Extended semen examinations in the sixth edition of the World Health Organization manual on semen analysis: contributing to the understanding of the function of the male reproductive system

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In the sixth edition of the World Health Organization manual for the examination and processing of human semen, extended examination methods to provide key diagnostics in the investigation of the male reproductive system function are elaborated. These go beyond the basic analysis of semen and may be useful in more specifically guiding the clinical characterization of fertile or infertile men. Among the extended examinations included in the chapter, the use of multiparametric scoring for sperm morphological defects, sperm DNA fragmentation, and the roles for computer-assisted analysis of sperm or semen are arguably those that will be the most widely used and may also cause the most debate. (Fertil Steril® 2021;■:■–■. ©2021 by American Society for Reproductive Medicine.)

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An important difference between the fifth and sixth editions of the "World Health Organization manual for the examination and processing of human semen" concerns the inclusion, in the latest edition, of a chapter on extended semen evaluations. The editorial board retains that such evaluations may be useful in

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certain circumstances for diagnostic or research purposes. Although most of the extended examinations described in the chapter have been shown to correlate to various degrees with semen parameters, these tests cannot be used for routine application until clear positive and negative predictive values are defined. These extended examinations may be useful in improving the characterization of fertile and infertile men.

MULTIPARAMETRIC SPERM ASSESSMENT

The concept of multiparametric sperm assessment involves using many scored variables (e.g., sperm number, motility, and morphology) with an aim to provide evidence-based increased specificity and sensitivity of diagnosis (1). In reality, this may, in the future, include any of the additional tests outlined in the manual and, for accurate fertility prognosis in more advanced models, would incorporate key female factors as well. For example, increasing evidence supports the fact that male age may play as important a role as female age in the prediction of success; therefore, the incorporation of such data, as that in the study by Horta et al. (2), into any model will also help guide patient expectation and choices (3).

The precise determination of sperm morphology is covered in basic semen analysis. However, the ability of a clinician to determine the meaning and prognosis is at best challenging. To improve the accuracy and consistency of the basic analysis of sperm morphology, a number of suggestions have been made—a key option highlighted in the extended analysis is the use of the basic data to compile a multiparametric assessment of morphology. It is also the simplest extended analysis method, as it uses the numbers that are already generated when the basic analysis is performed correctly, thus results can be instantly available. A useful example of the multiparametric output is the teratozoospermia index—this single number expresses the number of defects per abnormal sperm and, therefore, ranges between 1.0 and 4.0 in conventional use (4). Semen analysis has been widely discussed elsewhere as a measure of the health/quality of "the spermatogenic factory"—as such, the teratozoospermia index provides a potentially more meaningful assessment of how many mistakes are being made and may relate more accurately to the outcome (4).

SPERM DNA QUALITY AND FRAGMENTATION

As the DNA delivered by a sperm contributes 50% of the potential offspring genome, the occurrence of breaks within sperm DNA has been an area of significant attention because these breaks represent the most detectable frequent potential cause of paternal DNA anomaly transmission to the progeny. Breaks in the DNA strand (sperm DNA fragmentation [sDF]) are detectable in a large percentage of spermatozoa in the ejaculates of some subfertile/infertile men (5), raising concerns regarding the reproductive functions and health of the offspring of these men (6).

Several meta-analyses have indicated a role of sDF in the reproductive functions (in particular, increased rate of miscarriage and decreased pregnancy and live birth rates in in vitro fertilization programs) (7); however, whether the assessment of this type of damage should be used widely in the diagnostic process is still controversial, having generated an intense debate in the literature in the last few years. In particular, discussions have focused on the following: although all measures are discussed with respect to sDF, in fact, the separate techniques of measurement should be regarded as individual tests; the lack of standardization of the methods and poor details regarding their sensitivity and specificity; the lack of clear cutoff values for specific outcomes; and the lack of evidence for effective interventions to alter the prognosis.

In relation to generating cutoffs for use in individual patient cases, positive and negative predictive values must be identified for each test. For populations of men, correlations can indicate possible important factors; however, if the variability in the measurement technique is too high, the value for the individual man will be low. Thus, a cutoff constructed from, for example, a receiver operating characteristic analysis may be interesting for different variables; however, if the overlap between groups is too large the clinical value is too low to suggest routine use. To date, only a few studies have used receiver operating characteristic analysis to define cutoff levels for sDF (8–10).

Methods/Techniques Used

Put simply, different techniques use different properties of DNA and underlying scientific principles, meaning that the results are not interchangeable (11). As such, they must be considered separately, and their data should not be grouped or used to suggest clinical importance or validity (12). In particular, there are assays that evaluate the susceptibility of chromatin to be damaged after an insult (such as sperm chromatin dispersion and sperm chromatin structure assay) and those that directly evaluate the presence of breaks in the DNA (terminal deoxynucleotidyl transferase–mediated biotin–deoxyuridine triphosphate nick-end labeling and single-cell gel electrophoresis [also known as comet assay]). As such, each type of test must be used on the basis of its own evidence for the specific outcome that the evidence supports. This is possibly the most significant issue with the current use, interpretation, and deployment of these tests, and it thus poses a challenge to the field for future study and use. Recent meta-analyses (13–16) have grouped the different studies according to the method used to evaluate the damage and indicate some of the debate and differences of opinion. From these meta-analyses, it emerges that direct assays (such as terminal deoxynucleotidyl transferase–mediated biotin–deoxyuridine triphosphate nick-end labeling and comet assay) are, in general, better predictors of assisted reproductive technology (ART) outcomes or occurrence of miscarriage (13–16). It should be noted that the clinical studies included in these meta-analyses are highly heterogeneous, mostly employing nonstandardized assays to detect sDF, using different inclusion criteria, and in most cases, female factors were not considered in the statistical analysis.

Standardization of Methods and Cutoff Values

The lack of standardization of the methods among different laboratories represents an important problem emerging

from the current debate in the literature. An important consequence of the lack of standardization and heterogeneity of the assays regards the choice of threshold values to discriminate pathologic and normal conditions. Along with the use of tests without supporting evidence, this has generated confusion among clinicians and hindered the introduction of relevant sDF tests in the diagnostic management of infertile men. Defining a gold standard method to evaluate sDF in the couple infertility workup remains an important goal of the scientific community. Because the identification of the cutoff values depends strictly on the assay used to measure sDF and the indication for which the testing is occurring (11), the evaluation of each parameter for clinical purposes implies that each laboratory should define relevant cutoff levels using their own method. Alternatively, a test offered by a specialized laboratory/service, which is standardized by them for the outcome, can be used. In practicality, the standardization offered if all laboratories start to follow the precise methods outlined in the manual will allow an improved accumulation of supporting data from worldwide. We would hope that in the future, for a given assay, its threshold for detection of impaired fertility, miscarriage, change in ART modality/intervention, and ART outcome could be provided, in which these may be different values with different specificity depending on what is being discussed.

Decision Making and Treatments for "High" Results

The question that "is it possible to improve sperm DNA quality?" does not currently have a clear answer. Although the results of the test can be useful to the clinician for counseling of the couple regarding the chances of ART success, the available evidence regarding a therapeutic approach for men is scarce.

Studies investigating the mechanisms leading to sDF included testicular germ cell apoptosis, alterations in the spermatogenetic process, and oxidative stress as the main mechanisms generating damage (17). Therefore, sDF may be generated either in the testis or at any later stage. In the latter case, oxidative stress appears to be the main insult (17), although data suggest that there may be age-dependent mechanisms that are independent of oxidative stress levels (18). Many clinical studies investigate the effects of several antioxidants on sDF; however, these studies identify problems only within the male and do not consider susceptibility to damage during transit in the female tract or after laboratory procedures, which may be relevant. Most of these studies are performed in a small number of patients, do not report clear selection criteria, are limited by the specific assay employed, and are not placebo-controlled. A recent Cochrane review (19) on the role of antioxidants for male infertility included a meta-analysis of six studies reporting the effect of various antioxidants vs. placebo on sDF levels, without a clear conclusion; importantly, there is still a lack of high-quality data on whether this affects live birth or miscarriage.

A recent meta-analysis involving six studies with 383 men with idiopathic infertility treated with follicle-stimulating hormone (20) revealed a slight but significant decrease in sDF after 3 months of treatment. However, as in the case of antioxidant treatments, the heterogeneity of the studies and the lack of clear inclusion criteria in most of them do not allow the drawing of clear-cut conclusions.

In relation to patients with varicocele, an increase in the sDF levels in the ejaculate has been demonstrated (21). Several studies have shown the efficacy of varicocelectomy in decreasing the sDF levels and potentially increasing the chance of pregnancy (22).

Some recent clinical studies have suggested the use of testicular spermatozoa for intracytoplasmic sperm injection (ICSI) as an option for men with high sDF in semen (23). This strategy is based on testicular sperm retrieval procedures, as there is evidence that testicular spermatozoa have lower fragmented DNA (22). Although the initial data appear supportive, this approach has not been fully evaluated against other potential methods for reducing sDF, such as multiple fresh ejaculates, short abstinence, and lifestyle changes. As such and because of the possible occurrence of adverse effects of testicular sperm extraction and the associated discomfort for the patients, care should be taken in evaluating whether this should be considered (23). For patients with high semen sDF levels, advanced techniques for sperm selection for ICSI may be of some help, although more evidence is required (24, 25).

Finally, since the beginning of laboratory-based ART procedures, there have been attempts to select the best sperm from the ejaculate for therapeutic use with methods ranging from simple swim-up technologies, historically through methods such as glass wool, to density gradient centrifugation. More recently, techniques specifically aimed to improve the outcome through the selection of sperm with great DNA integrity have been commercialized, including magnetic-activated cell sorting and intracytoplasmic morphologically selected sperm injection; however, these have so far not been proven to improve the outcome (25). Physiologic ICSI has also not shown improved live birth rates, although, notably, miscarriage rates do appear to have been reduced (24, 26). The latest techniques in the field claim to be microfluidic but, in fact, bear similarities to the original swim-up technologies; high-quality evidence comparing these technologies with routine practice is still awaited (27).

In conclusion, although the introduction of sDF to the routine assessment of male infertility is probably unnecessary (28, 29), there are clinical conditions (such as previously failed intrauterine insemination/in vitro fertilization/ICSI cycles, repeated pregnancy loss, advanced paternal age, diabetes, and the presence of inflammatory signs of the lower genital tract) in which the assessment of sDF may be warranted to assist in the clinical decision (30). Such a conclusion appears in line with the recent American Urological Association/the American Society for Reproductive Medicine (formerly The American Fertility Society) male infertility guidelines regarding the potential use of sDF testing in the clinical management of infertile couples (28, 29). It should also be noted that, at present, limited data are available regarding possible therapeutic options to decrease sDF levels. It is likely that only a combined approach—which considers the background of female age and egg quality—will actually reveal the true prognostic value of sDF. In this, it is notable that sDF also appears to be male age–dependent (18). Female age being a key factor

as the underlying ability of the oocyte to correctly repair sDF (31, 32) is likely a major influencer that impacts the reproductive outcome (2). Recent arguments extend concern regarding the sperm DNA quality further to suggest that perhaps we should always be diagnosing and remediating this, whenever possible, as an aim to best protect the health of future generations (who may otherwise carry a deleterious mutational load due to aberrant repair of sDF) (33).

PRINCIPLES FOR COMPUTER-AIDED SPERM ANALYSIS

The use of computer-aided sperm analysis (CASA) systems in clinical assessment should be assessed in terms of its ability to perform tasks relating to basic sperm function analysis, live cell motility assessment, and fixed assays. Although CASA is used as a catch-all term for all computational analysis, this separation is important to clearly understand where it can have the most significant impact and, importantly, where it should not be used over expert manual analysis. Despite the emerging results of comparative studies (34–39), there is still not enough evidence that would currently allow the use of computer analysis in wide routine clinical practice. The lack of standardization of CASA algorithms and approaches is a significant barrier to this application (40); as with the other sperm functional tests laid out in this article, CASA requires extensive quality control procedures to validate and ensure the robustness of the assessment (41, 42).

The use of CASA for basic sperm functional analysis has often been a divisive issue, with critics maintaining that such instruments are unable to obtain sperm count and concentrations to the level of trained laboratory staff. This is likely correct; the small aliquot volumes used in CASA (often in the order of 2 μL) significantly restrict the likelihood of obtaining a statistically representative sample of cells in the examined droplet. Although classification algorithms are constantly improving, a complication lies in distinguishing sperm from debris or other cells present in semen. These nonsperm cells (debris and other extraneous objects) in undiluted semen can often be misclassified as sperm, contributing to errors in assessing the concentration and motility of a sample. To try to account for this, newer systems take note of the presence of a flagellum to help exclude debris from analyses. Further limitations include the inability of systems to assess and highlight agglutinated or aggregated spermatozoa (42).

Although many producers of CASA systems would claim that they can accurately perform sperm count and basic semen analysis, we (and other investigators (43)) believe that this is the wrong question to ask; instead, CASA should be considered in terms of where it can best provide information that is additional to, and not instead of, basic semen analysis and take advantage of the computational ability to make measurements that are unavailable to the human eye. Computer-aided sperm analysis can be (and has been) applied to a variety of tasks.

Sperm motility assessment is ideally suited for the use of CASA (also known as "CASA-Mot") (44). However, to do this effectively, the use of CASA (across all stages of preparation, imaging, and analysis) must be standardized (40, 45); however, the potential for this to reduce operator-dependent subjectivity of assessments should not be ignored. Current systems use a range of algorithms and approaches that render even measurements of the same name (e.g., the velocity of the average path) to be incomparable between systems (40).

Our approach in the sixth edition is, therefore, to develop consistent procedures for the use of CASA in motility analysis, emphasizing that the sample preparation, including the use of chambers for restricting sperm movement, is essential to obtain accurate results. One particular aspect of motility in which CASA may be able to provide significant added benefit over standard techniques is in the evaluation of sperm hyperactivation, in which the complex change to flagellar movement is difficult to reliably estimate by manual visual analysis (41). The ability of sperm to exhibit hyperactivated motility is a crucial component in enabling penetration into, and migration through, viscoelastic cervical mucus as well as to detach from the endosalpingeal epithelium (46). As a result, reliable quantification of the hyperactivating capability of a sample provides useful insight into whether the necessary working signaling systems are in place for fertilization. This may be in terms of natural fertility (47), or to detect such things as CatSper mutations that may affect medically assisted reproduction outcomes (48), without the requirement for complex technology such as calcium imaging or molecular biology.

A key issue for fixed assays, such as morphology analysis or sDF, is the inter- and intraoperator variation in making visual assessments. The use of CASA in such assays (e.g., computer-aided sperm morphometric assessment [CASMA]) has significant potential to address these issues, providing a consistently reproducible result that can be objectively tested to ensure accuracy. Care must still be taken for CASMA, however, to ensure that the same level of standardization and quality control is maintained as is for manual assessment. It is also worth noting that attempts to introduce CASMA have not yet achieved widespread clinical uptake, potentially in part due to the wide array of stains and staining still employed by laboratories.

Although CASA has existed in some form for the past few decades (49–51), the power of both sperm morphology and motility parameters to alone predict pregnancy outcomes in reproductive treatment is uncertain, with some studies suggesting low predictive power (52) and others highlighting that rapid motility may be predictive of outcome (53). Therefore, it is in the emerging technologies that have appeared in recent years that CASA may find most success. For this reason, the manual now aims to provide an overview of the most promising emerging technologies, which are classified as either computational or technological, covering a wide range of potential applications. Two significant areas highlighted by the new revision are algorithmic improvements to allow for the analysis of densely concentrated samples (54) and the introduction of flagellar waveform tracking (55). The former will allow for significantly more cells to be analyzed, providing greater statistical power and allowing for sperm to be classified into different subpopulations to improve understanding of the variety and changes in cell motility. Flagellar tracking has significant potential to go beyond

what is currently thought of as CASA (measuring quantities derived from head tracking) and instead provide a live cell readout of the internal metabolism and biochemical signaling of cells as they swim. As these are emerging technologies, their potential clinical significance is yet to be established. In particular, what is deemed to be a "normal" or "abnormal" flagellar beat remains to be characterized, and what can be done in the case of an abnormal result needs more investigation.

Cheaper and more portable CASA systems are beginning to become more widespread, particularly with improvements and access to mobile phone cameras (56–59). These portable approaches have additional potential to address health care disparities relating to the access to infertility care worldwide (57) and provide men with an ability to take ownership of their condition with affordable in-home testing (58). Although these systems do not currently reach the accuracy or confidence that fully featured CASA systems provide, they should be seen in the context of separation between tools for clinical judgments vs. clinical indicators. As an example of the latter case, the mobile phone–based system may provide a good quick check for the presence of spermatozoa in a sample and any gross features that may warrant further investigation, importantly supplementing, not replacing, the need or desire for a gold standard semen analysis where necessary (56–59).

Therefore, the future of CASA is not as a single technology that accurately replicates basic semen analysis but as a suite of techniques that individually may improve diagnostics for live cells (CASA-Mot) and stained or fixed cells (CASMA) or perform other relevant tasks. It is the belief that these emerging technologies will enable significant research findings in the near future and ultimately open the door for new therapies and diagnostics for male-factor infertility.

CONCLUSION

Following the standardized extended examination methods provided in the sixth edition is important for generating improved global diagnostic data in the investigation of the male reproductive system. Unfortunately, in these advanced investigations, almost all publications have individual variations in methodology; without some consensus, globally validated and appropriate techniques will struggle to emerge. The consensus methods provided will hopefully allow diverse international teams to generate high-quality, multicenter data that either justify or rebuff the use of individual tests, via the setout methods, for specific indications.

The future of male diagnostics will undeniably lie in the improved clinical characterization of fertile and infertile men, moving on from the poorer prognostics obtained in simple semen analysis (1).

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