Extended semen examinations in the sixth edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen

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DOI:
10.1016/j.fertnstert.2021.11.034

Document Version
Peer reviewed version

Citation for published version (Harvard):
Baldi, E, Gallagher, MT, Krasnyak, S, Kirkman-Brown, J & Editorial Board members of the World Health Organization laboratory manual for the examination and processing of human semen 2022, 'Extended semen examinations in the sixth edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen: contributing to the understanding of the function of the male reproductive system', *Fertility and Sterility*, vol. 117, no. 2, pp. 252-257. https://doi.org/10.1016/j.fertnstert.2021.11.034

Title: Extended semen examinations in the sixth edition of the WHO manual on semen analysis: contributing to the understanding of the function of the male reproductive system

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Capsule (30 words or less):

Beyond methods for basic semen examination, the WHO manual details precise methodology for extended examinations. This is provided to improve standardization of key additional tests that may aid specific diagnoses.

Running Title: WHO Extended Semen Examination 2021

Key words: multiparametric, sperm DNA, sperm motility, CASA, fertility

Short Narrative Abstract

In the 6th edition of the WHO 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 the fertile or infertile male.

Among the extended examinations included in the chapter: the use of multiparametric scoring for sperm morphological defects, sperm DNA fragmentation (sDF), and the roles for computer assisted analysis of sperm or semen (CASA) are arguably those which will be most widely used and may also cause most debate.

Introduction

An important difference between the 5th and the 6th editions of the "WHO 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 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, until clear positive and negative predictive values are defined these tests cannot be used for routine application. Where they may find use is 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 could in-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 being included. For example increasing evidence supports that male age may play an as important role as female in prediction of success, incorporation of such data as that of Horta *et al.* (2)into any model will therefore also help guide patient expectation and choices (3).

The precise determination of sperm morphology is covered in basic semen analysis. However, the ability for a clinician to determine 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 extended analysis is use of the basic data to compile a multiparametric assessment of morphology. It is also the simplest extended analysis method– as it uses numbers already generated when basic analysis is performed correctly, so results can be instantly available. A useful example of multiparametric output is the Teratozoospermia Index or TZI – 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 widely been discussed elsewhere as a measure of the health/quality of 'the spermatogenic factory' – as such TZI provides a potentially more meaningful assessment of how many mistakes are being made and may relate more accurately to oucome (4).

Sperm DNA Quality & 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. DNA strand breaks (sDF) are detectable in a large percentage of spermatozoa in the ejaculates of some sub/infertile men (5), raising concerns regarding the reproductive functions and the health of the offspring of these men (6).

Several meta-analyses have indicated a role for sDF on reproductive functions (in particular: increased rate of miscarriage, decreased pregnancy and live birth rates in in vitro fertilization programs (7)), but whether 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: (i) though all measures are discussed as sDF, in fact the separate techniques of measurement should be regarded as individual tests; (ii) the lack of standardization of the methods and poor details on their sensitivity and specificity, (iii) lack of clear cut-off values for specific outcomes, and (iv) lack of evidence for effective interventions to alter prognosis.

In relation to generating cut-offs for the use in individual patient cases, Positive and Negative Predictive Values need to be identified for each test. For populations of men, correlations can indicate possible important factors, but if the variability in the measurement technique is too high, the value for the individual man will be low. Thus, a cut-off constructed from, for example, a ROC-analysis may be interesting for different

variables, but if overlap between groups is too large the clinical value is too low to suggest routine use. To date, only a few studies have utilized ROC-analysis to define cut-off levels for sDF (8-10)

Methods / techniques used

Put simply, the different techniques utilize different properties of DNA and underlying scientific principles, meaning results are not interchangeable (11). As such they need to be considered separately, 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 [SCD] and sperm chromatin structure assay [SCSA]) and others that evaluate directly the presence of breaks in the DNA (terminal deoxynucleotidyl transferase nick end labelling [TUNEL] and single-cell gel electrophoresis (also known as COMET)). As such, each type of test must be utilized based on its own evidence for the specific outcome that evidence supports. This is possibly the most significant issue with current use, interpretation and deployment of these tests, and thus poses a challenge to the field for future study and utilization. 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 difference of opinion. From these meta-analyses it emerges that direct assays (TUNEL, COMET) are, in general, better predictors of ARTs outcomes or occurrence of miscarrage (13-16). It should be noted that the clinical studies included in these meta-analyses are highly heterogeneous, mostly employing non-standardized 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

Lack of standardization of the methods between 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 pathological and normal conditions. Along with the use of tests without supporting evidence, this has generated confusion among clinicians and has hindered the introduction of relevant SDF tests in the diagnostic management of the infertile male. Defining a gold standard method to evaluate SDF in the couple infertility work-up remains an important goal of the scientific community. Since identification of cut-off values depends strictly on the assay used to measure sDF and the indication for which the testing is occuring (11), evaluation of each parameter for clinical purposes implies that each laboratory should define relevant cut-off levels using their own method. Alternatively, a test offered by a specialized lab / 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 future, for a given assay, its threshold for detection of impared fertility; miscarriage; change in ART modality / intervention and ART outcome could be provided, where these may be different values with different specificity dependant upon what is being discussed.

Decision making and treatments for 'high' results

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

The 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 (9). Therefore, sDF may be generated either in the testis or any later stage. In the latter case, oxidative stress appears to be the main insult (9) though data suggests that there may be age dependant mechanisms that are independent of oxidative stress levels (17). There are many clinical studies investigating the effects of several antioxidants on sDF, but these studies only identify problems 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 (18) on the role of antioxidants for male infertility, included a meta-analysis of 6 studies reporting the effect of various antioxidants vs placebo on sDF levels, without clear conclusion and importantly there is still a lack of high-quality data on whether this affects live birth or miscarriage.

A recent meta-analysis including six studies with 383 men with idiopathic infertility treated with FSH (19) revealed a slight but significant decrease of sDF after three months of treatment. However, as for the case of antioxidant treatments, the heterogeneity of the studies and the lack of clear inclusion criteria in most of them does not allow the drawing of clear-cut conclusions.

In relation to patients with varicocele, an increase of sDF levels in the ejaculate has been demonstrated (20). Several studies have shown the efficacy of varicelectomy in decreasing sDF levels and potentially increasing the chance of pregnancy (21).

Some recent clinical studies have suggested the use of testicular spermatozoa for ICSI as an option for men with high sDF in semen (22). This strategy is based on testicular sperm retrieval procedures, as there is evidence that testicular spermatozoa have lower fragmented DNA (21). This approach, though initial data appears supportive, has not been fully evaluated against other potential methods of reducing sDF, such as multiple fresh-ejaculates, short abstinence, and lifestyle changes. As such and due to 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 (22). For patients with high semen sDF levels, advanced techniques for sperm selection for ICSI may be of some help, although more evidence is required (23, 24).

Finally, since the beginnings 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-ups, historically through such methods as glass wool, through to density gradient centrifugation. More recently techniques specifically aimed to improve outcome through selection of sperm with great DNA integrity have been commercialized including MACS and IMSI but these have so far not been proven to improve outcome (24). Physiological ICSI 'pICSI' has also not shown improved live-birth rates, though notably miscarriage rates do appear reduced (23, 25). The latest techniques to the market in the area claim to be microfluidic, but in fact bear similarities to the origal swim-up technologies, high-quality evidence comparing these technologies to routine practice is still awaited (26).

In conclusion, while the introduction of sDF to the routine assessment of male infertility is probably unnecessary (27, 28), there are clinical conditions (such as previous failed IUI/IVF/ICSI cycles, repeated pregnancy loss, advanced paternal age, diabetes, presence of inflammatory signs of the lower genital tract) where assessment of sDF may be warranted to assist in the clinical decision (29). Such a conclusion appears in line with recent AUA/ASRM male infertility guidelines regarding the potential use of sDF testing in the clinical management of infertile couples (31). It should be also 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 dependant (17). Female age being a key factor as the underlying ability of the oocyte to correctly repair sDF (30, 31) is likely a major influencer that impacts upon reproductive outcome (2). Recent arguments extend concern around sperm DNA quality further to suggest perhaps we should always be diagnosing and remediating this where possible, as an aim to best-protect the health of future generations (32) (who may otherwise carry a deleterious mutational load from aberrant repair of sDF).

Principles for CASA

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 (i) basic sperm function analysis; (ii) live cell motility assessment; and (iii) fixed assays. While CASA is used as a catch-all term for all computational analysis, this separation is important in order to clearly understand where it can have most significant impact, and importantly, where

CASA should not be used over expert manual analysis. Despite the emerging results of comparative studies (33-38), 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 (39); as with the other sperm functional tests laid out in this manuscript, CASA requires extensive quality control proceudres to validate and ensure robustness of assessment (40, 41).

The use of CASA for basic sperm functional analysis (i) has often been a divisive issue, with critics maintaining that such instruments are unable to obtain sperm counts and concentrations to the level of trained laboratory staff. This is likely correct; the small aliquot volumes used in CASA (often on the order of 2μl) significantly restrict the likelihood of obtaining a statistically representative sample of cells in the examined droplet. While classification algorithms are constantly improving, a complication lies in distinguishing sperm from debris or other cells present in semen. These non-sperm cells in undiluted semen (debris, other extraneous objects) 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 highlight agglutinated or aggregated spermatozoa (41).

Although many producers of CASA systems would claim that they can accurately perform sperm count and basic semen analysis, we (and others, (42)) 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, a basic semen analysis, and take advantage of the computational ability to make measurements that are unavailable to the human eye. CASA can be (and has been) applied to a variety of tasks.

Sperm motility assessment (ii) is ideally suited for the use of CASA (also known as CASA-Mot (43)). However, to do this effectively, the use of CASA (across all stages of preparation, imaging, and analysis) must be standardized (39, 44), but 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 [VAP]) to be incomparable between systems (39).

Our approach in the 6th edition is, therefore, to develop consistent procedures for the use of CASA in motility analysis, emphasizing that sample preparation, including the use of chambers for restricting sperm movement, is essential to obtain accurate results. One particular aspect of motility where CASA may be able to provide significant added benefit over standard techniques is in the evaluation of sperm hyperactivation, where the complex change to flagellar movement is difficult to reliably estimate by manual visual analysis (40). The ability of sperm to exhibit hyperactivated motility is a crucial component in enabling penetration into, and migration through, viscoelastic cervical mucous as well as to detach from the endosalpingeal

epithelium (45). As a result, reliable quantification of the hyperactivating capability of a sample provides useful insight into whether the necessary working signalling systems are in place for fertilization. This may be in terms of natural fertility (46), or to detect such things as Catsper mutations that may affect MAR outcomes (47), without the requirement for complex technology such as calcium imaging or molecular biology.

A key issue for fixed assays (iii), such as morphology analysis or sDF, is the inter- and intra-operator 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 QC is maintained as is for manual assessment. It is worth also 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 (48-50), the power of both sperm morphology and motility parameters to alone predict pregnancy outcomes in reproductive treatment is undertain, with some studies suggesting low predictive power (51) and others highlighting that rapid motility may be predictive of outcome (52). 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: (i) algorithmic improvements to allow for the analysis of densely concentrated samples (53); and (ii) the introduction of flagellar waveform tracking (54). 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 wide-spread, particularly with improvements and access to mobile phone cameras (55-58). These portable approaches have additional potential to address health care disparities relating to the access to infertility care worldwide (56), and provide men with an ability to take ownership of their condition with affordable in-home testing (57).

While these systems do not currently reach the accuracy or confidence that fully featured CASA systems provide, they should be seen in the context of a separation between tools for clinical judgements versus clinical indicators. An example of the latter case, mobile phone-based system may provide a good quick check for the presence of spermatozoa in a sample, and for any gross features that may warrant further investigation; importantly supplementing, not replacing, the need or desire for a gold standard semen analysis where necessary. (55-58). These portable approaches have additional potential to address health care disparities relating to the access to infertility care worldwide (56), and provide men with an ability to take ownership of their condition with affordable in-home testing (57).

The future of CASA, therefore, is not as a single technology that accurately replicates a basic semen analysis, but as a suite of techniques which individually may improve diagnostics for live cells (CASA-mot), stained or fixed cells (CASMA), or performs other relevant tasks. It is the hope 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.

Conclusions

Following the standardized extended examination methods provided in the sixth edition is key to 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 multi-centre data that either justifies or rebuffs use of individual tests, via the set-out methods, for specific indications.

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

Acknowlegments: *The development of the "6th Edition of WHO Laboratory Manual for the examination and processing of human semen" was funded by the UNDP-UNFPA-UNICEF-WHO-World Bank Special Programme of Research, Development and Research Training in Human Reproduction (HRP), a cosponsored programme executed by the World Health Organization (WHO).*

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