Semen Analysis in the Clinical Evaluation of Infertility

Properly done, as part of a complete workup, semen analysis is a powerful but complex tool for estimating fertility potential. Drs. Eric Seaman, Natan Bar-Chama, and Harry Fisch review the fundamentals and update methods for achieving accuracy.

Approximately 15% of couples attempting their first pregnancy meet with failure. Data available during the past 20 years have revealed that in about one third of these cases, significant abnormalities are found only in the male partner; in another 20% of cases, abnormalities are found in both the man and the woman. Therefore, in roughly 50% of all infertile couples, the male factor is at least partially responsible for the failure to conceive.

A physician wishing to address the problem of infertility should evaluate both partners. Routine assessment includes medical, surgical, and reproductive histories; physical examination; and laboratory evaluation. When focusing on the male factor, the semen analysis remains an essential component of the initial evaluation. This should not be an isolated test but, rather, considered in the context of the patient's clinical presentation and reproductive history. Because there are several potential sources of error in performing the semen analysis, this discussion will begin with a review of some basic principles and will then focus on techniques for counting spermatozoa.

BASIC PRINCIPLES OF SEMEN ANALYSIS

Before a baseline result can be established, two to three sperm analyses are usually performed. If there is a discrepancy in seminal parameters, additional specimens are needed to establish a reliable baseline.

For each analysis, patients are instructed to abstain from intercourse for two to three days. Abstinence for a shorter time can decrease ejaculate volume and sperm counts, whereas prolonged abstinence can increase these parameters. The complete ejaculate should be obtained in a nontoxic, wide-mouth container to avoid inadvertent loss of the specimen. Specimens must be brought to the laboratory for evaluation of sperm movement within two hours of collection.

Physical characteristics (viscosity)

Freshly produced semen is a coagulum that usually liquefies within one hour after ejaculation. The seminal constituents responsible for coagulation originate in the seminal vesicles; the proteolytic enzymes that initiate liquefaction are found in the prostate. Following liquefaction, seminal fluid viscosity can be quantified. Viscosity is considered normal when the specimen can be gently passed through a 21 G needle and exits the needle.
Semen volume

Nearly all of the ejaculate consists of seminal plasma, in which the sperm are suspended. This is made up of secretions mainly from the seminal vesicles and prostate. Normal ejaculate volume ranges from 1.5 to 5 cc, averaging approximately 3.5 cc. Low ejaculate volume may occur secondarily to collection errors, abnormal ejaculation, and/or abnormal seminal plasma secretion.

In cases of low ejaculate volume, examination of the post-ejaculate urine for sperm may reveal retrograde ejaculation. In these cases, sperm can be retrieved easily and processed for artificial insemination.

Low ejaculate volume may also indicate obstruction or agenesis of the seminal vesicles. Because fructose is the main disaccharide in semen and is specifically made and secreted in the seminal vesicles, assessment of seminal plasma fructose has been used in the past to evaluate seminal vesicle secretion. A low semen fructose concentration can be indicative of seminal vesicle blockage or dys-function. However, recent advances in transrectal ultrasonography provide a more detailed method of evaluating the anatomy of the pros-tate and seminal vesicles. Seminal vesicle agenesis, ejaculatory-duct cysts, and obstruction can be detected through transrectal ultrasonography. Obstructions may be treated by transurethral resection.

Excessive semen volume can also be a factor in infertility because of a dilutional effect that results in a lower concentration of sperm reaching the cervix. In these cases, treatment by sperm washing, followed by artificial insemination or split ejaculate, may be beneficial.

Sperm count

The sperm count is probably the most often cited but least well understood aspect of the sperm analysis. Most physicians recognize a sperm count of 20 million/mL as a critical number: greater than 20 million/mL equals fertility, less equals infertility.

Because ‘sperm count’ is an isolated parameter and not a definitive diagnosis, it is important to understand the origins of the 20 million sperm/mL cutoff.

In 1955, MacLeod evaluated sperm counts of 1,000 men whose wives were pregnant and 1,000 men whose marriages had been infertile. The greatest contrast between the two groups was seen at a concentration of 20 million sperm/mL. Of the fertile men, only 5% had sperm counts less than or equal to 20 million sperm/mL compared to 16% of the infertile men; 84% of the infertile men had counts greater than 20 million sperm/mL, suggesting that men with sperm counts above 20 million sperm/mL may also be infertile.

More recently, Lipshultz evaluated semen from a group of ‘normal’ men. Similar to the findings of MacLeod’s study, 6% of normal men had counts less than or equal to 20 million sperm/mL. The mean sperm count for these ‘normal’ men, however, was 80 million sperm/mL, and one third had counts greater than 120 million sperm/mL.

Based on similar clinical studies, the World Health Organization established a count of 20 million sperm/mL as the cutoff for ‘normal’ sperm count. As a practical point, great variability exists in a given individual’s sperm counts from one ejaculate to the next (Figure 1). A single individual’s count can vary from less than 20 million to more than 100 million sperm/mL. Because of this observation, it is important to repeat a semen analysis at least twice for verification of the result if a low sperm count is obtained.

Motility and forward progression As noted earlier, the motility of sperm should be evaluated within two hours after the specimen is produced. If the specimen is then promptly refrigerated to prevent bacterial overgrowth, sperm-density determinations can be delayed.

Sperm movement is evaluated both quantitatively and qualitatively. Quantitation motility (viability) is defined as the average percentage of sperm moving in ten random high-power microscopic fields. Estimates of motility by experienced individuals using this method are quite consistent. Qualitative assessment of sperm movement is based on the pattern displayed by the majority of motile spermatozoa, using the following scale of 0 to 4:

- 0: No movement
- 1: Movement, but no forward progression
- 2: Movement with slow forward progression
- 3: Movement in an almost-straight-line with good speed
- 4: Movement in a straight line with high speed

These two evaluations are combined: 50% viable sperm with forward progression of 3 or more is considered normal; lower values warrant investigation. Abnormalities in motility and quality of movement can arise from infection, the presence of antisperm antibodies, partial ejaculatory-duct obstruction, or the subtle testicular...
alteration that may be caused by gonadotoxins or varicoceles.

If none of the sperm are moving, the patient may have necrospermia. This is actually a misnomer, as metabolic studies and special vital stains have revealed that the immobile spermatozoa may not necessarily be dead.

**Morphologic characteristics**

Sperm morphology can be evaluated on stained or unstained specimens. While a simple hematoxylin or more complicated Papanicolaou technique can be used, Lipshultz et al reported using a small drop of fresh, well-mixed immobilized sperm and observing it under the phase microscope. Cells are categorized as normal (oval), amorphous (including large and small sperm), tapered, duplicated, and immature. According to a broad definition by the World Health Organization, a morphologically-normal semen sample contains 50% normal forms, defined as sperm with oval heads and no neck or tail abnormalities. The advanced technology of in vitro fertilization (IVF) has made it necessary to redefine sperm morphologic criteria that may correlate with fertilization outcomes.

According to the strict criteria of sperm morphology established by Kruger et al (Figure 2), a normal spermatozoon has an oval configuration with a smooth contour; an acrosome comprising 40% to 70% of the distal part of the head; no abnormalities of the neck, midpiece, or tail; and no cytoplasmic droplets of more than half of the sperm head. Borderline forms are considered abnormal. Kruger et al grouped together the normal and borderline forms to obtain what they called the 'morphology index'; they proposed that more than 4% morphologically normal sperm and a morphology index greater than 30% predict a good fertilization outcome. In couples undergoing IVF, a fertilization rate per oocyte of 7.6% was obtained in a group with less than 4% normal (strict) forms, in comparison to a fertilization rate of 64% in those with more than 4% normal (strict) forms.

**Leukocytes in semen**

Some infertile patients show numerous round cells in their semen. Round cells are classified as either germ cells (immature sperm cells) or leukocytes. The differentiation of a round cell as either germ cell or leukocyte is difficult with simple staining procedures. The peroxidase stain will identify polymorphonuclear leukocytes but not other white blood cells in the semen. Immunohistochemical staining of round cells that utilize monoclonal antibodies provide a precise means of identifying seminal leukocytes and their subpopulations.

Increased numbers of leukocytes may indicate a potentially treatable subclinical genital-tract infection. Leukospermia (excessive leukocytes in the semen) may adversely affect sperm movement and the fertilization capacity of the sperm. White blood cells (as well as their soluble products, cytokines) prove in vitro to have an adverse effect on sperm movement and on the ability of human sperm to penetrate hamster eggs.

**pH determination**

The normal seminal pH is 7.2-7.8. It is influenced by prostatic secretion, which is slightly acidic (pH = 6.6). In the presence of infection, the pH of prostatic secretion can increase, rising to more than 7.4. Therefore, excessive alkalization of the semen may indicate an infection such as prostatitis.

Another factor influencing the seminal pH is the amount of time elapsed before the semen sample is tested; a delay in testing results in a rise in pH because of a loss of carbon dioxide. However, excessive delay before testing semen may actually decrease the pH if lactic acid formation has been initiated.

In general, seminal pH determinations may provide insight about infection, but the seminal pH must be tested quickly and interpreted in light of other parameters such as motility, leukocytes, and sperm counts.
COUNTING SPERM: CHAMBERS AND TECHNIQUES

Several types of chambers can be used for performing sperm counts. Table 1 summarizes their features.

Hemacytometer
The improved hemacytometer consists of a thick glass slide with an H-shaped trough forming two counting areas (Figure 3). The edges of the trough are raised to support a clear cover slip 100 microns from the bottom of the chamber. A 3 x 3 mm ruled area, divided into smaller squares, is located on the glass slide.

Technique. Following semen liquefaction, an aliquot of the specimen is placed in a test tube and diluted 1:20 with distilled water to immobilize the spermatozoa. A drop of the mix-ture is then placed on a standard counting chamber. Five blocks of 16 squares each, forming one fifth of the red-blood-cell field, are observed, and all spermatozoa within the area, including those touching the lower and right sides of each block of 16 squares, are counted. This number is multiplied by 106. The process is repeated a second time; the average of two determinations represents the number of spermatozoa per milliliter. For specimens with visually-decreased density (less than 20 million/mL), less diluent should be used and the calculation adjusted accordingly.

Makier chamber
This chamber was specifically designed for determination of sperm concentration and percent motility of undiluted semen. It has a reported staged depth of ten microns, one tenth the depth of an ordinary hemacytometer. The chamber is constructed from two pieces of optically-flat glass; the upper layer serves as a cover glass, with a fine grid 1 square mm in the center subdivided into 100 squares of 0.1 x 0.1 mm each (Figure 4).

Technique. A small uncalibrated drop from a well-mixed undiluted specimen is placed in the center of the chamber and covered. Sperm heads within a ten-square area are counted in the same manner as blood cells in a hemacytometer. The number represents their concentration in millions/mL. In cases of oligospermic semen, spermatozoa in the entire grid are counted, representing their concentration in hundreds of thousands.

Cell-VU
Cell-VU consists of a dual-chamber glass slide patterned from a printed inert surface (Figure 5). The surface supports a 0.5-mm thick coverslip containing a laser-etched grid on the reverse side. The grid area is 1 x 1 mm, divided into 100 smaller squares each measuring 0.1 x 0.1 mm. The chamber has a depth of 20 microns, allowing visualization of cells in a monolayer. Cell-VU slides are disposable. In addition, the chamber ran be used with a slide holder, ensuring a tight fit between cover slip and slide.

Technique. Approximately 4 gL of undiluted specimen is placed in one of the counting chambers. As with the Makier, an uncalibrated drop from a well-mixed undiluted specimen may be used for examination purposes. Counting ten squares and dividing by two gives the count in millions/mL. For increased accuracy, it is recommended that cells in all 100 squares be counted.

MicrocellTm
The Microcell slide contains two independent chambers and uses a 0.5-mm fixed coverslip (Figure 6). Micro-cell slides are disposable. Slides are available with chamber depths of 12 and 20 microns. However, the Micro-
cell chamber has no grid and thus requires a reticle for manual use. Un-diluted semen samples are loaded at each end of the slide and enter the chambers by capillary action. Difficulties with the Microcell include the need for an additional eyepiece for the microscope when performing a manual sperm count and the fact that viscous samples may not fill the chamber easily.

QUALITY CONTROL
Variation in sperm count has been attributed to both technical difficulties and naturally occurring variation in particle distribution. Technical sources of error include differences in count by different technicians, the specific chamber used to perform the count, and poor pipetting technique.

Natural variation
A natural variation in particle distribution is predicted by Poisson's principle. By this principle, statistical errors in counting arise from the random distribution of cells in a counting chamber. These inherent, non-technical errors can be lessened only by counting larger numbers of cells. The standard deviation (SD) of the distribution of cells can be calculated from the formula: SD = ±√(M). (M is the number of cells counted). The larger the number of particles counted, the lower the coefficient of variance = (√(SD/M))x100. To achieve the most reliable results, then, large numbers of particles must be counted.

Chamber variation
Another source of error in performing sperm count comes from the variation in chamber depth and volume from chamber to chamber. One effective way to control for this variation is the routine use of a suspension of latex beads of a known concentration (Accubeads, Hamilton Thorne Research, Inc.). The bead suspension is vortexed before use, applied to the slide, and allowed to settle for several minutes. By comparing an observed count to the known concentration, a correction factor can be derived.

At our institution, we have compared the hemacytometer, Makier, Cell-VU, and Microcell chambers using latex beads of a known concentration (~35 million/mL). According to manufacturers' directions, the beads were counted. Counts were performed to obtain a mean and standard deviation of the counts for each chamber. Our results showed that the Cell-VU and Microcell disposable chambers were the most consistent and reliable (Table 2).

SUMMARY
When analyzed in the context of a patient's clinical presentation, semen analysis remains a powerful tool for estimating the fertility potential of the male partner of an infertile couple.

A summary of normal semen analysis parameters is given in Table 3. When done properly, however, semen

| Table 3 |
|----------------------|----------------------|----------------------|
| **Semen Analysis: Minimal Standards of Adequacy** (Apply to at least two specimens) |
| Ejaculate volume | > 1.5-5.0 mL |
| Sperm density | > 20 million/mL |
| Motility | > 50% |
| Forward progression | > 2 (scale 0-4) |
| Morphology | > 50% normal |
| pH | 7.2-7.8 |
| No significant sperm agglutination | |
| No significant leukocytospermia | |
| No hyperviscosity | |

* Standardized bead counts of 35 million/mL were used for all chambers (Accubead, Hamilton Thorne Research Inc).
analysis is complex and burdened with many potential sources of error. Utilization of reliable chambers, knowledge of Poisson's distribution, and quality control methods can mini- mize such errors.

Suggested Reading


Ginsberg KA, Arman DR: The influence of chamber characteristics on the reliability of sperm concentration and movement measurements obtained by manual and

In the Literature

Editor’s Comments by.-

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Using molecular biology in bladder cancer prognosis

Urinary cytologic examination, available since the 1940s, has been used successfully to screen individuals at high risk for transitional-cell carcinoma of the bladder. Cytologic detection techniques, however, depend on the cytopathologist for interpretation. This shortcoming may be overcome by techniques of molecular biology, particularly the polymerase chain reaction (PCR). This has been used to detect exfoliated malignant cells in body fluids and stool of patients with several types of malignancies.

To evaluate the efficacy of this new technique as a predictor of disease outcome in transitional-cell carcinoma of the bladder, the authors chose a patient of historic importance; they obtained blocks of paraffin-embedded tissue specimen from a former vice president of the United States, Hubert H. Humphrey, who died of the disease in 1978. Mr. Humphrey was a candidate for president in 1968. The series of specimens were screened for mutations in the p53 tumor suppressor gene, using PCR amplification of DNA. The authors also obtained Papanicolaou-stained filters from Humphrey's urine specimens that had been prepared for cytologic study; they then isolated DNA from the filters and amplified it by PCR.

Comparison of tumor cells in urine preparations from May 1967 and from the primary carcinoma resected in 1976 demonstrated similar p53 mutations. In 1967, however, at the time the urine specimens were examined cytologically, cancer was not identified grossly in an examination of the patient's bladder. It was two years before a biopsy revealed in situ carcinoma, and it was six years before he received any therapy for his bladder disease, when biopsy of the prostate urethra revealed 'borderline malignancy.'

Thus, two years before the diagnosis of carcinoma was established by biopsy, Humphrey's bladder probably harbored the malignancy. If he and his physicians had known of his malignancy at that time and opted for aggressive therapy of his tumor, the authors suggest, he may well have withdrawn from the 1968 presidential race and potentially undergone life-saving surgery years before his cancer spread.


Editor's Comment. The importance of this discussion is not limited to the historical figure of Hubert H. Humphrey and his earlier diagnosis of his transitional-cell carcinoma of the bladder. Such cases may have changed our postmortem history. More important is this brief report's showcasing of newer detection techniques utilizing molecular biology. It is apparent, however, that such new techniques are currently far too expensive and tedious for screening a large population- even for screening patients at risk for bladder cancer, which was diagnosed in more than 52,000 people in the US in 1993. It is also possible that p53 mutations are not limited to a single gene codon and may occur later in the evolution of some transitional-cell carcinomas of the bladder than was the case with Mr. Humphrey's. Furthermore, almost half of transitional-cell carcinomas of the bladder do not contain p53 mutations, so screenings of these tumors would be misleading. Despite these limitations, PCR-based techniques continue to evolve and may yield more powerful, more accurate methods for early diagnosis and prognosis of pa- tients with transitional-cell carcinoma of the bladder.
Ureteral imaging by fluoroscopy

Thirty-one consecutive patients were examined in a prospective study to determine how fluoroscopy improved ureteral visualization during intravenous urography. The percentages of ureter visualized with fluoroscopy and with plain radiographs after contrast administration were compared. Studies were evaluated by a single observer in a blinded fashion, comparing total visualized ureter with each technique and then comparing this with the calculated total ureteral length. Fluoroscopic-guided films demonstrated a mean of 87.8% of the right ureter and 80.4% of the left ureter. Spot films of the ureter with standard technique could identify 59.8% of the right ureter and 55.7% of the left ureter, using single frontal views, and 75.8% of the right ureter and 67.4% of the left ureter, using a three-view composite of films. The improvement in ureteral visualization achieved with fluoroscopy was statistically significant.

The ureter is the most difficult area of the upper urinary tract to visualize urographically, and invasive techniques such as retrograde uretero-opyography are frequently required to completely delineate areas of the ureter and identify suspicious areas. It is apparent that the addition of fluoroscopy to standard intravenous urography will decrease significantly the number of patients requiring invasive techniques. This improvement in visualization will not only add to the accuracy of excretory urography but by eliminating invasive studies in many patients, will also decrease costs and risks of studying the ureter with conventional radio-graphic technique. Similarly, unsuspected ureteral lesions may be identified earlier, permitting more-rapid therapeutic intervention.

It is unquestionably more difficult and time-consuming to perform fluoroscopic urography during intravenous urography, as most intravenous urography is performed in radiographic rooms not equipped with fluoroscopy. Patients must be transported to a fluoroscopy unit for these studies. It appears, however, that this is a small price to pay for increasing ureteral visualization from less than 60% to more than 80%.

Laser prostatectomy

A potato model and canine prostate were used in an attempt to define the dosimetry and optimal treatment parameters needed to obtain maximal tissue ablation using the UrolaseTM right-angle laser fiber and a standard neodymium/YAG laser source. Depth and volume of prostatic tissue ablation were measured after single, continuous Nd:YAG laser applications at power settings from 20 to 60 watts while maintaining a constant energy delivery of 3600 joules.

Peak tissue ablation in both the in vitro and in vivo models was observed at a power setting of 40 watts; mean tissue ablation in the canine prostate was 15.7 mm in depth and 5.5 cc in volume. Tissue penetration was more than 30% greater and the volume of tissue ablation was more than 60% greater with 40 watts than with 60 watts at similar total power delivery.

As treatment time was varied from 60 to 120 seconds, tissue ablation was seen to increase significantly from 60 to 90 seconds. Beyond 90 seconds, tissue ablation plateaued, with no significant increase in depth or penetration between 90 and 120 seconds. Interruption of laser application in these models significantly diminished both the depth of penetration and volume of prostate ablated.

The authors have used the Urolase right-angle fiber and have measured a variety of power settings, durations, and treatment techniques. It is apparent from their results that interrupted treatment produces diminished pros-tatic ablation despite the delivery of 3600 Joules of power. The experi-mental evidence strongly suggests that the lower power setting of 40 watts for a longer duration of 90 sec-onds may be optimal for performing VLAP with the Urolase fiber. While the authors have not investigated other fiber designs, powers beyond 60 watts, or durations beyond 120 seconds, their conclusions are quite clear and helpful.

Editor's Comment

The authors have added significant information to the empirical parameters previously used to establish laser power settings and application times for the new technique of right-angle laser pros-tatectomy termed 'visual laser ablation of the prostate' (VLAP). Laser prostatectomy continues to gain in popularity because of its effectiveness and low morbidity in selected patients with moderate-sized pros-tate tases. VLAP continues to compete with other laser techniques for a place in this emerging technology.

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See no increased risk of postvasectomy testicular Ca

Copenhagen-A cohort study of almost 74,000 Danish men vasectomized between 1977 and 1990 has found no increased incidence of testicular cancer and an incidence of prostate cancer close to expected, although the short follow-up period makes the latter finding inconclusive, according to the investigating epidemiologists.

On the basis of their study, they conclude 'it is most likely that vasectomy neither induces testicular tumorogenesis nor accelerates the growth or diagnosis of noninvasive precursor lesions or clinically unrecognized testicular cancers.'

Writing in the British Medical Journal (1994;309:295–299), Henrik Møller, Elsebeth Lynge, and Lisbeth B. Knudsen of the Danish Cancer Society note that vasectomy is an increasingly popular form of contraception in many parts of the world. Although several large cohort studies have failed to show any negative effect on overall mortality or hospitalization rate, there have been conflicting reports concerning the postvasectomy risk of testicular and possibly prostate cancer. The epidemiologists point out some difficulties in interpreting results of the available studies, such as bias in case-control studies, use of self-reports by cohort members and their relatives, and the small numbers of cases.

Only 70 cases of testicular cancer were documented among the 73,917 men (standardized morbidity ratio 1.01-95% confidence interval 0.79-1.28). During the first year of follow-up the incidence was also close to that expected-nine cases (standard-ized morbidity ratio 0.80-95% confidence interval 0.36-1.51). There were 165 cases of prostate cancer (standardized morbidity ratio 0.98-95% confidence interval 0.84-1.14) and a total of 1811 cancers of all types.

Tie genetic mechanism to subfertility in men Leeds, United Kingdom—An autosomal recessive mode of inheritance, probably involving several genes, may account for as much as 60% of subfertility in men, concludes a team of researchers at the University of Leeds. Their case-control study focused on 163 subfertile men involving several genes, may account for as much as 60% of subfertility in men, concludes a team of researchers at the University of Leeds. Their case-control study focused on 163 subfertile men and 327 fertile controls, with information on vasectomy being obtained from the medical records. The researchers point out in the British Medical Journal (1994;309:570-573) that four of the subjects had more than one involuntarily childless brother, and six other brothers were believed to be involuntarily childless, bringing the total prevalence of sub-fertility to 16%. In addition, 19 (1.7%) of the subfertile men and 10 (5.1%) of the controls reported an uncle or cousin to be subfertile. Ex-amination of sperm from 'affected' brothers showed similarities within but not between families.

In trying to sort out the possible genetic factors or factors, the research team, headed by Professor Richard Lilford, an epidemiologist, used segregation analysis. This is a statistical technique by which the proportion of affected members among different types of relatives is compared with that predicted by the laws of genetics. If, for example, all subfertility in men was due to an autosomal recessive gene, fathers would only rarely be subfertile but one quarter of brothers would be. The model best fits the data when 60% of cases are assumed to be due to a recessive gene and 40% to random nongenetic factors, and according to the authors, their data did indeed fit this model.

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