Chapter 23
How is sperm chromatin structure quality assessed? What is the value of doing such assessments?

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International standards to assess semen parameters have been developed and updated over several decades by the World Health Organization (WHO), with the most recent revision appearing in 2021. As discussed in Chapter 22, these parameters are useful in population studies; when parameters such as total sperm number, concentration or sperm motility are very high or very low it is clear that there is a strong correlation with fertility outcome. However, the semen sample parameters of many individuals fall well below the WHO reference values, yet they can father children; on the other hand, men with semen parameters well above the WHO reference values may experience infertility. Thus, a very large proportion of unexplained (idiopathic) infertility may be due to factors other than those determined in standard semen analysis.

The parameters used in routine semen analysis are not designed to assess the "quality" of the spermatozoa, other than their ability to swim and "appear" normal. What matters for successful fertility is not just getting a single spermatozoon to an oocyte but also delivering its nucleus (chromatin package) that is not damaged and capable of supporting the development of normal, healthy progeny. There are many animal studies showing that spermatozoa that are damaged due to exposure to chemicals, drugs or radiation, but that otherwise appear normal, do not have a disadvantage in fertilizing eggs and can deliver their damaged chromatin to the oocyte. Therefore, assessing the quality of the chromatin of spermatozoa has become of growing interest in the last two decades.

Although the most recent WHO manual does not include assessment of sperm chromatin integrity as part of routine semen analyses, it does include an extensive section on the value of extended examination of semen and concludes that: "Clinically, there is growing awareness that chromosomal anomalies and gene

mutations underlie a diverse spectrum of male infertility that underlie many of the anomalies seen in a semen analysis." (p.83, WHO Manual 2021).

There are many aspects of the components of the nuclei of spermatozoa that can be assessed. These include not only the integrity of the DNA (single or double strand breaks, cross-links), but also the way it may be modified (DNA methylation, histone modifications), how it is packaged with non-coding RNAs, associated with the nuclear matrix, and condensed with protamine cross-links and histones.

Starting more than fifty years ago, techniques such as alkaline elution were developed to assess aspects of chromatin quality such as DNA breaks and cross-links. Some of these assays, such as the nuclear decondensation assays (either in vitro or in vivo) or the DNA template function assay, are used less frequently now thanks to the development of more rapid and efficient new methods. During the past twenty years a number of these newer methods have been developed using light, florescence and confocal microscopy, flow cytometry, real time PCR and high throughput screening. There are a number of ways of groupings these many assays. We will provide here a highlight of some of the assays that are more commonly used or under active development.

Assays used to evaluate genomic integrity

TUNEL Assay. The quantity of open DNA 3'-OH ends can be assessed using the terminal deoxytransferase mediated dUTP nick end-labeling (TUNEL) assay in which the terminal deoxytransferase enzyme incorporates a fluorescent UTP at 3'-OH free ends; fluorescence increases proportionally with the number of DNA strand breaks. This assay can be run as either a slide-based or a flow-cytometry assay.

Comet Assay. This electrophoresis-based assay evaluates DNA strand breaks in a single spermatozoon. Small, broken pieces of DNA migrate away from the DNA core of the cell, creating the tail of the comet figure. The percentage of the DNA in the tail of the comet and the tail length are measured using specific image analysis software. Depending on whether the assay is run at basic or neutral pH, the Comet Assay will assess single or double strand breaks, respectively. Although a novel high-throughput assay (HT-COMET) has been developed, this very sensitive procedure requires the use of expensive equipment. While providing a powerful index of DNA

damage, the Comet assay is time consuming and the results are variable among labs.

Gene-specific DNA Damage and Repair Assay. Using quantitative PCR, the presence of single/double strand DNA breaks and/or the extent of bulky DNA adducts are assessed. This assay depends on the principle that increased bulky adduct formation or DNA breaks prevent the polymerase from completing amplification of the complimentary strands, thus causing less PCR product to be amplified. An advantage of this assay is that it requires very small (nanogram) amounts of DNA, but it is time consuming and can be difficult to do accurately.

Fluorescence in situ Hybridization (FISH) Assay. By using probes specific to individual chromosomes, the frequency of chromosomal aneuploidy (duplication or deletion of whole or part of chromosomes) can be assessed in spermatozoa after proper decondensation. Multiple probes are available for human spermatozoal chromosomes, but until this method is automated, having to score thousands of sperm manually for accurate results renders this method still primarily a research tool.

Assays used to evaluate sperm chromatin conformation and proteins

Acridine Orange Flow Cytometry or SCSA® Assay. This commonly used flow-cytometry based assay assesses the susceptibility of sperm chromatin to denaturation in acid and detergent as determined by acridine orange binding to double- or single-stranded DNA; this dye gives a green fluorescence for unfragmented DNA and a red fluorescence for fragmented DNA. After denaturation, measurement of fluorescence at both wavelengths assesses the percentage of fragmented DNA (DNA fragmentation index: DFI). This assay allows for rapid assessment of a large number of cells; it may also be run as a "slide assay", where the color reading is made on microscope slides, but where only a few cells are monitored.

Sperm Chromatin Dispersion Test. In this assay, the extent of halo formation of single spermatozoa, representative of the decondensation of their nuclei, is determined for cells that are embedded in agarose, lysed and acid treated. The larger the halo, the greater the extent of DNA breaks. Even though the results obtained using this technique have been correlated with double strand breaks, it is not a direct measure of DNA breaks but a reflection of the overall chromatin structure.

CMA3 Fluorometric Assay. This assay indirectly measures the amounts of protamine present, thus reflecting the extent of chromatin condensation. The dye, CMA3, binds to sites in DNA where protamines would normally bind. This assay was first developed as a slide based assay but is now available as a FACS (fluorescence-activated cell sorting) assay.

Monobromobimane (mBBr) Thiol Labeling Assay. The objective of this assay is to determine the amount of free sulphydryl groups in spermatozoa. Less mature sperm have more free sulphydryls, whereas more mature sperm have fewer. Thiol labeling is done to quantify the total thiol, free thiol and disulfide levels in the nuclei of spermatozoa. As for the two assays above, this may be run as a slide-based or a FACS assay.

Proteomic Analysis. The specific chromatin structure of the sperm is essential for proper fertility and is, in part, due to the proteins that are bound to the DNA, including the protamines, histones and components of the nuclear matrix. With the recent advances in proteomics technology, using 1 or 2 dimensional SDS polyacrylamide gels coupled with mass spectrometry, various components of the sperm are being identified. This, in turn, increases our knowledge of the sperm chromatin structure composition and opens the possibility of new criteria to be assessed when assessing sperm quality.

Assays used to evaluate the epigenetic status of spermatozoa

DNA Methylation. DNA methylation of cytosine residues is one of the major epigenetic marks established during spermatogenesis. Both single site and genome wide methylome studies have revealed alterations in the methylome under various conditions, including smoking and advanced age. Indeed, the sperm methylome can be used to accurately predict a man's age. The identification of specific changes in DNA methylation patterns with specific causes of infertility is under investigation (Chapter 13).

Histone Modifications. Most histones are removed during spermatogenesis but a significant proportion, up to 15% in man, remains in mature sperm. The role of these histones is still under debate, but recent studies indicate that they mark genes that are activated early after fertilization. Alterations in either the amount and distribution of histones or in their numerous marks, e.g.,

acetylation, methylation, sumoylation, appear to affect the functions of spermatozoa (Chapter 14).

Long and Small Non-coding RNAs. The third major pillar of epigenetic regulation is the expression and function of a wide range of non-coding RNAs that include both long (mRNA, lncRNA, and circRNA) as well as short, noncoding RNAs (miRNAs, piRNAs, tsRNAs, and snRNAs) (Chapter 15). The exact functions of these various RNAs are actively being pursued. However, there is clear evidence that some of them play an essential role in the development of the post-fertilization embryo. It is likely that their relationship to the quality of spermatozoa will become evident as our knowledge grows about these molecules.

Conclusions

In an effort to help standardize some of these sperm chromatin structure assays, the WHO 2021 manual has provided protocols for the TUNEL assay, sperm chromatin dispersion test, acridine orange flow cytometry assay (*SCSA*®), and for fluorescence in situ hybridization.

No consensus has yet emerged regarding the value of any one or a group of these tests in assessing the fertility of an individual, but their usefulness in certain circumstances such as recurrent miscarriages, or unexplained fertility is now appreciated (Chapter 32). Furthermore, the growing understanding of the complexity of what is in a sperm nucleus and how it is packaged, as well as the increased precision and accuracy of newer tests, makes it likely that new clinically useful tools to accurately assess the quality of spermatozoa will be used clinically in appropriate subgroups of patients.

Suggested reading

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