An Assessment of New Sperm Tests for Male Infertility

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The routine semen analysis, although used for more than 50 years, fails to accurately distinguish between fertile and infertile men. As a consequence, many tests of sperm function (TSF) have been developed. This review discusses both older and newer diagnostic TSF. It outlines the principles underlying each assay and reviews aggregate clinical data to determine its current relevance and utility. It concludes that the relevance of many older TSF is questionable, with the wide acceptance of intracytoplasmic sperm injection (ICSI). Newer TSF have the potential to deliver more clinically relevant information but require more extensive study to better understand their predictive role in the ICSI era. UROLOGY 77: 1027–1034, 2011. © 2011 Elsevier Inc. All rights reserved.

In addition to the medical history and physical examination, the conventional semen analysis has been an essential laboratory test for the evaluation of male fertility for at least 50 years. However, the idea that fertility can be defined by threshold values of semen parameters is a concept that is fundamentally flawed.[1] Although not a true measure of fertility, the semen analysis, if abnormal, suggests that the probability of achieving fertility is lower than normal.[2] Because of biological variability, two semen analyses are generally needed, performed with two to three days of sexual abstinence and evaluated in a standardized fashion.[2] Recognized normal values for semen parameters are given in Table 1 in agreement with the latest recommendations by the World Health Organization (WHO).[2] Traditionally, (except for the latest WHO recommendations), these references are derived by expert consensus and not by prospective clinical trials and thus their true relationship to male fertility is unclear. In addition, the definition of what constitutes “normal” semen parameters is constantly challenged. A meta-analysis of 29 US studies of semen quality from 9612 fertile, or presumably fertile, men suggested that a sperm concentration of 98 million/mL is normal.[3] Although sperm motility is considered the “best” predictor of fertility, normal sperm motility ranges from 53% to 62%.[4] Thus, simply deriving “normal” semen parameters has been a prohibitively lengthy and inconclusive process to date.

Finally, other biological variables affect the clinical utility of the semen analysis. In addition to wide intra-individual variation, seasonal[5] and geographic variation[4] further complicate the performance of the semen analysis as a fertility measure. As examples, within-subject, interejaculate coefficients of variation for sperm concentration and motility are estimated at 44.7% and 15% in one study.[1] Thus, even with excellent quality control, wide biological variation in semen quality profoundly challenges the notion that the conventional semen analysis can accurately assess male fertility.

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Submitted: July 25, 2010, accepted (with revisions): October 11, 2010

OLDER ADJUNCTIVE SPERM TESTS

Because of the need to more precisely characterize normal fertile semen, adjunctive semen testing has become popular (Figure 1). The concept behind developing adjunctive sperm tests is that the functional competence of sperm matters for fertility. Although such testing has fallen out of favor in the last decade with the rise of intracytoplasmic sperm injection (ICSI), the idea that not all sperm used with ICSI are the same has reignited interest in adjunctive sperm testing.

Sperm Morphology

One of the oldest adjunctive sperm tests is the formal evaluation of sperm shape, termed *morphologic assessment*.[6] Several descriptive systems exist to evaluate morphology, and within each system, sperm are designated normal or abnormal based on specific criteria. It is believed that sperm morphology may correlate with a man’s fertility potential as reflected by in vitro fertilization (IVF) success in case cohort studies.[7] More recently, however, the ability of sperm morphology to distinguish candidates who are at risk of failing IVF has been called into question.[8] In addition, test reliability and reproducibility are low and normal biological variation may be high, all of which complicate and reduce the performance of sperm morphology as a diagnostic test.

Sperm Penetration Assay (SPA)

This bioassay, first described by Yanagimachi and colleagues in 1976, examines the ability of sperm to pene-

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0090-4295/11/$36.00
doi:10.1016/j.urology.2010.10.005

Table 1. Lower references limits (5th centile) for semen characteristics (WHO 2010)

Volume (mL)	1.5
Concentration (10^6 sperm/mL)	15
Total sperm number ($10^{6/\text{ejaculate}}$)	39
Motility (% motile)	40 (a + b + c)
Forward progression	32 (a + b)
Morphology (% normal)	4
Viability/Vitality (% alive)	58%
White blood cells (10^6 sperm/mL)	<1.0

trate zona-free hamster eggs.[9] The assay was designed to analyze four steps in the fertilization process: capacitation, acrosome reaction, fusion with the oocyte vitelline membrane, and sperm decondensation. In limited studies, the SPA has been shown to correlate with fertilization rates in vitro. In a study by Soffer et al., in which 241 infertile couples scheduled for IVF were assessed, the SPA predicted fertilization with high negative (74%) and positive (82%) predictive values and with excellent specificity (0.96).[10] Limitations to the clinical utility of the SPA include the use of hamster oocytes, its labor-intensive nature and expense, the fact that it is technically demanding to perform, and that it has significant issues of assay standardization.[1]

Hemizona Assay (HZA)

The HZA, first described by Burkman et al. in 1988, was introduced to examine the binding of sperm to the human-oocyte zona pellucida.[11] Quantification of sperm-zona binding uses salt-stored human hemizonae and is a relatively sophisticated and involved bioassay. It was developed to predict sperm fertilization potential for IVF treatment. Arslan and colleagues evaluated the value of the HZA to predict pregnancy in 82 couples with unexplained or male factor infertility after 313 intrauterine insemination (IUI) cycles.[12] The main outcome measures evaluated were hemizona index (HZI) and clinical pregnancy rate. Overall, patients with a HZI value of <30 had a significantly lower pregnancy rate compared with patients with a HZI of ≥30 (11.1% vs 40.6%, respectively; $P < .05$), and the relative risk for failure to conceive was 1.5 (CI, 1.2–1.9). User receiver operating characteristics curves over the range of HZI scores (0–60), the duration of infertility, and the HZI score were significant determinants of conception. In addition, the negative and positive predictive values of the HZA for clinical pregnancy were 93% and 69%, respectively. This assay, although complicated, is significant in that it can predict sperm binding to the oocyte and can delineate sperm deficiencies of this nature. This matters because defective sperm-zona binding is a common cause of IVF failure. HZA has not been sufficiently evaluated to know whether it correlates with natural conception.[12]

Acrosome Reaction (AR)

This acrosome reaction is an exocytic secretory event within the sperm head that releases proteolytic enzymes for zona pellucida penetration and remodels the sperm surface in preparation for oocyte fusion.[1,13] It is triggered by sperm-zona binding. The AR status of sperm is assessed using fluorescent lectins that bind to the acrosomal membrane or to the acrosomal contents. To differentiate nonspecific from physiological ARs, the assay is combined with a cell viability assessment. One limitation of AR assays is that there is a measurable prevalence of spontaneous AR in human sperm (<4%), making correlations to pregnancy outcomes difficult.[1] Because of this, AR induction with ionophores has been reported to improve the dynamic range of the assay. Unfortunately, using ionophores is a nonphysiological maneuver and avoids the normal receptor-signal activation system that is important to assess.

Progesterone, a hormone present in follicular fluid and in the oocyte cumulus matrix, induces the acrosome reaction and the development of hyperactive motility patterns.[14] This “physiological” agent has been used by Krausz et al.,[14] who reported a significant correlation between acrosome reaction induction with progesterone and fertilization rate in vitro. In addition, impaired responsiveness of human sperm to progesterone has been associated with reduced fertilization.[15] These data suggest sperm responsiveness to progesterone, as assessed by the acrosome reaction, may predict fertilization.[16] In summary, induced AR assays have value in explaining fertilization failure in IVF cases, but have not been shown to correlate with natural pregnancy rates.

Hyposmotic Swelling Test (HOS)

The HOS test, first described by Jeyendran in 1984, was developed to evaluate the integrity of the sperm plasma membrane.[17] The test is based on the biology of osmosis and the fact that fluid transport occurs across an intact cell membrane until osmolar equilibrium is reached. In test conditions, in which sperm are exposed to hypo-osmolar fluid, the sperm expand, especially in the tail, and produce characteristic morphologic changes. In the original assay, the ability of sperm to swell correlated well with its capacity to penetrate denuded hamster oocytes.[17] Although simple and economical, HOS has not proven useful clinically in the assessment of the fertilizing capacity of sperm.[18] It has however been valuable in noninvasively selecting viable sperm from a nonmotile population for ICSI.[19]

Conclusions

Although innovative, older assays of sperm function are not widely used in clinical medicine today. The combination of complexity, expense, lack of standardization, and poor correlation to reproductive outcomes severely limit their clinical utility. In addition, the information learned may not significantly affect clinical decision mak-

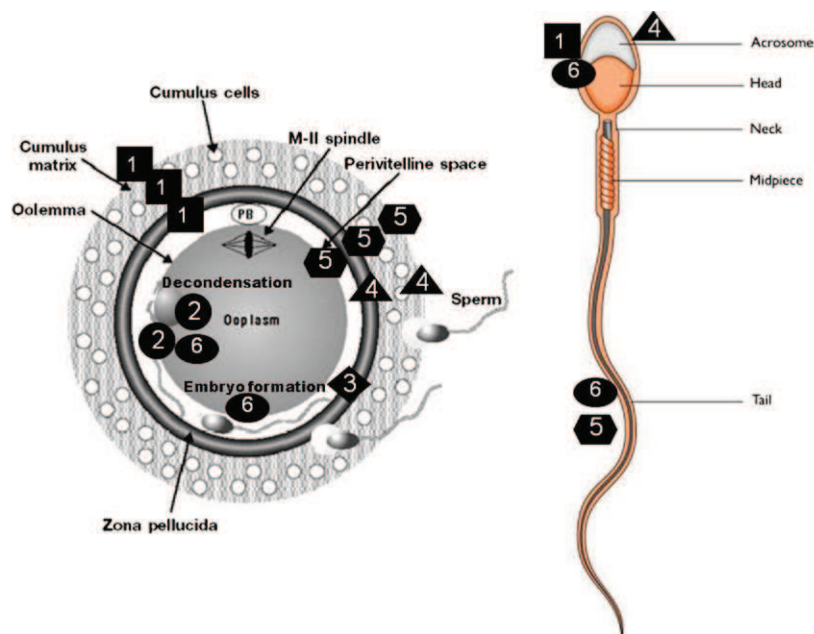

Figure 1. Schematic illustration of the steps of the egg fertilization process that the older sperm function tests assess. 1. Sperm morphology correlates with stages of egg cumulus and egg binding, and egg penetration and fertilization at IVF. 2. Sperm penetration assay assesses the ability of sperm to bind and penetrate the egg and decondense within it. 3. Hemizona assay examines sperm binding to the zona pellucida. 4. Acrosome reaction and the progesterone test assess the ability of sperm to penetrate cumulus and bind and penetrate the zona pellucida. 5. Hypo-osmotic swelling correlates with cumulus and egg binding and egg penetration and fertilization at IVF. 6. Reactive oxygen species evaluation correlates with sperm membrane, motility, and DNA integrity.

ing with the wide use of ICSI. In bypassing many of the sperm requirements needed for egg fertilization (Fig. 1), such as the AR, capacitation, sperm-zona binding and penetration, and oocyte binding, ICSI effectively treats most sperm deficiencies described in these assays. Despite this, the development of new functional sperm assays is important, becasue accurate, simple, and reliable tests of sperm function can be valuable in predicting infertility that may benefit the most from ICSI.

NEWER ADJUNCTIVE SPERM TESTS

Sperm DNA Fragmentation

Recently, the integrity of DNA packaging within sperm chromosomes has been suggested as a biological correlate of fertility.[20] DNA fragmentation is characterized by both single and double DNA strand breaks, and is particularly frequent in the sperm of subfertile men. However, it is also true that oocytes and early embryos can repair sperm DNA damage.[21] Thus, the biological effect of damaged sperm DNA depends on both the degree of sperm chromatin damage and the capacity of the early embryo to repair it (Fig. 2).

Abnormal sperm chromatin structure or DNA damage is thought to arise from four sources: (1) deficiencies in recombination during spermatogenesis, (2) abnormal spermatid maturation caused by protamination disturbances, (3) abortive apoptosis, and (4) oxidative stress.[20] Several assays have been developed to evaluate sperm DNA integrity, and exactly what is measured with each assay differs.[20] In general, however, the assays can be divided into three types: (1) assays to determine sperm chromatin structure, (2) tests of sperm DNA fragmentation, and (3) assays that assess the sperm nuclear matrix.[20]

Probes to sperm chromatin structure. Chromatin structural probes apply sensitive nuclear dyes to examine DNA integrity. Their cytochemical performance, however, is rather complex, because several factors influence the DNA staining of chromatin by these dyes: (1) the secondary structure of DNA, (2) the regularity and density of chromatin packaging, and (3) the binding of DNA to chromatin proteins. Assays in this category include:

Acridine Orange (inexpensive, simple). Measures in situ DNA susceptibility to acid-induced conformational helix-coil transition[22] (Fig. 3).

Aniline Blue (inexpensive, simple). Stains proteins in loosely condensed chromatin[23]

Chromomycin a (inexpensive, simple). Competes with protamines for association with DNA and staining relates to the degree of protamination in mature sperm[24]

Toluidine blue (inexpensive, simple). Stains phosphate residues of loosely packed and fragmented sperm nuclear DNA[25]

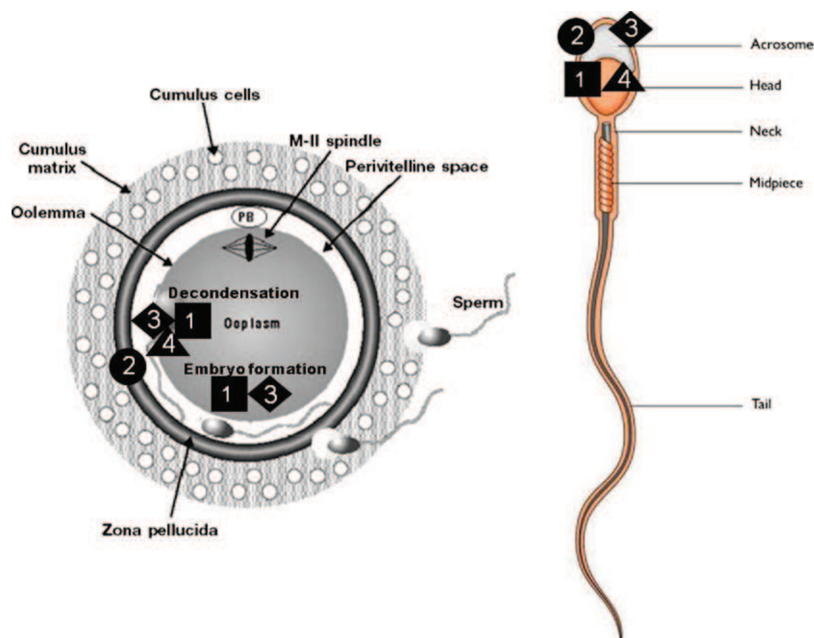

Figure 2. Schematic illustration of the steps of the egg fertilization process that the newer sperm function tests assess. 1. Sperm DNA fragmentation correlates with sperm decondensation and form embryos. 2. Sperm HA binding examines the ability of sperm to bind to the zona pellucida. 3. Ultrafine morphology correlates with the ability of sperm to decondense within the egg and form embryos. 4. Chromatin decondensation assesses the ability to sperm to decondense within the oocyte.

Figure 3. A common method of assessing sperm DNA fragmentation is to observe acridine orange (AO)-stained sperm that is exposed to 488-nm laser light. AO intercalated into double-stranded DNA fluoresces green and AO bound to single-stranded DNA fluoresces red (color figure available online).

Sperm chromatin structural assay (SCSA) (expensive, complex). Measures in situ DNA susceptibility to acid-induced conformational helix-coil changes with acridine orange fluorescence using automated cell sorting[20] (Fig. 3). SCSA is the most widely used assay, because associations with clinical outcomes after natural conception and assisted reproductive have been reported. The advantages of SCSA are its robustness and small intra- and interassay variation.

Tests of sperm DNA fragmentation. These tests focus on the identification of single- or double-stranded DNA strand breaks within sperm DNA. Assays included in this category are:

In situ Nick translation. This assay quantifies the incorporation of biotinylated deoxyuridine triphosphate (dUTP) at single-stranded DNA breaks (SSBs) within sperm DNA in a reaction catalyzed by DNA polymerase I.[24] Not widely used clinically, this assay lacks sensitivity and correlative studies with fertility outcomes are lacking.[24]

Terminal deoxynucleotidyl transferase mediated dUTP Nick end labeling (TUNEL). This assay accurately detects mainly double-stranded breaks (DSBs) in DNA through the incorporation of dUTP at DNA breaks catalyzed by terminal deoxynucleotidyl transferase. TUNEL is performed with flow cytometry, which allows efficient de-

tection of DNA fragmentation in a large population of sperm.[26] However, TUNEL evaluation by flow cytometry may underestimate actual sperm DNA fragmentation rates. Combining TUNEL and propidium iodide nuclear staining can distinguish sperm from other round cells in the semen, termed *M540 bodies*, that contain small but contaminating quantities of interfering fragmented DNA.[27] A major disadvantage of TUNEL is that it is relatively labor-intensive; standardized, clinical kits are now available.[28] In addition, there is no clear clinical pregnancy outcome data that correlate well with test results[28].

Single cell gel electrophoresis assay (COMET). This approach quantifies DNA SSBs and DSBs in single sperm after electrophoresis of fluorochrome-stained DNA.[29] COMET is highly sensitive, but labor intensive and it can be difficult to standardize comet tail length for reporting. In addition, assay correlation to clinical infertility outcomes is less apparent than with SCSA or TUNEL methods.[30]

Sperm nuclear matrix assays. These assays are based on the degree to which intact DNA deprived of chromatin proteins is able to loop around the sperm nucleus matrix.[30] In support of this approach, it has recently been shown that germ line mutations in nuclear matrix proteins may lead to deficient DNA repair and chromatin disorganization.[31] Two assays are allocated to this category:

Sperm nuclear matrix stability. This assay assesses the high-level DNA organization within the sperm nuclear matrix and can detect aberrations in the ability of matrix to organize DNA into loop-domains.[30] This test is in the developmental stages and clinical information regarding outcomes is extremely limited.

Sperm chromatin dispersion. In this assay, fragmented DNA fails to produce the characteristic "halo" when it is mixed with aqueous agarose after acid/salt treatment to remove nuclear proteins.[32] This assay is recent and has limited verification in male infertility.[32]

Correlation of DNA integrity to ART outcomes. Assays of sperm DNA damage have the potential to discriminate among sperm of different quality. In general, the mean percentage of sperm with abnormal DNA denaturation and abnormal DNA fragmentation in infertile men is 25% and 28%, respectively, compared with fertile men, at 10% and 13%, respectively.[33] Assuming that DNA damage arises solely from a failure to repair DNA breaks introduced during spermatogenesis, it could be expected that DNA damage would correlate well with low sperm concentration or poor morphology. Alternatively, if sperm DNA damage results mainly from the adverse effects of reactive oxygen species (ROS), then reduced sperm motility might be expected. Currently, the relative contribution of these two sources of DNA damage to sperm remains unknown.[33]

Several studies have examined the association of sperm DNA integrity on reproductive outcomes after intrauterine insemination (IUI), IVF and IVF/ICSI. To date, there is little correlation between sperm DNA damage and fertilization rates with either IVF or IVF/ICSI.[34] This makes sense, because neither fertilization nor early embryo development are dependent on sperm DNA integrity, because the embryonic genome is expressed after the second cleavage division at the four-cell embryo stage.[35] However, high levels of sperm DNA damage appear to correlate inversely to successful pregnancy with IUI, IVF, and IVF/ICSI.[36] In the largest retrospective study to date, Bungum et al.[37] did not find increased pregnancy loss with high sperm DNA fragmentation rates with IVF/ICSI but did find a ten-fold lower risk of pregnancy with IUI. This study also demonstrated significantly higher clinical pregnancy rates (52.9% vs 22.2%) and delivery rates (47.1% vs 22.2%) with IVF/ICSI compared with IVF in couples with high sperm DNA damage. Indeed, when the DNA fragmentation rate exceeded 27%, the odds ratio for a positive reproductive outcome after ICSI compared with IVF was 8 for biochemical pregnancy, 4 for clinical pregnancy, and 3 for delivery. These data confirm earlier observations that IVF outcomes are affected more profoundly than are IVF/ICSI results by DNA damage.[36] Thus, as the literature on this assay accrues, the assessment of sperm DNA integrity may become an important predictor of pregnancy success in the setting of assisted reproduction.

In what clinical scenarios should DNA fragmentation assays be used? They can be considered in the following cases:

- Unexplained or idiopathic infertility[26]
- When deciding between IUI or IVF/ICSI as therapeutic options[37]
- In the setting of varicocele-associated infertility[38]
- Recurrent pregnancy loss[39]

Reactive Oxygen Species

Oxidative stress is a consequence of free radicals generated from cellular metabolism.[40] Naturally occurring antioxidants abound in the testis and semen, but cellular damage can occur when homeostatic mechanisms are perturbed. Excessive ROS have been detected in seminal plasma and are produced by both sperm and neutrophils. ROS production is directly measured by a chemiluminescence assay using luminol (5-amino-2,3-dihydro 1,4-phthalazinedione) as the probe and assessed by a luminometer and reported as counted photons/min/U sperm concentration.[41] Because of their confounding effect on ROS measurements, it is critical to evaluate leukocyte concentrations in semen to determine the ROS contribution by these cells. Seminal free radicals can also result from chronic disease, environmental exposures, infections, and immune responses. It is thought that excessive

ROS damages sperm membranes, reduces sperm motility, and induces sperm DNA damage.[40] Although antioxidant treatments may improve ROS balance, increases in naturally pregnancy rates have not been as obviously forthcoming. For these reasons, semen ROS levels are not a commonly performed sperm function assay.[40]

Sperm Hyaluronic Acid Binding

It is clear that except for rare instances, such as globozoospermia and multiflagellated forms, sperm shape does not predict underlying chromosomal aneuploidy. Thus, selection of sperm for ICSI by "normal" morphology does not ensure the absence of chromosomal aneuploidy.[42] A sperm selection technique based on sperm membrane binding to hyaluronic acid (HA), the main substrate of the oocyte zona pellucida, could improve the likelihood of obtaining chromosomally normal sperm for ICSI.

Noting an association between the presence of sperm membrane HA receptors and various upstream features of sperm maturity, Huszar et al.[43] hypothesized that mature sperm would selectively bind to HA. They also surmised that sperm of diminished maturity have lower levels of the 70-kd testis-expressed chaperone (HspA2), increased chromosomal aberrations, and, because they have not undergone membrane remodeling, are unable to bind to solid-state HA.[43] Lastly, based on these attributes, Huszar et al. postulated that HA binding would facilitate the selection of individual mature sperm with lower levels of chromosomal aneuploidy.[44] In addition, Parmegiani et al. suggest that HA-selected ICSI sperm produce equivalent fertilization rates and good quality embryos compared with visually selected sperm, but may improve live baby rates substantially by decreasing miscarriage rates.[45]

One limitation of this sperm selection technique is that because sperm are firmly bound to HA, they must be manually removed and cannot be obtained in suitable numbers for IUI or IVF without ICSI. In addition, substantial clinical trial confirmation of these study findings are lacking, including a precise definition of which patients will benefit most from this technology. However, this technique, termed PICSI ("Physiological" ICSI), has the potential to mimic sperm selection at the traditional evolutionary level and may improve IVF/ICSI outcomes in selected patients by reducing the abnormal paternal contribution of sperm.[45]

Ultrafine Morphology

For 20 years, since the first report of a relationship between sperm morphology and IVF outcomes by Kruger,[6] the value of sperm morphology as a predictor of pregnancy has been debated. Although correlating with IVF success, the relationship between morphology and pregnancy success, either naturally or with IUI are, at best, controversial associations.[46] What is clear is that no relationship exists between sperm strict morphology and IVF/ICSI outcomes.[47]

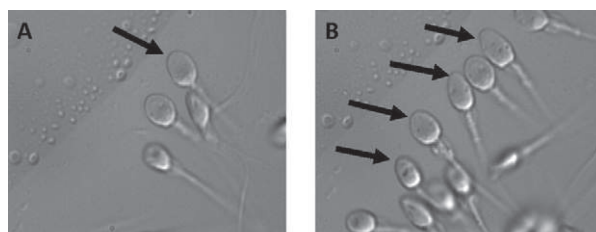

Figure 4. Sperm ultrafine morphology. (**A**) Arrow delineates a motile sperm with normal sperm nucleus by ultrafine morphology (good contour, no vacuoles). (**B**) Arrows delineate motile sperm with abnormal ultrafine morphology (abnormal contour or presence of vacuoles or both). Magnification 6600x. (Courtesy Dr. Benjamin Bartoov.)

Based on the concept that there may be ultrastructural features of sperm that could predict improved IVF/ICSI outcomes but that are not currently detected by strict morphology, Berkovitz et al. have proposed the examination of ultrafine sperm morphology.[46] It might be that the low power magnification (200x or 400x) used for visual sperm selection for ICSI is relatively insensitive and unable to detect subtle sperm organellar malformations that might affect pregnancy rates.[46]

To increase the "gain" on sperm morphology with the goal of improving IVF/ICSI outcomes, Berkovitz and colleagues developed a method for the detailed morphologic evaluation of motile sperm in real time that is termed MSOME (motile sperm organellar morphology examination).[46] MSOME is performed with an inverted light microscope equipped with high-power Nomarski optics enhanced by digital imaging to achieve a magnification of 6300x (Fig. 4).[46] In theory, the normalcy of the sperm nucleus, as observed by MSOME, reflects sperm nuclear DNA content and organization, which in turn could influence the outcomes of IVF/ICSI procedures.[46]

Based on research with MSOME, Bartoov et al. introduced a therapeutic procedure termed *intracytoplasmic morphologically selected sperm injection* (IMSI).[48] IMSI is performed in association with ICSI and uses MSOME criteria to precisely select sperm with morphologically normal nuclei for ICSI. They have also shown that the use of IMSI leads to significantly higher pregnancy rates compared with conventional IVF/ICSI in patients who have failed to achieve a clinical pregnancy in at least three IVF/ICSI cycles.[49] The study design used by Bartoov's group was able to exclude the special sperm preparation technique needed for IMSI as the reason for higher implantation and pregnancy rates and lower miscarriage rates.[50] Performing diagnostic MSOME or therapeutic IMSI has several clinical limitations: (1) significant time and effort is needed for IMSI, because sperm selection can take several hours; (2) expertise and training is needed for these techniques; and (3) the cost of upgrading laboratory equipment to perform IMSI is significant.[50] Taking into account these issues, a diagnostic MSOME is advised in advance of IMSI to

determine in which patients IMSI is likely to improve ICSI outcomes.

Chromatin Condensation and Sperm Epigenetics

The replacement of somatic histones by protamines is important for sperm nuclear chromatin compaction, sperm maturation and fertility.[51] Condensation and stabilization of sperm chromatin occurs in elongating spermatids, during epididymal transport, and with seminal fluid contact after ejaculation.[52] Furthermore, chromatin stability increases with time after ejaculation likely because of the formation of disulfide bridges.[51] As a consequence, mature sperm DNA is transcriptionally inactive. During oocyte fertilization, the paternal genome is restored to its transcriptionally active conformation. This is accomplished through the chromatin decondensation process characterized by the degradation of protamines, the synthesis of histones, and binding of the histones to DNA, leading to restoration of the paternal genome.[51]

A large variable in this process is that the histone to protamine exchange process can be incomplete, with 5% to 15% of the genome bound to nucleosomes.[51] In humans, the proportion of protamine 1(P1) to protamine 2 (P2) is approximately 1:1, and changes to this ratio are associated with altered sperm quality and decreased embryo quality and IVF outcomes.[53]

Diagnostic tests now under development seek to determine the quality of the sperm decondensation process and its role in male infertility.[53] Human sperm heads can be decondensed in vitro using either oocytes from animals (e.g., frog) or with detergents. However, precise determination of the degree of sperm head decondensation is time-consuming and time sensitive, because the process is dynamic.[54] Because of this, classification schema that attempt to examine sperm decondensation are subjective and imprecise.[54] Despite this, attempts have been made to correlate the quality of sperm decondensation with routine semen parameters, DNA integrity, and IVF fertilization rates, but no consistent relationship has been observed.[55] Thus, the role of diagnostic sperm nuclear decondensation in the male infertility evaluation is currently speculative.

CONCLUSIONS

In summary, older established tests of sperm function, including morphology, do not necessarily deliver better clinical information regarding the fertility potential of sperm than that obtained from the semen analysis. In addition, the relevance of many of these tests is questionable with the wide acceptance of ICSI, a technique that can bypass many abnormalities of sperm function. Newer diagnostic tests of sperm function have more potential to deliver clinically relevant information in the era of ICSI. However, they require more extensive study to better understand their predictive role in reproductive medicine.

It is exciting to speculate how newer diagnostic tests of sperm function relate to each other and to the older assays. For example, will ultrafine morphology be the physical correlate of abnormal sperm chromatin packaging and DNA fragmentation? Will sperm chromatin decondensation tests correlate with the SPA in some way, and does the HA-binding assay reflect similar findings as the HZA assay? The development of such assays should be encouraged in the future to rationalize clinical decision-making in the heavily technological field of human reproductive medicine.

Acknowledgments The authors are grateful to Prof. Csilla Krausz (University of Florence, Italy) for scientific assistance with preparation of this manuscript.

References

1. Aitken RJ. Sperm function tests and fertility. *Int J Androl*. 2006; 29:69-75.
2. World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th ed. Geneva: World Health Organization; 2010.
3. Saidi JA, Chang DT, Goluboff ET, et al. Declining sperm counts in the United States? A critical review. *J Urol*. 1999;161:460-462.
4. Haugen TB, Egeland T, Magnus O, Magnum O. Semen parameters in Norwegian fertile men. *J Androl*. 2006;27:66-71.
5. Levine RJ. Seasonal variation of semen quality and fertility. *Scand J Work Environ Health*. 1999;25;Suppl 1:34-37.
6. Kruger TF, Swanson RJ, Hamilton M, et al. Abnormal sperm morphology and other semen parameters related to the outcome of the hamster oocyte human sperm penetration assay. *Int J Androl*. 1988;11:107-113.
7. Kruger TF, Acosta AA, Simmons KF, et al. New method of evaluating sperm morphology with predictive value for human in vitro fertilization. *Urology*. 1987;30:248-251.
8. Keegan BR, Barton S, Sanchez X, et al. Isolated teratozoospermia does not affect in vitro fertilization outcome and is not an indication for intracytoplasmic sperm injection. *Fertil Steril*. 2007;88: 1583-1588.
9. Yanagimachi R, Yanagimachi H, Rogers BJ. The use of free animal uova as a test—system for the assessment of the fertilizing capacity of human spermatozoa. *Biol Reprod*. 1976;15:471-476.
10. Soffer Y, Golan A, Herman A, et al. Prediction of in vitro fertilization outcome by sperm penetration assay with TEST-yolk buffer preincubation. *Fertil Steril*. 1992;58:556-562.
11. Burkman LJ, Coddington CC, Franken DR, et al. The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil Steril*. 1988;49:688-697.
12. Arslan M, Mahmood M, Ebru OA, et al. Predictive value of the hemizona assay for pregnancy outcome in patients undergoing controlled ovarian hyperstimulation with intrauterine insemination. *Fertil Steril*. 2006;85:1697-1707.
13. Florman HM, Jungnickel MK, Sutton KA. Regulating the acrosome reaction. *Int J Dev Biol*. 2008;52:503-510.
14. Krausz C, Bonaccorsi L, Luconi M, et al. Intracellular calcium increase and acrosome reaction in response to progesterone in human spermatozoa are correlated with in-vitro fertilization. *Hum Reprod*. 1995;10:120-124.
15. Falsetti C, Baldi E, Krausz C, et al. Decreased responsiveness to progesterone of spermatozoa in oligozoospermic patients. *J. Androl*. 1993;14:17-22.
16. Krausz C, Bonaccorsi L, Maggio P, et al. Two functional assays of sperm responsiveness to progesterone and their predictive values in in-vitro fertilization. *Hum Reprod*. 1996;11:1661-1667.
17. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, et al. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil*. 1984;70:219-228.

18. Van der Ven HH, Jeyendran RS, Al-Hasani S, et al. Correlation between human sperm swelling in hypoosmotic medium (hypoosmotic swelling test) and in vitro fertilization. *J Androl*. 1986;7:190-196.
19. Smikle CB, Turek PJ. Hypoosmotic swelling can accurately determine the viability of nonmotile sperm. *Mol Reprod Dev*. 1997;47: 200-203.
20. Erenpreiss J, Spano M, Erenpreisa J, et al. Sperm chromatin structure and male infertility biological and clinical aspects. *AJA*. 2006; 8:11-29.
21. Genesca A, Caballin MR, Miro R, et al. Repair of human sperm chromosome aberrations in the hamster egg. *Hum Genet*. 1992;89: 181-186.
22. Hoshi K, Katayose H, Yanagida K, et al. The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. *Fertil Steril*. 1996;66:634-639.
23. Auger J, Mesbah M, Huber C, Dadoune JP, Blue A. Staining as a marker of sperm chromatin defects associated with different semen characteristics discriminates between proven fertile and suspected infertile men. *Int J Androl*. 1990;13:452-462.
24. Manicardi GC, Bianchi PG, Pantano S, et al. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biol Reprod*. 1995; 52:864-867.
25. Beletti ME, Mello ML. Comparison between the toluidine blue stain and the feulgen reaction for evaluation of rabbit sperm chromatin condensation and their relationship with sperm morphology. *Theriogenology*. 2004;62:398-402.
26. O'Brien J, Zini A, Sperm DNA. Integrity and male infertility. *Urology*. 2005;65:16-22.
27. Muratori M, Porazzi I, Luconi M, et al. Annexin V binding and merocyanine staining fail to detect human sperm capacitation. *J. Androl*. 2004;25:797-810.
28. Henkel R, Kierspel E, Hajimohammad M, et al. DNA fragmentation of spermatozoa and assisted reproduction technology. *Reprod Biomed Online*. 2003;7:477-484.
29. Hughes CM, Lewis SE, McKelvey-Martin VJ, et al. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Mol Hum Reprod*. 1996;2:613-619.
30. Ankem MK, Mayer E, Ward WS, et al. Novel assay for determining DNA organization in human spermatozoa: implications for male factor infertility. *Urology*. 2002;59:575-578.
31. Sjakste N, Sjakste T. Nuclear matrix proteins and hereditary diseases. *Genetika*. 2005;41:293-298.
32. Fernandez JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. *J Androl*. 2003; 24:59-66.
33. Zini A, Bielecki R, Phang D, et al. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril*. 2001;75: 674-677.
34. Bungum M, Humaidan P, Axmon A, et al. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Hum Reprod*. 2007;22:174-179.
35. Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature*. 1988;332:459-461.
36. Larson-Cook KL, Brannian JD, Hansen KA, et al. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil Steril*. 2003;80:895-902.
37. Bungum M, Humaidan P, Spano M, et al. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod*. 2004;19:1401-1408.
38. Zini A, Blumenfeld A, Libman J, Willis J. Beneficial effect of microsurgical varicocelectomy on human sperm DNA integrity. *Hum Reprod*. 2005;20:1018-1021.
39. Carrell DT, Wilcox AL, Lowy L, et al. Elevated sperm chromosome aneuploidy and apoptosis in patients with unexplained recurrent pregnancy loss. *Obstet Gynecol*. 2003;101:1229-1235.
40. Tremellent K. Oxidative stress and male infertility—a clinical perspective. *Hum Reprod Update*. 2008;14:243-258.
41. Athayde KS, Cocuzza M, Argawal A. Development of normal reference values for seminal reactive oxygen species and their correlation with leukocytes and semen parameters in a fertile population. *J Androl*. 2007;28:613-620.
42. Celik-Ozenci C, Jakab A, Kovacs T, et al. Sperm selection based on shape properties alone does not preclude the presence of numerical chromosomal aberrations. *Hum Reprod*. 2004;19:2052-2059.
43. Huszar G, Ozenci CC, Cayli S, et al. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. *Fertil Steril*. 2003;79:1616-1624.
44. Huszar G, Jakab A, Sakkas D, et al. Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects. Repr. Bio. *Medicine* online;**14:**650-663, 2007.
45. Parmegiani L, Cognini GE, Bernardi S, et al. Physiologic ICSI" : hyaluronic (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. *Fertil Steril*. 2010;93:598-604.
46. Berkovitz A, Eltes F, Soffer Y, et al. ART success and in vivo sperm cell selection depend on the ultramorphological status of spermatozoa. *Andrologia*. 1999;31:1-8.
47. Nagy ZP, Verheyen G, Tournaye H, Van Steirteghem AC. Special applications of intracytoplasmic sperm injection: the influence of sperm count, motility, morphology, source and sperm antibody on the outcome of ICSI. *Hum Reprod*. 1998;13:143-154.
48. Bartoov B, Berkovitz A, Eltes F. Selection of spermatozoa with normal nuclei to improve the pregnancy rate with intracytoplasmic sperm injection. *N. Engl. J. Med.*. 2001;345:1067-1068.
49. Bartoov B, Berkovitz A, Eltes F, et al. Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. *Fertil Steril*. 2003;80:1413-1419.
50. Berkovitz A, Eltes F, Yaari S, et al. The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. *Hum Reprod*. 2005;20:185-190.
51. Carrell DT, Hammoud SS. The human sperm epigenome and its potential role in embryonic development MHR 16. *Mol Hum Reprod*. 2010;16:37-47.
52. Bjorndahl L, Kjellberg S, Roomans GM, Kvist U. The human sperm nucleus takes up zinc at ejaculation. *Int J Androl*. 1986; 9:77-80.
53. Aoki VW, Liu L, Jones KP, Hatasaka HH, et al. Sperm, P1/P2 ratio are related to IVF PR and predictive of fertilization ability. *Fertil Steril*. 2006;86:1408-1415.
54. Huret JL. Variability of the chromatin decondensation ability test on human sperm. Arch. *J Androl*. 1983;11:1-7.
55. Lin MH, Lee RKK, Li SH, et al. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. *Fertil Steril*. 2008;90:352-359.