New concepts in operative andrology: a review*

SHERMAN J. SILBER

Infertility Center of St. Louis, St. Luke's Hospital, St. Louis, MO 63017, USA

Summary

Several recently published, or about to be published, controversial issues in operative andrology are clarified and reviewed in this paper. The microsurgical technique for sperm retrieval for nonobstructive azoospermia, the round spermatid controversy and the varicocoele dilemma (why does everybody keep doing varicocoelectomy for male factor infertility?) are presented with salient points that have recently been presented elsewhere and referenced. Finally, at the end, we review briefly what is known about the likelihood of genetic transmission of infertility from male factor patients to their offspring as a result of the new ICSI technology.

Keywords: testicular sperm extraction, ICSI, microsurgical

Introduction

This review summarizes recent developments in operative andrology. In 1993, we first introduced testicular sperm extraction (TESE) and microsurgical epididymal sperm aspiration (MESA) with intracytoplasmic sperm injection (ICSI) for the treatment of obstructive azoospermia (Schoysman et al., 1993; Devroey et al., 1994; Silber et al., 1994, 1995a). It was soon discovered that this technique could also be used for azoospermic men with deficient spermatogenesis, i.e. nonobstructive azoospermia (Devroey et al., 1995; Silber et al., 1995b, 1996, 1998a). In the majority of cases of non–obstructive azoospermia, occasional mature spermatids are noted in the testis biopsy of men who might have been thought to have had no spermatogenesis. We discovered that there is a certain threshold of a minimum amount of spermatogenesis that is necessary for spermatozoa to 'spill over' into the ejaculate (Silber et al., 1995a, 1997; Silber & Rodriguez-Rigau, 1981).

Extensive multiple biopsies from every area of the testis are often performed in an effort to find sufficient sperm for TESE (Tournaye et al., 1994). This can result in a great deal of testicular damage, and may even limit 'successful' patients to only one attempt (Schlegel et al., 1997). Some try to limit damage by using a needle rather than open biopsy to obtain

sperm for ICSI (Craft et al., 1997). However, control studies have shown that for difficult cases of non-obstructive azoospermia, where spermatogenesis is very meager, needle biopsy is much less likely to find the rare foci of spermatogenesis for ICSI than open biopsy (Friedler et al., 1997, 1998; Rosenlund et al., 1998)

In similar studies, Ezeh also showed a dramatic improvement in success using open biopsy vs. needle with TESE-ICSI in non-obstructive azoospermia (Ezeh et al., 1998). Yet some andrologists prefer not to risk future attempts at TESE with a massive open biopsy procedure.

We studied the distribution of spermatogenesis in azoospermic men, and have outlined a microsurgical approach to TESE that minimizes tissue loss and pain, and makes TESE very easily repeatable for an indefinite number of cycles. Knowledge of the distribution of spermatogenesis and use of microsurgical technique will make TESE more successful in difficult cases, and will prevent testicular damage and postoperative pain, making multiple repeat procedures simple and reliable (Silber, 2000).

The diagnosis of testicular failure is based on the finding of azoospermia, the absence of obstruction and a quantitative analysis of testicular histology (Steinberger & Tjioe, 1968; Zukerman et al., 1978; Silber & Rodriguez-Rigau, 1981; Silber et al., 1990, 1997). Both the number of tubules containing sperm (i.e. mature spermatids) and the number of sperm (i.e. mature spermatids) per tubule are counted.

Men with non-obstructive azoospermia caused by germinal failure have a mean of only 0–3 mature spermatids/

seminiferous tubule seen on a diagnostic testicle biopsy, compared with 17–35 mature spermatids/tubule in men with normal spermatogenesis and obstructive azoospermia (Silber & Rodriguez-Rigau, 1981; Silber *et al.*, 1990, 1997). More than half of azoospermic patients with germinal failure thus have some minute foci of spermatogenesis, which are not of sufficient quantity to produce spermatozoa in the ejaculate. When spermatogenesis exceeds three mature spermatids per tubule, the patient has sperm 'spill-over' into the ejaculate, and then is oligospermic rather than azoospermic. The methodology for these cases is described in Figs 1 and 2 (Silber, 2000).

The issue of 'spermatid' vs. 'sperm' extraction needs some clarification. There has been a great deal of confusion generated by the use of expressions like 'mature spermatids' per tubule. What the pathologist refers to on histological sections as 'mature spermatids' are, in fact, what appear to the embryologist at TESE to be sperm. On histological sectioning, the tail of the sperm is seldom seen, and only the thicker sperm head shows up in thin sections. But it is not a tailless sperm. It is just that only the oval-shaped head is observed on histology. By mature spermatid, we do not mean 'round cell'. The solution to cases where there are no sperm to be seen on TESE is not to look for 'round spermatids' (Silber & Johnson, 1998). When we refer on histology to 'mature spermatids per tubule', we are talking about what appear on TESE simply to be sperm.

Microsurgical TESE procedure

All microsurgical cases are performed under local anesthesia only. This involves both cord block and local infiltration of the incision line in the scrotum. The procedure is truly painless. The tunica vaginalis is opened and the testicle exteriorized. The operating microscope is then used under 16–40 × magnification for the rest of the procedure. This technique has been used either for multiple testis biopsy samplings from different regions of each testis, or for removal of large contiguous strips of testicular tissue, after microdissection and evaluation of tubular dilation. It is also used for tiny microscopic removal of single dilated tubules.

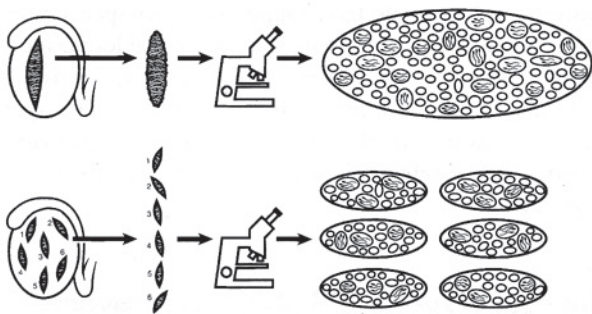First, an attempt is made to remove tubules that appear ectatic and more opaque, and then if no sperm are found

with this approach, then large strips of tissue (no greater than the total amount of tissue that would have been removed in the conventional 'blind' TESE technique) can be excised if necessary. Quantitative histology is performed for mapping and compared with microsurgical observation. The tunica albuginea is then closed with 9–0 nylon interrupted sutures, after meticulous hemostasis with microbipolar forceps. This prevents any increase in intratesticular pressure, resulting in minimal pain and no subsequent atrophy.

Microsurgical TESE observations

With maturation arrest tubule size and opacity were uniform, just as with normal spermatogenesis (Silber, 2000). Therefore, it was impossible with the operating microscope in maturation arrest to pick out the few tubules that exhibit full maturation to mature sperm, and distinguish them from those tubules without sperm.

In cases of Sertoli cell only, the tubules were collapsed, and yellow staining on the outside of the tubules, representing Leydig cell hyperplasia, was clearly visible. If sperm is present, it can be determined by the surgeon under the operating microscope by observing tubules that are not collapsed. Of the total cases subjected to microsurgical TESE for nonobstructive azoospermia, 68% have sperm recovered. In Sertoli cell only, microsurgical dissection allows removal of a very minuscule amount of testicular tissue to find this sperm. In maturation arrest, often a larger amount of tissue had to be removed.

None the less, even in those microsurgical cases where relatively large amounts of tissue have to be removed, minimal damage is incurred because blood supply is not interrupted, microscopic bleeders are meticulously coagulated, tunica albuginea is not encroached upon because of the closure with 9–0 nylon interrupted stitches, and consequently there is no increase in intratesticular pressure. This results in no testicular damage, and minimal pain.

There are varying concentrations of spermatogenic foci in the different patients with non-obstructive azoospermia. Some are so sparse as to readily explain cases where a random open biopsy might miss the spermatogenic foci in 15% of cases (Silber *et al.*, 1997), and why random needle
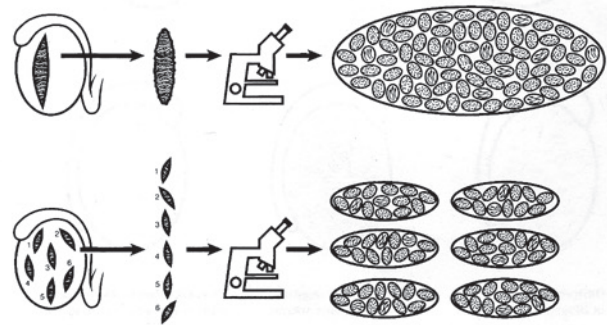

Figure. 1 Depiction of experimental methodology: azoospermic (Silber, 2000).

Figure. 2 Depiction of experimental methodology: oligospermic (Silber, 2000).

biopsy would miss these foci in about 86% of cases (Friedler *et al.*, 1997; Friedler, 1998). Figures 3 and 4 summarize the distribution of spermatogenesis in various types of azoospermic patients.

Our direct mapping gives evidence for a diffuse rather than regional distribution of spermatogenesis in non-obstructive azoospermia (Silber *et al.*, 1997). Furthermore, the variation in sparseness of spermatogenesis verified by observation of contiguous strips of testicular tissue, explains why a single random biopsy may or may not yield sperm, and why removal of very small amounts of tissue blindly with a needle has a high success rate with obstructive azoospermia, but a low success rate with non-obstructive azoospermia. Microsurgical preselection of a small spermatogenic focus observed under the operating microscope truly trivialized the testicular damage. The latter is obviously preferable.
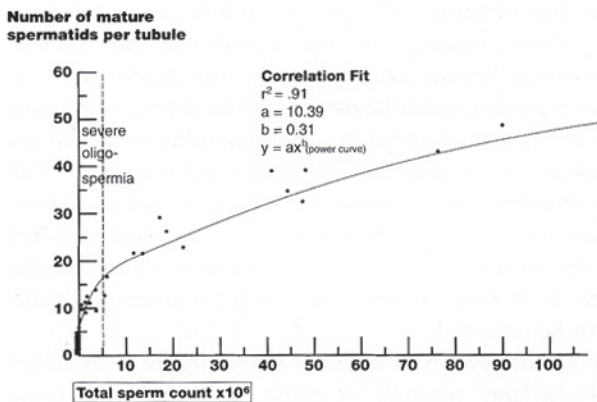
Number of mature spermatids per tubule

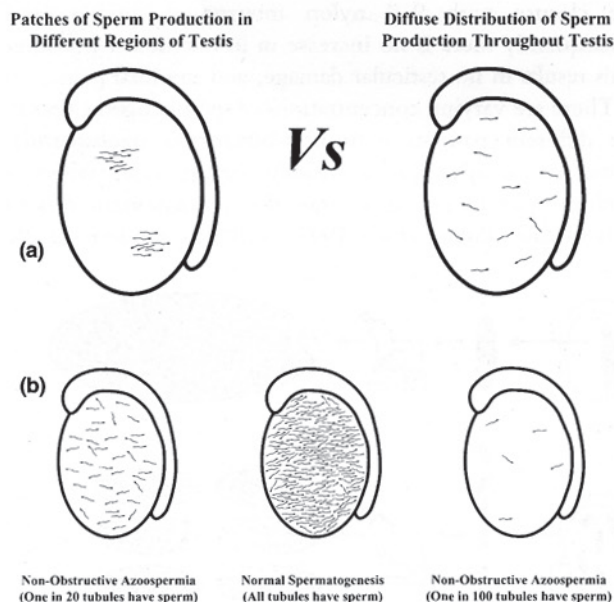**Figure. 3** Quantitative testicle biopsy and sperm count (Silber *et al.*, 1997).

Figure. 4 (a) Distribution of spermatogenesis in the testis. (b) Degrees of azoospermia (Silber *et al.*, 1997).

However, even in cases where the only solution is removal of a larger amount of testicular tissue, microsurgery still provides a major advance. The formidable testicular deterioration that has been observed with overly aggressive TESE procedures is caused by either direct interference with microvascular supply of the seminiferous tubules or even more commonly, increased intratesticular pressure caused by minor amounts of bleeding within the enclosed tunica albuginea. The tunica albuginea is a very non-flexible enclosure. A small degree of intratesticular bleeding causes a noticeable increase in intratesticular pressure. This can be readily observed by anybody doing conventional, multiple testicle biopsy samplings for TESE. Furthermore, the closure of open biopsies with the usual non-microsurgical suture, particularly in a running fashion, further compromises the intratesticular volume and thereby adds to the increased pressure. With a single, routine, conventional diagnostic testicle biopsy, the damage may not be readily noticeable. But with multiple or extensive biopsies, the damage can be considerable (Schlegel & Su, 1997).

A microsurgical approach to TESE in all cases we have performed results in no sign of increased testicular pressure for three major reasons. The first is that it is easier to avoid the interruption of blood supply to different regions of the seminiferous tubules. A second reason, which is extremely important when larger amounts of tissue need to be removed, is that meticulous hemostasis can be achieved with microbipolar forceps by having proper microsurgical visualization of the cut areas. A third reason that microsurgery allows minimal tissue damage even when larger pieces of the testis have to be removed, is microsurgical suturing of the tunica albigunea with 9–0 nylon interrupted stitches. This microsuturing technique, particularly when using interrupted stitches, allows for an accurate closure of the tunica albuginea without any compromising of the intratesticular space. Thus, whatever amount of tissue that is removed is the only tissue loss that the patient need suffer from his TESE procedure.

An awareness of the quantitative distribution of spermatogenesis can improve the efficiency of TESE. In cases with extremely sparse foci of spermatogenesis, a random open biopsy may miss spermatogenic focus even though a more extensive sampling might have found occasional sperm. Our routine therefore is to first attempt microsurgical localization in order to remove the smallest amount of testicular tissue, and if this is not successful, resort to removing larger amounts of tissue. If the latter approach is necessary, however, microsurgical technique will still minimize the damage incurred.

The round spermatid (ROSI) controversy

We have previously expressed skepticism about round spermatid injection (ROSI) in the current human IVF

setting (Verheyen *et al.*, 1998; Silber & Johnson, 1998). We do not find round spermatids in TESE specimens in which we do not also find elongated sperm with tails. We find many 'round cells' in all TESE specimens, but in the absence of sperm, these are not round spermatids (Silber *et al.*, 1997).

The classic histologic appearance we see in human maturation arrest reflects no normal progression beyond spermatocyte. There are also apoptotic cells with clumped chromatin. The abundant round-appearing cells along the basement membrane of the tubule are simply Sertoli cell nuclei with their typical prominent nucleoli. These are the 'round cells' typically seen in the majority of cases of non-obstructive azoospermia (Silber & Johnson, 1998). We have only found round spermatids when elongated forms are also present (Silber *et al.*, 1997).

Our observation is based not only on phase contrast evaluation of TESE microdroplets, but also on detailed stained histology of thousands of testis specimens, and on the previous literature on the histology and pathology of maturation arrest (Johnson *et al.*, 1981, 1992; Schulze *et al.*, 1998; Soderstrom & Suominen, 1980; Silber *et al.*, 1996, 1997). The acrosomal vesicle under phase contrast provides an easy identification marker for round spermatids on wet microdroplet ICSI preperations (Holstein & Roosen-Runge, 1981; Verheyen *et al.*, 1998). the absence of round spermatids in wet TESE preparations based on phase contrast visualization of the acrosomal vesicle is conformatory in the ICSI setting to what has already been demonstrated in fixed tissue stained specimens over many years.

We question whether 'human round spermatids can be identified in the native state by simply respecting the criteria of cell size (*approximately* that of red blood cells) and by detecting the presence of a round nucleus surrounded by a rim of cytoplasm' in wet preps with Hoffman optics (Tesarik, 1998). The notion that round spermatids can be readily distinguished from Sertoli cell nuclei, Leydig cells, spermatogonia and other TESE components, with standard Hoffman optics, and without any need to identify an acrosomal vesicle, just by looking at size, is very doubtful. The Sertoli cell nucleus has a diameter of about 10 μm on average; red blood cells and round spermatids have an average diameter of about 8 μm; spermatogonia average 9 μm; and pachytene spermatocytes on average are a much larger 12 μm. However, the intrinsic variability of several microns in many of these cells makes size alone a very unreliable way to distinguish (for human clinical ICSI) which cells to inject into the human egg.

Many serious IVF centres have been struggling in vain to reproduce the clinical success reported by just a few authors (Tesarik, 1998; Antinori *et al.*, 1997; Barak *et al.*, 1998; Fishel *et al.*, 1995; Tesarik *et al.*, 1995; Tesarik, 1998; Amer *et al.*, 1997). Some centres have experienced and reported consistently negative results with ROSI with desperate patients in whom no mature sperm or elongated spermatids could be found (Ghazzawi *et al.*, 1998; Ghunaim *et al.*, 1998). Many other centres perform ROSI regularly but understandably fail to report their negative results. We have visited these centres and seen the confusion regarding which round cells to inject. We are aware of the very low success rate (1%) with round spermatid injection even in the successful mouse model with normal spermatogenesis (Ogura & Yanagimachi, 1993; Ogura *et al.*, 1993, 1994). Furthermore, the mouse, whose centriole does not derive from the male, may be more favourable than primates such as the human for round spermatid fertilization.

The varicocoele dilemma

The lack of effect of varicocoele on pregnancy rate following vasovasostomy

In 1989, we reported a 10-year follow-up of men who had sperm in the vas fluid (i.e. without secondary epididymal blowouts), undergoing vasovasostomy (Silber, 1989b). This experience was the origin of my scepticism regarding the value of varicocoelectomy. Out of 282 patients undergoing vasovasostomy 10 or more years earlier, 42 (14.8%) had a discernible varicocoele upon physical exam, and 240 (85.2%) had no varicocoele on physical exam. These men had no other medical or surgical treatment over more than a 10-year period from 1976 to 1989 other than their vasovasostomy, 78.5% of those men with varicocoele (not operated upon) impregnated their wives, and 81.2% of those without varicocoele impregnated their wives. There was no statistically significant difference between the 78.5% pregnancy rate with varicocoele vs. the 81.2% pregnancy rate without varicocoele over a period of greater than 10 years. There was also no difference in postoperative semen parameters. Our conclusion from this study was that the presence of a varicocoele did not have any discernable long-term effect on fertility. In 1997, Mulhall *et al.*, performed varicocoelectomy simultaneously with vasovasostomy in vasectomy reversal patients who had varicocoele and also noted no difference (Mulhall *et al.*, 1997).

Evidence-based practice of medicine

Nieschlag stated in 1995 a basic axiom that needs to be followed in male infertility treatment, 'Therapeutic interventions in male infertility should be based on properly controlled clinical trials' (Nieschlag *et al.*, 1995). In 1993, Hargreave reported that despite alarmingly low sperm counts, women can get pregnant without treatment of the male (Hargreave, 1993), verifying concepts that have been clear for many years. The interesting thing about his observation is that 33% of the men in this category had a varicocoele and did not have time to undergo varicocoelectomy surgery before their wife became pregnant.

Furthermore, contrary to popular myth, there is no 'characteristic morphologic stress pattern in infertile men with large left varicocoeles' (Baker *et al.*, 1985). These concepts were discussed earlier by Steinberger and his group (Zukerman *et al.*, 1977; Smith *et al.*, 1977; Steinberger & Rodriguez-Rigau, 1983).

To understand the importance of a controlled study in evaluating the validity of varicocoelectomy, one only has to look at the spontaneous conception in the wives of men with various low sperm counts. The major interest in these papers is not the issue of varicocoelectomy, but the issue of just 'what is male infertility' (Hargreave & Elton, 1983; Silber, 1989a). Even in men with sperm counts as low as 1 000 000 per cm^3, with a duration of infertility of 4 years, in 9% the wives eventually have a spontaneous conception without ever having any improvement in the sperm. In men with sperm counts of 5 000 000 per cm^3 with only 1 year of infertility, 36% impregnate their wives successfully without any treatment. If one had performed a varicocoelectomy on such men prior to their wife's conception, we might very well conclude that the varicocoelectomy is what caused the pregnancy even though it was simply a spurious, unrelated event. Nieschlag concluded in 1998, as did Mordel in 1990, 'Studies since 1952 advocating varicocoelectomy have been uncontrolled and not evidence-based' (Mordel *et al.*, 1990; Nieschlag *et al.*, 1998).

Controlled studies on varicocoelectomy

Nieschlag performed one of the most meticulously controlled studies ever attempted to determine the effect of varicocoelectomy, and reported it in 1995 and again with greater numbers in 1998 (Nieschlag *et al.*, 1995, 1998). They very meticulously studied 125 infertile couples with varicocoele. Sixty-two of those couples underwent varicocoelectomy and 63 underwent counselling. There was no significant difference in pregnancy rate measured over time between those couples that simply underwent varicocoelectomy and those couples that underwent psychological counselling. Furthermore, Nieschlag's group found no relationship of pregnancy to semen parameters, hormone levels, grade of varicocoele or the age of the male. The only relationship to pregnancy rate was the age of the wife and that was the only factor that could help predict what the chances were for pregnancy. We discovered a similar phenomenon in the treatment of obstructive and non-obstructive azoospermia with sperm retrieval and ICSI. The only factor that significantly affected the variation in pregnancy rate in couples undergoing ICSI with retrieved sperm was the age of the wife (Silber *et al.*, 1997). The most important confounding factor aside from duration of infertility, which probably does not matter in azoospermic couples, is the age and ovarian reserve of the wife.

In the controlled varicocoele study by Baker *et al.* (1985), there was no significant difference in the sperm concentra-tion or motility after varicocoelectomy (Baker *et al.*, 1985). There was also no difference in pregnancy rate after varicococoelectomy.

The meticulous studies of Nieschlag's group in 1995 and 1998, and the study a decade earlier by Baker *et al.* (1985), seemed to negate any overwhelming enthusiasm for varicocoelectomy on the part of infertility physicians, even though there is still registered throughout the urology world a strong defensive posture regarding varicocoelectomy. Earlier controlled studies have also shown negative results (Vermeulen *et al.*, 1986; Rodriguez-Rigau *et al.*, 1978; Nilsson *et al.*, 1979; Rageth *et al.*, 1992).

Varicocoelectomy and sperm count

MacLeod and Gold as far back as 1953 first demonstrated that sperm concentration and motility tend to increase with time with repeated testing in oligospermic and asthenospermic men despite no treatment being given (MacLeod & Gold, 1951). Baker *et al.* were the first to clearly and mathematically explain this phenomenon of 'regression toward the mean' (Baker *et al.*, 1981, 1984). When patients have a controlled period followed by a treatment period, there is likely to be a significant improvement even if the treatment is ineffective. 'In a similar fashion, sperm motility increased in men with varicocoeles whether or not they had testicular vein ligations performed' (Baker *et al.*, 1985). No matter what the treatment, whether erythromycin or watchful waiting, clomiphene citrate or varicocoelectomy, an initially low sperm count because of intrinsic variability, will always gravitate higher because of 'regression toward the mean'.

Studies that favor varicocoelectomy

There have been three 'control' studies that suggested a beneficial effect of varicocoelectomy. Marmar *et al.* in 1994, reviewed retrospectively a series of 455 varicocoelectomies and only 19 controls (Marmar *et al.*, 1994). The pregnancy rate in the 455 couples that underwent varicocoelectomy was 35.6%. The pregnancy rate in the small number of 19 'controls' that did not undergo varicocoelectomy was 15.8%. The enormous difference in the size of the varicocoelectomy group and the 'control' group certainly suggests a great deal of bias. This heavily skewed population would make it very likely that the 'controls' were simply people whose semen was so poor that there was no desire to undergo surgery, or possibly there may have been a problem with the wife that made surgery also very problematic.

Another so-called 'control' study often referred to is that of Girardi and Goldstein, in which 1500 infertile males underwent varicocoelectomy, and only 47 controls did not undergo varicocoelectomy (Girardi & Goldstein, 1997). Again bias must have affected such a huge difference in the size of control and varicocoelectomy population. They reported a 43% pregnancy rate in couples in whom the

husband had a varicocoelectomy and a 17% pregnancy rate in those whose husbands did not have a varicocoelectomy. They also noted an improvement in sperm count from 40 000 000 per cm^3 to 47 000 000 per cm^3. This is not a very dramatic increase in mean sperm count and is most likely simply related to 'regression toward the mean'.

The WHO study from 1997 was an attempt to settle the varicocoele issue employing 238 couples who were split off from thousands that were originally enrolled in a multicentre trial design. This study was never published in its original form because of problems with protocol deviations. It is very difficult in multicentre studies with a highly controversial subject to be certain that all programmes that are serving their patients in the way that they think is best can stick to a rigid protocol. However, such a rigid protocol would be necessary in order to give credibility to this WHO study (WHO, 1992). The group that pulled out of the WHO study did publish their results for only 45 of these 238 couples who were split off from the original group of 7000. These authors maintained that varicocoelectomy did have a beneficial effect. But such a splitting off from the original study group of five times as many patients as originally started, after a previous splitting off from 30 times the original group that started, obviously suffers from a great risk of both selection and observer bias (Madjar et al., 1995).

Goldstein suggested that in 22 initially azoospermic men, the varicocoelectomy was able to bring up their sperm count from zero to very low levels that allowed for an occasional spontaneous pregnancy, without ICSI being necessary. The problem with this study again is that there is no control group, no longitudinal follow-up and no recognition to the phenomenon of 'regression toward the mean'. For example, with patients who are initially azoospermic, a small number (maybe as many as 5% or 10%) will eventually, in subsequent semen analyses, have sperm in the ejaculate without any treatment. Without a control group to compare, one should not be terribly surprised at a spontaneous pregnancy rate of 9%–23% without any treatment, particularly if the couple has had a short period of infertility, and/or if the wife is very young (Hargreave & Elton, 1983; Schoysman, 1983; Silber, 1989a).

Does varicocoele cause a progressive decline in fertility?
Uehling et al. as far back as 1968 studied the fertility of 440 men in the military coming in for routine physical examination not officially complaining of infertility, normal men both with and without varicocoeles. There was no difference in fatherhood of those young military recruits who had varicocoele vs. those who had varicocoele and who were married. The presence or absence of a varicocoele in these young men had no influence on whether or not they were able to impregnate their wife (Uehling, 1968). But could a varicocoele cause a progressive decline in fertility later in life?

It has been commonly thought that secondary infertility was due to increased age and declining fertility of the female

(Nieschlag et al., 1995, 1998; Silber et al., 1997). Gorelick and Goldstein, however, have suggested that a varicocoele is found in 35% of men with primary infertility, and in 81% of men with secondary infertility (Gorelick & Goldstein, 1993). This is a huge incidence of finding a varicocoele in infertile couples. These authors have suggested that this finding meant that over time the presence of a varicocoele causes a diminution in sperm quality and indeed is the major cause of the secondary infertility. Therefore, they recommend varicocoelectomy for virtually all young men with a varicocoele in order to prevent subsequent decline of testicular function. However, our vasovasostomy follow-up study already referred to argues against that proposition (Silber, 1989a). There was no difference in pregnancy rate or sperm parameters with long-term follow-up of older vasovasostomy patients who did or did not have a varicocoele.

It should be noted that the sperm counts of men with secondary infertility in Gorelick and Goldstein's study were for the most part normal. Thus, there was no demonstrated decline in sperm count caused by the varicocoelectomy, but rather simply an increased incidence of varicocoele found in this group of couples. Furthermore, most patients with 'secondary' infertility have normal semen parameters, but the wife is older, and that is considered the major cause of secondary infertility.

It thus appears fairly conclusive that varicocoelectomy does not do much, if anything, to help the average infertile couple. That should not be controversial. I also think it is very unlikely that varicocoele has a long-term damaging effect on fertility. However, the latter is a fascinating concern, and our minds must be open to further study of that conjecture.

Y chromosome deletions in azoospermic and severely oligospermic men

The Y chromosome plays an important role in male germ cell development. We have always suspected a genetic etiology to most cases of male infertility (Silber et al., 1995a; Silber, 1989a, 1998b). Only recently has this concept become widely accepted. Y deletions encompassing the AZFc region were originally reported by us to be found in 13% of azoospermic men and 7% of severely oligospermic men (Reijo et al., 1995). Because of the effectiveness and widespread adoption of ICSI, we wished to determine what impact such Y deletions might have on the severity of testicular defects in infertile men undergoing TESE and ICSI, what effect these Y deletions might have on the results and whether this common genetic cause of male infertility would be transmitted to offspring as a result of ICSI (Silber, 1998a). In fact, we suspect that these Y deletions represent only the 'tip of the iceberg', and that the current success with ICSI in treating male infertility may result in greater infertility in future generations (Silber, 1999).

Studying these gross microdeletions on the Y chromosome has implications beyond these few cases that are easy to discern with current methods. There are probably at least 36 genes, only one-third of which are primarily on the Y chromosome that affect spermatogenesis, some on autosomes and some on the X chromosome. Because of multiple copies of these genes, particularly on the Y, the more common point mutations are currently difficult to find (Chao et al., 1999).

In azoospermic or oligospermic men who are Y-deleted, deletions limited to AZFc are generally associated with the presence of small numbers of testicular or ejaculated sperm (Silber et al., 1998a). Larger Y deletions, including but extending beyond the AZFc region and encompassing more Y genes, tend to be associated with a total absence of testicular sperm. These results support the concept that several genes on the Y chromosome in addition to those of the AZFc region impinge on spermatogenesis. Multiple spermatogenesis genes on the Y chromosome may contribute to and modify the severity of the spermatogenic defect in men with deletions encompassing the AZFc region. However, in those cases where sperm are found, the presence of Y deletions has no impact on fertilization or pregnancy rate.

In our laboratory, about 13% of men with non-obstructive azoospermia, and 7% of men with severe oligospermia, are deleted for a particular portion of the Y chromosome, the AZFc region, which contains the DAZ gene cluster (Reijo et al., 1995, 1996; Saxena et al., 1996). A number of laboratories have subsequently confirmed these submicroscopic deletions of certain portions of the Y chromosome in azoospermic and severely oligospermic men (Pryor et al., 1997; Vogt et al., 1996, 1997; Ma et al., 1993; Girardi et al., 1997; Mulhall et al., 1997; Kremer et al., 1997; Vereb et al., 1997; van der Ven et al., 1997; Foresta et al., 1997; Chai et al., 1997; Elliot et al., 1997; Nakahori et al., 1996; Qureshi et al., 1996; Najmabadi et al., 1996; Simoni et al., 1997). These deletions have been shown to be new mutations not present in fathers or brothers, nor in normal fertile controls. All of these results suggest that genes on the Y chromosome play an important role in spermatogenesis.

Over the course of the last 350 million years of mammalian evolution, and the evolution of the X and Y chromosomes, spermatogenesis genes have transposed, or retropositioned themselves from autosomes to the Y and there amplified into multiple copies. These include DAZ and CDY. Other genes have persisted from their original position on the X and achieved greater prominence on the Y, such as RBM, and indeed even the SRY gene, which was originally SOX-3 on both the X and the Y. Genes associated with SRY, on its evolving Y chromosome, that were specific for male function, flourished on the Y because of the preference on the Y for 'male benefit genes' (Silber, 1999; Lahn & Page, 1997, 1999a, b).

Using the polymerase chain reaction (PCR), we searched for Y chromosome deletions in azoospermic or severely oligospermic men by testing genomic DNAs for the presence or absence of as many as 52 DNA landmarks (STSs, or sequence tagged sites) distributed across the entirety of the Y chromosome. All tests were performed on DNAs purified from blood leukocytes or lymphoblastoid cell lines. All Y-DNA markers employed had been placed previously on a physical map of the chromosome; the markers represented all known genes and gene families in the non-recombining region of the Y chromosome (Vollrath et al., 1992; Foote et al., 1992; Vogt et al., 1997; Lahn & Page, 1997).

Y deletions limited to intervals 6D–6F (the AZFc region) were generally associated with finding some sperm at TESE, and larger Y defects (extending more proximally and/or more distally) were associated with finding no sperm at TESE (Silber et al., 1998a). Also, all Y deletions in severely oligospermic (not azoospermic) men were strictly limited to the AZFc region. There was a clear trend toward larger deletions causing more severe spermatogenic defects. The oligospermic men who were Y-deleted also all had small deletions limited to intervals 6D–6F. When the deletion extended beyond AZFc, encompassing additional testis-specific genes, no sperm could be found.

When sperm were recoverable in azoospermic or oligospermic men, there was no significant difference in pregnancy rate with ICSI whether the man was Y-deleted or Y-intact. In azoospermic men, the pregnancy rate was 29% for Y-intact and 20% for Y-deleted cases. In oligospermic men the pregnancy rate was 46% for Y-intact and 50% for Y-deleted cases (Silber et al., 1998a).

The histological classification of the azoospermic men did not appear to correlate in a straightforward way with the extent or site of deletion. Of the 10 patients with deletions limited to AZFc, three had maturation arrest, five had Sertoli cell only and two had a combination of maturation arrest and Sertoli cell only. Of the five patients with deletions extending beyond AZFc, four were Sertoli cell only and one was maturation arrest.

The irony of Y mapping and ICSI coming to prominence concurrently

The history of our work on the Y chromosome and male infertility is very revealing. Cytogenetic studies dating back to 1976 have shown in a very small number of azoospermic men, large defects visible in the long arm of the Y chromosome with simple karyotyping, implying the existence of an azoospermic factor somewhere in that region (Tiepolo & Zuffardi, 1976). However, smaller defects (i.e. 'microdeletions') could not be discerned with those limited cytogenetic methods. Therefore, such defects were considered rare in azoospermic men.

In 1992, comprehensive Y chromosomal maps constructed using yeast artificial chromosomes (YACS) and

sequenced tagged sites (STS) created the possibility for more detailed study of the Y chromosome in infertile men (Foote *et al.*, 1992; Vollrath *et al.*, 1992). Our Y chromosomal mapping study of a large series of severely infertile men with clearly identified phenotypes revealed deletions in as many as 13% of azoospermic males. The commonly deleted region was location in the distal portion of interval 6, subsequently referred to as AZFc (Reijo *et al.*, 1995; Vogt *et al.*, 1996). The fertile fathers of the Y-deleted, infertile men were shown to have intact Y chromosomes, demonstrating that the deletions had arisen de novo and providing strong evidence that the deletions were the cause of the spermatogenic failure observed in these men. The DAZ gene cluster was identified within this small, commonly deleted region (Reijo *et al.*, 1995; Saxena *et al.*, 1996). These DAZ genes have been shown to be transcribed specifically in spermatogonia and primary spermatocytes (Menke *et al.*, 1997).

Shortly after we began our Y chromosomal mapping study of infertile men, ICSI with testicular and epididymal sperm retrieval methods were developed for the treatment of couples with azoospermia (Devroey *et al.*, 1994; Schoysman *et al.*, 1993; Silber *et al.*, 1994, 1995c; Tournaye *et al.*, 1994; Silber, 1998a). TESE and ICSI could thus be utilized to achieve pregnancy for the wives of men with azoospermia caused by deficient spermatogenesis (Silber *et al.*, 1995a, b, 1996; Devroey *et al.*, 1995). Men with the most severe spermatogenic defects causing complete azoospermia could now have children.

The presence or absence of Y deletions has no effect on fertilization or pregnancy rates. Thus, at the very moment in time that we finally had a treatment for male infertility that actually worked, the reality that most male infertility is of genetic origin finally became generally recognized. In fact, if varicocoelectomy or clomiphene citrate were to have any effect on male infertility (which they do not), then they also would provoke as great a concern for future generations as does ICSI.

Our results with ICSI suggest that multiple spermatogenesis genes on the Y chromosome may contribute to and modify the severity of the spermatogenic defect in men with deletions encompassing the AZFc region. Though deletions limited to intervals 6D–6F remove the entire DAZ gene cluster, in most individuals such deletions are apparently not sufficient to completely eliminate all spermatogenesis. Deletions of DAZ and AZFc alone were not generally sufficient to completely prevent all spermatogenesis. However, there are several genes on the Y chromosome that impinge on spermatogenesis (Vogt *et al.*, 1997; Lahn & Page, 1997). It may be the total effect of all these spermatogenesis genes on the Y chromosome that determines sperm production rates.

ICSI offspring of Y-deleted men

In addition to summarizing our experience with ICSI involving Y-deleted and non-deleted azoospermic and severely oligospermic men, the follow-up on their children may actually be of greatest interest today.

Seventeen (8%) Y-deleted non-obstructive azoospermic or severely oligospermic men ($< 2 \times 10^6$ sperm per ejaculate) and 205 similar men who were not Y-deleted underwent TESE-ICSI or ICSI. There were 27 cycles (20 with sperm) involving these 17 Y-deleted men, and 312 cycles involving the 205 non-Y-deleted men. Fathers, brothers and paternal uncles of the infertile men, whenever possible, were also examined for Y deletions and fertility. All male offspring from ICSI procedures involving these Y-deleted men underwent Y deletion testing as well, and all offspring, male and female, were evaluated for clinical abnormalities. Y DNA analysis was performed as has already been described (Page *et al.*, 1999; Silber *et al.*, 1998a; Vogt *et al.*, 1997; Reijo *et al.*, 1995).

Microdeletion of the AZFc or AZFb/AZFc regions of the long arm of the Y chromosome did not appear to adversely affect the fertilization results or resulting pregnancies from severely oligospermic men nor from azoospermic men (from whom sperm were successfully retrieved via TESE). Ten babies (five male and five female) resulted from Y-deleted men (20 cycles), and 99 babies (43 males and 56 females) resulted from non-deleted men (312 cycles).

No Y deletions were found in patients with $> 2 \times 10^6$ sperm per ejaculate (that were not also found in fertile paternal relatives). All seven fertile fathers (who were tested) of Y-deleted infertile men who underwent ICSI were shown to carry intact Y chromosomes. However, all of the ICSI-derived sons of these infertile men were shown to carry the same Y chromosome microdeletions as their infertile fathers. All of the offspring (boys and girls) of Y-deleted men had a normal karyotype. One of the 10 children (male) had a severe cardiac abnormality (right ventricular and pulmonary atresia) and died within 1 week of birth. Another (female) survived and is healthy, but required cardiac reconstruction.

Conclusions from our experience with Y-deleted men undergoing ICSI regarding transmission of infertility via ICSI

(1) The presence of Y deletions does not decrease the fertilization or pregnancy rate for azoospermic and severely oligospermic ($< 2 \times 10^6$) men. (2) The Y deletion (and presumably infertility) is transmitted to the male offspring. (3) The sex ratio of offspring is not affected by the father's Y deletion. (4) The children are karyotypically normal.

There are most likely many spermatogenesis genes involved in male infertility, and we have barely scratched the surface with what are at present very crude mapping techniques on the Y chromosome. Whether or not these gross 'microdeletions' are found in an infertile male patient does not obviate the likelihood of there being a genetic cause for his azoospermia or severe oligospermia. If the defective

gene or genes is on his Y chromosome, then his male offspring will surely inherit his problem. If they are on the X chromosome (probably one-third of cases), then his sons will be fine, but his daughters will be carriers, and his grandsons will have a 50% chance of being infertile. Purely autosomal male infertility will be less common (for purely evolutionary reasons), and in those cases the sons will be at minimum risk.

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