

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

### *Range of methods that assess sperm chromatin quality*

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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 2010. As discussed in Chapter 12, these parameters are useful in population studies. At the extremes of parameters such as sperm number, concentration or sperm motility, it is clear that there is a strong correlation with fertility outcome. However, the semen samples of many individuals fall well below the WHO standards, yet they can father children and many men with samples that meet the WHO standard are not fertile. A very large proportion of unexplained (idiopathic) infertility may be due to factors other than those classically measured using the methods described in the WHO manual.

The parameters used in standard semen analysis are not designed to assess the “quality” of the spermatozoa, other than their ability to swim and “appear” normal. But what matters for successful fertility is not just getting to the oocyte but also delivering a 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.

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) or how it is packaged and associated with the nuclear matrix. In addition to DNA, sperm nuclei contain small non-coding RNAs (pi and micro RNAs) and proteins (protamines, some histones, and nuclear matrix proteins).

Starting more than forty 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 because of 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, fluorescence 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 include:

- **Comet Assay.** This electrophoresis-based assay evaluates DNA strand breaks in a single cell. 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 it is run at basic or neutral pH, the Comet Assay will assess single or double strand breaks, respectively. This is a very sensitive assay, but it is time consuming and variable among labs.
- **TUNEL Assay.** The quantity of 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 flow-cytometry assay.
- **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 complementary 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 several thousand of sperm manually for accurate result render this method still primarily a research tool.

Assays used to evaluate sperm chromatin conformation and proteins include:

- *Acridine Orange or SCSA® Assay.* A commonly used flow-cytometry based assay wherein the susceptibility of sperm chromatin to denaturation in acid and detergent is determined by using acridine orange, a dye that binds to double- or single-stranded DNA, giving a green or red fluorescence, respectively. After denaturation, measurement of fluorescence at both wavelengths assesses the percentage of fragmented DNA (DNA fragmentation index: DFI). This assay may be run as a “slide assay”, where the color reading is made on microscope slides, or as a FACS assay, where large numbers of sperm can be analyzed rapidly.
- *CMA3 Fluorometric Assay.* This assay has been developed to indirectly measure the amounts of protamine present. 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 assay.
- *Monobromobimane (mBB) Thiol Labeling Assay.* The objective of this assay is to determine the amount of free sulphhydryl groups in spermatozoa; less mature sperm have more free sulphhydryls 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.
- *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.
- *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, but also opens the possibility of new criteria to be looked at when assessing sperm quality.

Assays used to evaluate the epigenetic status of spermatozoa include:

- *DNA Methylation.* DNA methylation of cytosine residues is one of the major epigenetic marks established during spermatogenesis. Methods for assessing changes in methylation at specific sites are well established, but several methods are under rapid development that will allow accurate assessment of changes in DNA methylation throughout the entire sperm genome.
- *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 their numerous marks, e.g., acetylation, methylation, sumoylation, may affect the functions of spermatozoa.
- *Small Non-coding RNAs.* The third major pillar of epigenetic regulation is the expression and function of small non-coding RNAs. The exact functions of the various RNAs (mRNAs, piRNAs, antisense and miRNAs) are not known, but they clearly play an essential role in spermatogenesis and potentially 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.

No consensus has yet emerged regarding the value of any one or group of tests in assessing the fertility of an individual (Chapters 12,19). However, the growing power, precision and accuracy of newer tests, based on the molecular structure of sperm nuclei, makes it likely that new clinically useful tools to accurately assess the quality of spermatozoa will emerge.

### Suggested reading

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