end-arteries that spread over the testicular parenchyma, multiple biopsies should be avoided to minimize the risk of devascularization of the testis. The use of optical magnification may also minimize the risk of testicular injury. We have seen two men who have had significant devascularization of the testis with atrophy after TESE performed at other centers using multiple biopsy technique (Schlegel & Su, 1997).

Fresh or Frozen?

Unfortunately, no preoperative parameter absolutely predicts the chance of sperm retrieval by TESE in men with non-obstructive azoospermia. Testicular volume and FSH levels have no predictive value, and as discussed above, even the histology of a diagnostic biopsy cannot absolutely predict sperm retrieval when the rest of the testis is sampled. Unfortunately, ovarian hyperstimulation is necessary for all women, even though there is, on average, only a 60% chance of sperm retrieval. Many couples choose to have donor sperm ready to use, in case sperm is not retrieved, to maximize each IVF cycle. . There are several good reasons to use simultaneous TESE with ICSI:

1. *Sperm retrieval is difficult in men with non-obstructive azoospermia (NOA.)*

A single testicular biopsy is inadequate to retrieve sperm in most men with NOA. We found that less than one-half of successful sperm retrievals were accomplished on the first random biopsy. Careful dispersion of the testicular specimen and simultaneous evaluation by an experienced embryologist is needed to determine how many biopsies are needed. We have noted that sperm may not be found until the fourteenth random testicular biopsy in men with NOA (Ostad et al, 1998.) Microdissection TESE will increase the proportion of men with NOA who have sperm found over that achieved with random biopsies alone. As noted above, microdissection TESE will often succeed despite previous failed multi-biopsy TESE. A controlled comparison of microdissection versus random biopsies demonstrated that approximately one-third of men with sperm present in the testes have unsuccessful treatment with random biopsies alone. In addition, far less tissue is removed with microdissection, compared to random biopsies, despite having a higher yield of sperm (Schlegel, 1999.) Despite a single larger incision, less intratesticular reaction has been seen (on ultrasound) after microdissection when compared to a multiple biopsy approach (Ramasamy et al, Urology 65:1190, 2005). Therefore, it is now incorrect to consider TESE effectively performed with only a single (or even multiple) biopsy(ies) per testis.

2. *Testis biopsy before the ICSI cycle is often an unnecessary operation.*

Many men who have had repeated azoospermic semen analyses will actually have enough sperm present in their ejaculate on the day of sperm retrieval to cancel a planned TESE procedure. For men at Weill Cornell, 10-20% of planned TESE-ICSI cases for "non-obstructive azoospermia" are cancelled because adequate numbers of viable sperm are found on the morning of the planned TESE procedure. Ron-El et al. have reported that up to 35% of men with NOA have sperm found on an extended sperm preparation, allowing men to avoid the unnecessary testicular procedure. It is not clear whether comparable pregnancy results would occur for these rare, and/or non-motile sperm from the ejaculate.

3. *Testicular sperm from men with NOA will often not survive freeze-thaw.*

Our experience is that testicular spermatozoa retrieved by biopsy from men with well-documented NOA will usually be non-viable after thawing. A total of 95 attempted ICSI cycles using frozen-thawed testicular sperm specimens were evaluated. All men with sperm retrieved in this series had non-obstructive azoospermia. Motile sperm were documented prior to cryopreservation. Out of the 95 cycles, only 33% (31/95) had viable sperm from the frozen-thawed sample. All other patients required repeat TESE. For the 31 cycles with frozen-thawed sperm alone, a clinical pregnancy rate of only 39% and a live birth rate of only 26% were observed. The clinical pregnancy rate with fresh sperm is 47%, and live birth rate is 31%. Not only do sperm frozen from men with non-obstructive azoospermia usually not survive freeze-thaw, but they appear to be less successful than freshly-retrieved sperm in effecting pregnancies.

Another group from Germany reported that *only 42% (178/426) of attempts at using frozen testicular tissue had enough motile sperm present after thawing to inject all oocytes.* A total of 38% (161/426) of attempts had no motile sperm present for injection, and the remainder of the cycles required use of immotile spermatozoa because inadequate motile sperm were present in the thawed specimen to inject all oocytes (Fisher et al., [abstr O-190] ESHRE annual meeting, 1998).

4. *Testicular sperm from men with NOA have impaired function*

Fisher et al. further noted a dramatically lower pregnancy rate of 8% (+hCG/transfer) when immotile (frozen-thawed?) spermatozoa were used for injection for immotile sperm cycles, versus a pregnancy rate of 30% when motile frozen-thawed testicular spermatozoa where used. A higher spontaneous abortion rate after implantation was also seen for immotile sperm. Of additional concern, Ron-El et al. noted poor embryo cleavage after use of immotile spermatozoa, despite reasonable fertilization rates, suggesting that testicular sperm from men with NOA may have impaired function after freeze-thaw (abstract O-181, ESHRE annual meeting, 1998). These findings suggest that **use of immotile frozen-thawed spermatozoa (the only sperm available for ICSI in most cases) causes poor embryo development with lower implantation and pregnancy rates, even if fertilization is effected.** The poor results with these sperm suggest that the initial TESE cycle may be wasted (again, a potentially unnecessary operation) since most sperm don't survive freeze-thaw with motility.

5. *Subsequent ICSI cycles may need to await the potential for repeat TESE retrieval.*

Since poor results are obtained with non-motile frozen-thawed sperm, and most samples do not have enough motile spermatozoa to inject all oocytes, the back-up option of repeat TESE retrieval should be available in most cases to allow optimal results. Unfortunately, **repeat sperm retrieval is not certain, and may require up to 6 months after the initial TESE to optimize the chance of obtaining sperm.** In our experience, repeat TESE cycles retrieve sperm in only 25% of cases if performed within 6 months, versus 80% of cases if performed after 6 months of an initially successful TESE for NOA. Note, however, that the 80% success rate means that repeat TESE does not always yield sperm, even if they were obtained previously. (Schlegel & Su, 1997.) In occasional cases where better sperm quality is seen, intentional use of the frozen sperm could be attempted. Whether sperm can be used after freeze-thaw may be determined based on a test-thaw sample or on the initial sperm number and quality.

Although simultaneous sperm retrieval-ICSI carries the risk of ovarian hyperstimulation without the availability of testicular sperm, the above observations clearly suggest that the best results will be obtained using fresh testicular sperm for men with NOA. Unsuccessful retrieval attempts may be salvaged with donor sperm if couples choose to avail themselves of that option.

TESE (Testicular Sperm Extraction)

On the day before oocyte retrieval, scrotal exploration is performed through a median raphe incision under local or general anesthesia, and sperm are retrieved using an open testicular biopsy technique. In order to confirm accurate identification of the testis and to avoid any injury to the epididymis, delivery of the testis is routinely performed. Testicular blood vessels in the tunica albuginea are identified with 8-15x optical magnification. An avascular region near the midportion of the medial, lateral or anterior surface of the testis is chosen, and a generous incision in the tunica albuginea, avoiding any capsular testicular vessels, is created to directly examine a wide area of testicular parenchyma. The greatest risk of damage to testicular tissue is not exposure or devascularization of tissue during the procedure but bleeding that occurs inside the testis after the operation. Therefore, wide exposure is used to facilitate very careful hemostasis using bipolar cautery and prevent postoperative bleeding within testicular tissue that leads to scar formation and loss of testicular function.

Microdissection TESE

The technique that we developed allows the removal of tiny volumes (2-3 mg) of testicular tissue with improved sperm yield. This technique requires use of an operating microscope and there is a learning curve to optimize the technique of safe dissection within tissue as well as to identify which tubules contain sperm. The tubules containing sperm can often be visually identified under an operating microscope after opening the testis, when 15-20x magnification is used to assist the biopsies. This approach 1) improves the yield of spermatozoa per biopsy, 2) results in less tissue removal (and loss of testicular function), 3) makes the embryologist's job easier in finding sperm, since less tissue has to be examined and 4) allows identification of blood vessels within the testicle, minimizing the risk of vascular injury and loss of other areas of the testis (Schlegel, 1999). Our observations of better sperm yield when using microdissection TESE (versus multiple random biopsy TESE) has been confirmed by several other investigators (Amer et al., 2000; Okada et al., 2002; Okubo et al., 2002).

The excised testicular biopsy specimen is placed in human tubal fluid culture medium supplemented with 6% Plasmanate. With this approach, sperm yield can be enhanced 2-fold per biopsy specimen (from 64,000 to 164,000 sperm/specimen), despite excision of 70-fold less testicular tissue (722mg versus 9.4mg). These results were demonstrated for a series of men with non-obstructive azoospermia who underwent standard testicular biopsies as controls in the same testes that were used for microdissection TESE. In a sequential series of patients who had sperm retrieval attempted with standard multi-biopsy TESE procedures vs. TESE with microdissection, the proportion of non-obstructive azoospermic men with sperm retrieved increased from 36% (4/11) to 68% (15/22) with the application of microdissection. For five men, 33% (5/15 men with sperm found with TESE), sperm could only be found in the testis with microdissection. Microdissection is of no therapeutic benefit for approximately 40% of attempted TESE cases, because no enlarged (normal) tubules are seen. Unfortunately, most of these cases also failed to yield spermatozoa with multiple standard biopsies. The use of microdissection, however, does still expedite sampling of testicular tissue, since multiple samples of seminiferous tubules can be rapidly examined and excised.

Handling of biopsy samples

When standard (larger volume) testicular biopsy samples are obtained, isolation of individual tubules from the mass of coiled testicular tissue is achieved by initial dispersal of the testis biopsy specimen between two sterile glass slides, which compress the testicular parenchyma to isolate individual seminiferous tubules. Subsequently, mechanical disruption of the tubules is accomplished by mincing the extended tubules with sterile scissors in HTF/Plasmanate medium (Schlegel et al., 1997). Additional dispersion of tubules is achieved by passing the suspension of testicular tissue through a 24-gauge angiocatheter (Ostad et al., 1998). For minimal tissue specimens, limited dissection is performed in the operating room, because the tissue sample is so small. Since spermatozoa are normally within the tubules, at least some of the tubules are cut and passed through a 24-gauge angiocatheter. These samples are then immediately examined in the operating room under phase contrast at 100-200x magnification to determine whether adequate numbers of sperm have been found for ICSI.

Dissection is stopped when it has been reliably determined that enough sperm for ICSI are retrieved. This process involves evaluating (and therefore discarding) a very small proportion (usually 5-7 microliters of each 300-500 microliter sample) of the tissue that has been removed to see if sperm are present in that sample. Although the sperm identified on that slide are lost because it's examined with phase-contrast microscopy under a cover slip, so the sperm cannot be retrieved, this process is the most efficient way to identify if sperm are present and If no spermatozoa are seen, then 1) additional samples of tissue are obtained through the same tunical incision, and 2) contralateral samples are obtained, if needed. The initial sample of testicular tissue identifies sperm in nearly ½ of cases. Dissection of all areas of the initially exposed tissue is successful in identifying sperm for about 60% of the cases where sperm are found, but dissection through the remainder of the testicular tissue is necessary to find sperm for about 30% of cases, and sperm are found in the contralateral testicle for about 8% of men, even if no sperm are found anywhere in the first testicle examined. Extended efforts to remove large numbers of sperm from the testis is of

limited value, since the sperm may not survive freeze-thaw. After ICSI has been performed, excess aliquots of tissue are then processed for cryopreservation.

Testicular tissue processing in the laboratory

If no sperm are seen on initial examination of the testicular samples in the operating room, then further processing and examination of the tissue is done in the IVF laboratory. The sample is allowed to sediment and the supernatant is then centrifuged at 1,800xg to identify any rare spermatozoa in the liquid portion of the sample. The tissue portion is then enzymatically digested to identify rare sperm. The suspension of tissue is pretreated with an erythrocyte-lysing (hypo-osmotic) buffered solution for 5 minutes at 37ºC before digestion. Collagenase/DNAse (0.1%) is prepared and mixed 1:1 (v:v) with the testicular tissue suspension for one hour at 37ºC. The tubes are agitated every 10-15 minutes to facilitate digestion.

After digestion is complete, low speed centrifugation (50xg) for 5 minutes is used to pellet the undigested tissue and separate the supernatant. The enzymatic solution is removed by adding an equal volume of sperm wash medium to the suspension and centrifugation at 1,800xg with removal of the supernatant. Resuspended pellets are mixed thoroughly and microdroplets are made for sperm searching and ICSI. Appropriate dilution of the suspension is necessary to allow enough dilution to identify sperm within the cellular material, but excess dilution creates a very large volume of tissue to search. Multiple embryologists search for an appropriate period of time (up to several hours each) to identify any sperm and prepare the sperm for ICSI.

IVF and ICSI

The general ICSI procedure is well described in standard textbooks. Aggressive immobilization of spermatozoa, a technique that increases fertilization rates for immature sperm, is often used for testicular spermatozoa.

Results

Results with ICSI are primarily dependent on the availability of viable sperm and age of the female member of the couple being treated. Encouraging experience has been obtained at Weill-Cornell with TESE-ICSI in the past 1,414 attempted treatment cycles for couples in whom the man had NOA. The mean age of patients entering treatment was 35.5 years for men and 30.4 years for women. In men, the initial mean serum FSH level was 25.3 IU/L (normal, 1 to 8 IU/L), and average testicular volume 13 ml. During the past 1,414 attempted TESE-ICSI cycles, sperm were retrieved for injection in 794 (56%) cycles (52% (607/1176) retrieval rate per-patient). For those cycles in which sperm were retrieved, the fertilization rate per injected oocyte was 51% (4,423 of 8,705). Our embryo transfer rate was 94 %. Clinical pregnancies were established in 48% of the cycles, and live deliveries occurred in 41% of couples. With multiple gestations were seen in 10% of pregnancies.

No etiology of azoospermia provided an absolute predictor for the presence or absence of sperm within the testes, except for AZFa and AZFb deletions. Testicular volume and serum FSH levels did not predict sperm retrieval.

The results for treatment of 127 men with classic and mosiac KS (47,XXY, or mosaic patterns that do not include 46,XY) who underwent attempted sperm retrieval during simultaneous 155 ICSI cycles at our institution are presented herein. Sperm were found in 65% (100/155) of the fresh retrieval attempts. Our per-patient success rate of sperm retrieval for these 127 men was (77/127) 61%. Embryo transfer occurred in 83% cases, with clinical pregnancy and fertility of 40% and 40 children born to-date. A multiple gestation rate of 31% has been seen in these pregnancies. Results did not differ for mosaic or non-mosaic patients. All children have been healthy (46,XX girls and 46,XY boys.). These findings illustrate the potential for TESE-ICSI to provide fertility despite underlying genetic abnormalities. Pre-treatment testicular biopsy histology was not helpful in distinguishing who would succeed with microdissection TESE for patients with Klinefelter syndrome. Although a majority of men had Sertoli cell-only on diagnostic biopsy, 70% of these patients had sperm found on subsequent microdissection TESE. Even though two of the patients treated had previously undergone multiple random biopsy TESE with no sperm found, sperm were retrieved in a subsequent procedure using the microdissection TESE technique. These findings illustrate the potential for TESE-ICSI to provide fertility despite underlying genetic abnormalities.

Another treated subset of men with nonobstructive azoospermia includes the cohort of men with a history of chemotherapy administered for a variety of diagnoses who underwent sperm retrieval attempts with TESE for persistent nonobstructive azoospermia. All men were azoospermic and at least six years post-chemotherapy at the time of treatment. In a cohort of 93 men with a history of chemotherapy administered for a variety of diagnoses underwent 114 sperm retrieval attempts for persistent NOA. Thirty of the 93 (32%) patients had also received extragonadal radiation. Sperm were successfully retrieved in 48% (55/114) of micro-TESE attempts, with clinical pregnancy occurring in 40% of couples. Per-patient sperm retrieval rate was 42% (39/93). Men treated for lymphoma had a sperm retrieval rate per cycle of 44%, whereas after treatment for germ cell tumor the retrieval rate was 70%. Diagnostic biopsy was not helpful in determining the prognosis for sperm retrieval. Most patients had predominantly Sertoli cell-only pattern, even if germ cells were present in some seminiferous tubules. No correlation was noted between the outcome of TESE-ICSI and the specific chemotherapeutic agents used.

Several case reports have described successful pregnancies after TESE with ICSI for men with NOA associated with cryptorchidism. While the undescended testicle results in a loss of germ cells, including spermatogonia with subsequent NOA. The treatment with an orchiopexy, have multiple risk factors and might cause ischemic insult to the testicle. It is believed that orchiopexy has no benefit for seminiferous tubules that have undergone irreversible degeneration. However, it serves to preserve the foci of germ cells capable of normal spermatogenesis. Micro-TESE allows for the harvesting of such foci that may have otherwise gone undetected. In 152 men, 181 micro-TESE procedures were performed. At Weill Cornell, spermatozoa were successfully retrieved in 116/181 (64%) attempts at sperm retrieval, pregnancy rate was 50%, and the delivery rate was 38% with 8 spontaneous abortions. In this subgroup, the per-patient sperm retrieval rate was 62% (94/152).   In our cohort, 39% had bilateral cryptorchidism, and 4 patients with history of other genetic abnormality. The sperm retrieval rate in men with a history of bilateral cryptorchidism was 62%.

Genetic testing for Y chromosome microdeletions is of prognostic significance for TESE procedures. For men with complete deletions of the AZFb region the chance of sperm retrieval during TESE is severely impaired. In our experience, 0 of 23 men with Y chromosome partial deletions involving all of AZFb had sperm retrieved with TESE, whereas the sperm retrieval rate in a contemporary series of men with non-obstructive azoospermia but no AZFb deletions was 67% (85/126). The presence of a complete deletion of AZFa appears consistently associated with Sertoli cell-only and a poor chance of sperm retrieval (Kamp et al., 2001). Of the men with complete deletions of the AZFa region, who had diagnostic biopsies or TESE at our institution, zero out of ten had sperm found. Therefore, for men with complete AZFa or AZFb deletions, we do not recommend proceeding with TESE (Hopps et al., 2003).

Pregnancy rates for ICSI using sperm from men with Y microdeletions appear to be very similar to those obtained for couples with similar sperm production. For men with AZFc deletions alone (the only Y-deleted patients who have had sperm in our experience), most (75%) men will have at least rare sperm in the ejaculate. For those men who are AZFc-deleted and azoospermic, most (50-75%) will have sperm found with biopsy or microTESE (Hopps et al., 2003). We recently reviewed a series of 27 IVF cycles involving men with AZFc microdeletions who were azoospermic (12 cycles) or severely oligospermic (15 cycles.) The clinical pregnancy rates per cycle were comparable to that obtained for non-affected men from azoospermic (TESE cycles) and oligospermic men (ejaculated sperm.) All children born were phenotypically normal, but we expect all boys to have deletions involving the AZFc region, with resulting impairments in spermatogenesis (Choi et al., Fertil Steril 81:337, 2004).

Should sperm retrieval be done in men who have sperm in the ejaculate?

In general, evidence from most ICSI studies has indicated that fertilization and pregnancy results are independent of sperm quality or number. However, more recent data has suggested that fertilization and pregnancy rates can be affected by sperm source or sperm quality (Palermo et al., 1999.) Several studies have shown that fertilization and pregnancy rates are lower when testicular sperm from men with non-obstructive azoospermia are used, when compared to results obtained with ejaculated sperm or epididymal sperm obtained by MESA from men with obstructive azoospermia (normal production). In addition, sperm from men with cryptozoospermia (so few sperm in the ejaculate that none are seen on an initial evaluation, but only after centrifugation and concentration of the sample) have lower fertilization and pregnancy rates than that obtained for men with higher numbers of spermatozoa in the ejaculate. Indeed, the results with cryptozoospermia are worse than those for men who have testicular sperm retrieved in non-obstructive azoospermia. Since most men with cryptozoospermia have sperm readily retrieved from the testis, it is worth considering sperm retrieval to obtain testicular sperm for ICSI in at least some men with cryptozoospermia who have had poor fertilization, embryo development and pregnancy results.

For couples with a prior failed IVF cycle where there is concern about a male factor contributing, we will evaluate the man for correctable abnormalities (toxin exposures, hormonal abnormalities, varicoceles, etc) and test sperm for excess aneuploidy (FISH or sperm aneuploidy testing) as well as sperm DNA fragmentation tests (TUNEL or SCSA.) If significant sperm DNA

abnormalities are detected, then retrieval of sperm from the testis may be considered. We have done testicular sperm extraction for abnormal ejaculated sperm DNA integrity in 25 men as part of a repeat attempt at IVF at Cornell. Whereas the mean testicular sperm DNA fragmentation rate was 25% in the ejaculated sample, it was only 5% in these same men when testicular sperm was analyzed. Whereas the prior IVF attempt had failed, the pregnancy rate was 53% using testicular sperm from these men. So, in select cases where abnormal sperm DNA fragmentation is identified in ejaculated sperm, testicular sperm may provide better results with ICSI.

We continue to use viable ejaculated sperm for any initial ICSI attempt at Weill Cornell rather than proceeding to surgical retrieval. However, for selected couples with cryptozoospermia, or evidence of partial obstruction, who have had poor fertilization and/or embryo development using ejaculated sperm in a prior cycle AND abnormal sperm DNA fragmentation, sperm retrieval may be worthy of consideration. If sperm extraction is done, then we always prefer to compare the ejaculated sample (obtained on the day of sperm retrieval) to testicular sperm DNA fragmentation rates (using the TUNEL assay) to confirm that better sperm quality was obtained and to guide us in selecting sperm to use for any future ICSI attempts.

What complications can occur after testicular sperm extraction?

Although testicular sperm extraction using a microdissection TESE technique is invasive, it appears to be a very safe and well-tolerated procedure. The procedure is typically done under general anesthesia because it may take several hours and patient movement under the microscope can disrupt the operation. The risk of internal bleeding or hematoma (which can present as an appearance of infection after 1-2 weeks or longer) is 2-3%, depending on how many prior scrotal surgical procedures a patient has had. Since many men have low or borderline testosterone levels, the effect of surgery on testosterone levels is of significant concern postoperatively. Indeed, many men with non-obstructive azoospermia have such low testosterone levels before any intervention that testosterone replacement therapy may be indicated. After surgery, testosterone levels drop on average by 5-10%, typically returning to baseline over 6-18 months. Testosterone levels decrease enough after surgery to require testosterone replacement in 5-10% of men who undergo microTESE at our center, typically only if multiple or extensive dissection procedures were required. Testosterone replacement will typically restore these patients to normal health without any symptoms, but life-long treatment may be required.

How can this procedure be made easier for the patient?

We have carefully studied different approaches to microTESE surgery to make the operation effective and limit pain, complications and other potential side effects of the operation. Prevention of postoperative bleeding within the testicle (by intraoperative use of bipolar cautery) is one of the most important interventions to facilitate patient recovery. Preoperative use of anti-inflammatory medications is also very effective in reducing postoperative pain and effects of surgery. Celecoxib (200 mg, twice a day), starting the night before surgery, is highly effective at limiting pain, reducing need for pain medications and speeding recovery of patients in a randomized, controlled trial of patients undergoing TESE at our institution.

Summary

Sperm retrieval is an effective adjunct for treatment of azoospermic men, including men with ejaculatory failure. At Weill Cornell, retrieval of sperm from men with ejaculatory failure or obstructive azoospermia is nearly certain. Using these sperm, pregnancy rates of 56% and 73% have been achieved for ejaculatory failure and obstruction, respectively, when advanced assisted reproduction, ICSI, is applied.

Sperm retrieval for use with ICSI, is now also possible for most men with non-obstructive azoospermia. Men with NOA may have unique genetic defects that should be evaluated prior to an attempt at conception. We have now attempted TESE for nearly 1,600 men during programmed IVF cycles at our institution. At New York-Presbyterian Hospital-Weill Cornell Medical Center, sperm is retrieved from the testis with TESE in 56% of couples despite non-obstructive azoospermia, and 48% of couples achieve a clinical pregnancy using this sperm and ICSI. Many of the patients we treat at Cornell have previously failed an attempt at testicular sperm extraction. The chance of sperm retrieval in non-obstructive azoospermia is enhanced with the application of a microdissection technique. Since some couples will not have sperm retrieved with TESE, the potential use of frozen donor spermatozoa should be discussed with couples as a back-up, prior to simultaneous TESE-ICSI attempts. Couples in which men have obstructive azoospermia tend to have higher fertilization rates, and subsequent pregnancy rates than do couples in which men have non-obstructive azoospermia, in part because of the better genetic composition and quality of their sperm, a subject that will be discussed in later presentations.

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