

Review

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Male infertility: biomolecular aspects

Abstract: Male infertility is a problem that faces increasing interest, and the continuous development of assisted reproduction techniques solicits attempts to identify a precise diagnosis, in particular for idiopathic infertile couples and those undergoing assisted reproductive technique cycles. To date, diagnosis of male infertility is commonly based on standard semen analysis, but in many cases, this is not enough to detect any sperm abnormality. A better understanding of biomolecular issues and mechanism of damaged spermatogenesis and the refinement of the molecular techniques for sperm evaluation and selection are important advances that can lead to the optimization of diagnostic and therapeutic management of male and couple infertility. Faced with a growing number of new proposed techniques and diagnostic tests, it is fundamental to know which tests are already routinely used in the clinical practice and those that are likely to be used in the near future. This review focuses on the main molecular diagnostic techniques for male infertility and on newly developed methods that will probably be part of routine sperm analysis in the near future.

Keywords: biomolecular sperm markers; male infertility; molecular sperm diagnostic; sperm analysis; sperm parameters.

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Introduction

Male infertility is attracting increasing interest due to evidence of decline in semen quality of young healthy men worldwide (1, 2). It has been estimated that couple

infertility affects 10%–15% of the general population, and male factor is responsible, alone or in combination with female factors, in about half of the cases (3–5). Many factors adversely affect sperm quality including lifestyle, diabetes, obesity, hormonal diseases, testicular trauma, cryptorchidism, varicocele, genitourinary infections, ejaculatory disorders, chemo/radiotherapy, or surgical therapies (6–8). Moreover, it is well established that genetic causes account for 10%–15% of infertility cases (9), including chromosomal abnormalities and single-gene mutations that influence at different levels many physiological processes involved in male reproduction, such as hormonal homeostasis, spermatogenesis, and sperm quality (10). However, in many cases, the cause of infertility is not identified and is therefore considered idiopathic or unexplained, suggesting little knowledge about the basic mechanism regulating spermatogenesis and sperm function. Semen analysis has an important role in the routine evaluation of idiopathic male infertility resulting from ductal obstruction due to congenital abnormal development or infection or testicular damage (11), and the World Health Organization standardized the procedures for semen analysis by producing a guide manual for semen analysis (12). Semen analysis alone is not sufficient to distinguish fertile subjects from infertile ones, and for example, having a normal sperm count is not synonymous with fertility, as having a reduced count does not indicate that a man will be unable to father a child (13). Figure 1 shows our representation of the distribution of fertile subjects in relation to sperm count. From these observations, it appears that standard semen analysis cannot clearly distinguish fertile from infertile populations and fails to detect any abnormality in many cases, and this is particularly evident in cases of infertility or repeated assisted reproduction failure with normal routine semen parameters (14). Therefore, assuming in these cases the presence of abnormal sperm function or molecular defects, it is mandatory to take into account other sperm characteristic and trigger further research aimed at identifying new potential biomolecular markers. In particular, it is important to consider sperm DNA alterations such as DNA integrity, defective chromatin packaging, apoptosis, oxidative stress, DNA fragmentation, and aneuploidy. In fact,

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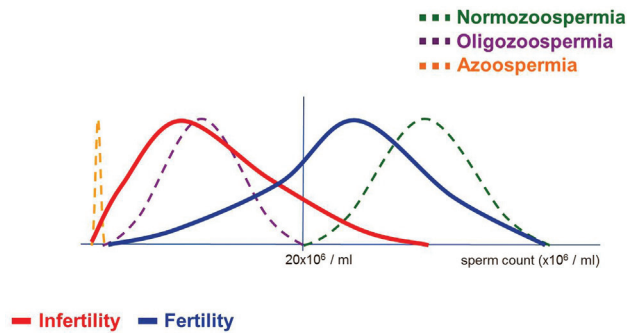


Figure 1 Fertile subjects distribution in relation to sperm count.

it is increasingly evident that the integrity of sperm DNA is of vital importance for sperm function and embryo development (15, 16), while damaged DNA can have negative impact in fetal development, health of offspring, blastocyst development, leading to failed implantation and miscarriages (17–19). This review focuses on the most widely considered biomolecular aspects and possible future development in male infertility.

DNA integrity and fragmentation

Sperm DNA integrity is considered an increasingly important parameter in the diagnosis of male infertility because DNA quality is essential in maintaining reproductive potential in men (20). In addition to the ability of DNA to resist damage, and to the limited capacity to repair certain types of damages, natural selection allows only sperm with intact DNA to fertilize (21). The increasing use of assisted reproductive techniques (ARTs), which bypasses natural selection, made it necessary to understand the pathogenesis of DNA damages and to identify the best methods to assess DNA integrity. Different mechanisms of sperm DNA damage have been described: abortive apoptosis during meiosis I (22), oxidative stress and reactive oxygen species (ROS) production (23), caspase and endonuclease activity inducing DNA damage (24), iatrogenic, environmental, and occupational factors (25–27), advanced male age (28), and cryopreservation (29). Many techniques have been developed to evaluate sperm DNA integrity: acridine orange staining assay, sperm chromatin structure assay (SCSA), comet assay, sperm chromatin dispersion (halo) test, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (TUNEL) test, γ H2AX evaluation, and *in situ* nick translation test. Acridine orange (Figure 2) is a metachromatic dye that has a different fluorescent property in the presence of

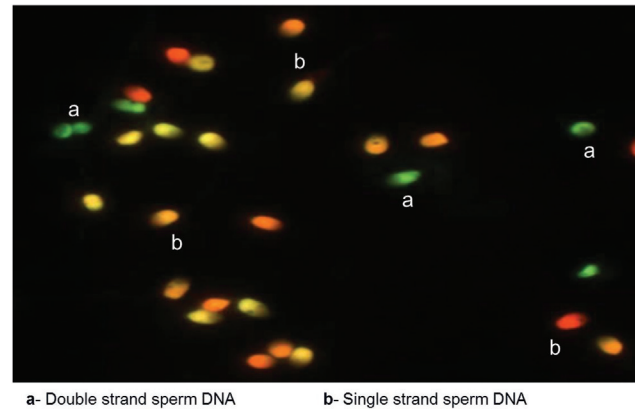


Figure 2 An example of acridine orange staining assay.

single- or double-stranded DNA. It is easy and fast, but it is limited by inter-observer subjectivity and rapid fading of the fluorescence (30). SCSA is the flow cytometric version of the acridine orange test and measures the susceptibility of sperm DNA to breakage after mild acid treatment. It is simple and fast and allows the analysis of a high number of cells. However, it gives only the percentage of sperm with higher susceptibility to DNA breaks but not much information about the amount of DNA damage in a single sperm (31). Comet assay consists of single-cell gel electrophoresis, performed under neutral or alkaline conditions. DNA damage is quantified by measuring the displacement between the genetic material of the nucleus comet head and the resulting tail by specific image analysis software. With this method, it is possible to analyze many cells and the percentage of single and double DNA breaks is easily detectable, but the technique setup is labor-intensive, needs a dedicated software to analyze the results, and the DNA damage can be overestimated (32). The sperm chromatin dispersion (halo) test, similarly to the comet test, involves agarose gel electrophoresis of single sperm that is then treated with an acid or alkaline denaturing solution. After cell lysis, a halo around the head is generated in cells with low levels of DNA breaks, whereas the cells with more extensive DNA breaks show a small halo or no halo because the DNA loops do not diffuse. The halo test can easily detect the number of DNA breaks for single spermatozoa in a large number of cells, but as with comet, the setup is not easy, a dedicated software is necessary, and DNA damage can be overestimated (33). TUNEL is based on the TdT-mediated incorporation of fluorescently labeled nucleotides at the 3'-OH ends of single- and double-strand DNA breaks to create a signal that increases with the number of DNA breaks. This technique analyzes both single- and double-strand DNA breaks and allows

the analysis of thousand of cells in few times (it is a fast process and only a short period of time is needed to finish the process), but it is not standardized (34). γ H2AX plays a major role in the mechanisms of recognition and repair of DNA double-strand breaks involving some protein kinase, like ATM (ataxia telangiectasia mutated protein), for the phosphorylation and activation of H2AX histone (35, 36). These aspects are still poorly investigated in mature sperm, but it has been demonstrated that H2AX is activated during the remodeling process of chromatin that occurs in spermatids during the final stages of spermatogenesis (37). This method allows the measurement of DNA double-strand breaks, and it can be evaluated by fluorescence microscopy or flow cytometry, but only a few studies have been published; thus, standardization and normal value are still under consideration.

In situ nick translation is similar to TUNEL, consisting of an enzymatic labeling method that incorporates biotinylated dUTP at single-strand DNA breaks with template-dependent DNA polymerase I (38). The measured parameter is proportional to fluorescent spermatozoa with incorporated dUTP. This technique is simple, inexpensive, and requires only a fluorescence microscope for analysis, but because it uses a template-dependent polymerase, it has a low sensitivity compared with other techniques and identifies only single-strand breaks.

Protamination and DNA packaging

The protamination process consists of the substitution of the nuclear protein histones with protamines during the transition from spermatids to mature sperm, and in human spermatozoa, it is not complete and a fraction of DNA (10%–15%) remains bound to histones. During spermatogenesis, the elongating spermatid chromatin undergoes a gradual process of condensation, which is initiated in the round spermatids and extends to elongated spermatids. This mechanism is an elaborate process that encompasses several biochemical and biological aspects, culminating in the deposition of protamine in DNA grooves. The protamination of sperm chromatin involves the expression and storage of proteins involved in the condensation, removal, and degradation of nuclear histones and their replacement by transition proteins and protamine 1, transcriptional silencing and DNA repair, reduction of nuclear volume, repackaging of protaminated chromatin in torroids, and development of a characteristic head shape and perforatorium (39, 40). Protamine expression acts as checkpoint mechanism and guarantees

sperm quality, and compromised or damaged sperm chromatin may affect fertilization, embryogenesis, and fetal development (41, 42). The main causes of damage to the protamination process are genetic variation and mutation, endocrine disruptors, failed checkpoint mechanism, and other endogenous and/or exogenous injuries occurring during spermatogenesis (43). The methods used for the detection of chromatin damage are aniline blue, toluidine blue, and chromomycin A3 (CMA3). Aniline blue is an acidic dye that has a greater affinity for the basic groups of the nucleoprotein in the loose chromatin of sperm nucleus. Sperm nuclei with normal chromatin packaging are nearly colorless, whereas increased aniline blue staining indicates loose chromatin packing (44). This technique is simple, inexpensive, and requires a simple bright-field microscope for the analysis, but its efficacy is limited by inter-observer subjectivity in establishing classification groups and by heterogeneous slide staining. Toluidine blue is a basic stain that evaluates the phosphate residues of the sperm DNA with loosely packed chromatin and fragmented ends. Sperm heads with normal chromatin packaging are light blue, whereas in sperm with defective protamination, the stain attaches to the lysine-rich regions of histone and produces an intense bluish violet coloration (45). The method is simple, inexpensive, and requires an ordinary microscope for the analysis, but inter-observer subjectivity represents a main drawback. Moreover, its results are very precise, but when cytometer evaluation is used, it becomes expensive. Lastly, CMA3 is a fluorimetric assay that indirectly measures the amounts of protamines present in the sperm nucleus. It is a specific GC-rich sequence dye, and it interacts at the same site where protamine binds to the DNA. A greater intensity of CMA3 staining indicates protamine deficiency or aberrant chromatin packing (46). It represents a simple and inexpensive technique, and it only requires a simple fluorescence microscope for the analysis, but, as in the previous case, inter-observer subjectivity is a prominent limit.

Aneuploidies

Human sperm cells are haploid cells containing 22 autosomes and 1 sex chromosome. Chromosome number variations give rise to aneuploidy, a condition for which a cell has one or more additional chromosomes or a defect in basal disposition. Aneuploidy is caused by a lack of disjunction of sister chromatids during mitosis or of homologous chromosomes during meiosis (47). Aneuploid gametes represent a very important risk factor not only to

infertility but also to the occurrence of spontaneous abortions and fetal pathologies (48). It is possible to obtain data on aneuploidy frequencies in large populations of sperm using the emerging technology of fluorescence *in situ* hybridization (FISH) analysis, an assay that uses chromosome-specific DNA probes to detect numerical chromosomal abnormalities in decondensed sperm (Figure 3) (49, 50). Sex chromosomes and chromosomes 13, 18, and 21 represent the most common aneuploidies detected at birth in humans (Klinefelter syndrome, Turner syndrome, trisomy 13, 18, and 21) and are the most important causes of congenital abnormalities, developmental disabilities, mental retardation, and infertility in humans (51). To detect aneuploidies for these chromosome abnormalities, five-color FISH is usually performed. To standardize the analysis and minimize inter-individual differences, automated systems can be used instead of manual scoring. The advantage of this method is the possibility to analyze a large number of sperm cells, especially with automated systems, but at the same time, only few chromosomes can be analyzed and normal values (percentage of sperm with specific aneuploidy) are still debatable.

Mitochondrial function and apoptosis

Mitochondrial status is an important trait of sperm physiology, as they generate a major part of the ATP required for sperm metabolism, membrane function, and motility. Moreover, the loss of mitochondria membrane potential is one of the earliest apoptosis mechanisms in the cell systems (52). The mitochondrial stain JC-1 (5,5,9,6,6'-tetrachloro-1,9,3,3'-tetraethylbenzimidazolyl-carbocyanine iodide) allows distinguishing between spermatozoa with

poorly and highly functional mitochondria (53). JC-1 accumulates in the cytosol of healthy sperm as a green fluorescence monomer, whereas in the presence of high mitochondrial membrane potential, the monomers accumulate as aggregates inside the mitochondria, emitting red fluorescence. When spermatozoa are dying and the mitochondrial membrane potential is no more persistent (collapses), JC-1 exists only in monomeric form and emits green fluorescence (54). This technique is simple and represents the only method clinically available for evaluating mitochondrial function, but it may be affected by many variables and it requires careful preparation when adjusting the cytometer.

Another method used to evaluate apoptosis is annexin V (55). One of the early steps during apoptosis is the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, and it can be detected by an annexin V-labeling dye that shows early plasma membrane degeneration. The combination of annexin V-FITC with propidium iodide to detect sperm vitality in cytofluorimetry is able to simultaneously distinguish live spermatozoa from those in apoptosis and from those that are dead (56). It is easy and fast, and cytofluorimetric analysis allows the analysis of thousands of cells in few times (it is a fast process and only a short period of time is needed to finish the process), but it requires the use of adequate control in adjusting the cytometer.

Future perspectives

The molecular aspects of spermatogenesis and male infertility have gained increasing interest in the recent years, and Table 1 summarizes the main molecular techniques currently applied in diagnostics. New possible diagnostic techniques are continuously proposed, and the following are some methods that are not yet part of routine investigations but will most likely play a role in the diagnostic workup of infertile patients in the near future. Raman microspectroscopy provides information on DNA packaging at the single sperm cell level in living cells and will likely be used more in the near future (57). It is based on the principle of inelastic scattering, which results from the interaction between light and matter and can be used to obtain images of the spermatozoa cell shape together with a chemical analysis of the sperm cell contents. By this method, it is possible to obtain detailed information about the conformation, composition, and intermolecular interactions of macromolecules such as DNA and proteins in sperm (58). Moreover, it is noninvasive

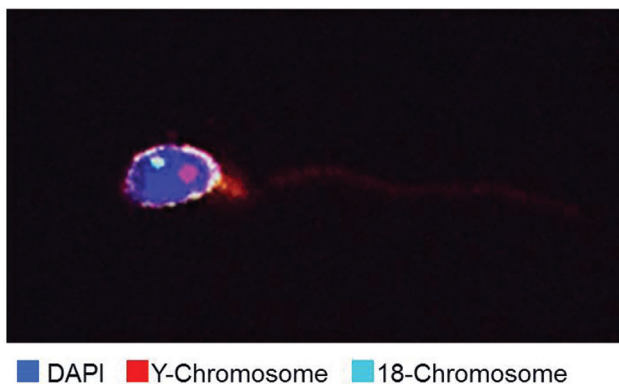


Figure 3 An example of FISH analysis.

Table 1 Main molecular techniques currently applied in male infertility diagnostics.

Evaluation	Technique	Pros	Cons
DNA integrity and fragmentation	Acridine orange	Easy and fast	Inter-observer subjectivity Rapidly fading of fluorescence
	SCASA	Simple and fast	Not information about amount of damaged DNA
		Many cells analyzed	
	Comet	Many cells analyzed	Labor-intensive setup
		Easy detection of DNA breaks	Dedicated software necessary DNA damage can be overestimated
Protamination and DNA packaging	Halo test	Many cells analyzed	Labor-intensive setup
		Easy detection of DNA breaks	Dedicated software necessary DNA damage can be overestimated
	TUNEL test	Many cells analyzed	Not standardized
	γ H2AX	Easy detection of DNA breaks	Not standardized
	Aniline blue	Simple and inexpensive	Inter-observer subjectivity
Aneuploidies	Toluidine blue	Simple and inexpensive	Inter-observer subjectivity
	Toluidine blue with cytometer detection	Simple	Very expensive
Mitochondrial function and apoptosis	CMA 3	Very precise	
		Simple and inexpensive	Inter-observer subjectivity
Mitochondrial function and apoptosis	FISH	Many cells analyzed	Few chromosomes analyzed Normal threshold debatable
		Simple	Only method for mitochondrial evaluation Many variables
Mitochondrial function and apoptosis	Annexin-V	Easy and fast	Careful preparation
		Many cells analyzed	Careful preparation

and nondestructive at moderate photon energies; it can work *in vitro* and *in vivo* under a wide range of environmental conditions, and combined with the use of image analysis, it could represent a possible label-free and rapid identification of normal sperm cell (57, 58). Comparative genomic hybridization (CGH) array is considered a potential method for the simultaneous analysis of all chromosomes of sperm cells, up to single spermatozoa (59). This procedure allows obtaining a molecular cell karyotype detecting both aneuploidies and structural chromosomal alterations (60). To date, this analysis is mostly used in research, mainly due to the high cost, but it seems to give important information on the biology and pathophysiology of spermatogenesis and sperm chromosome aberrations in normal subjects and in patients at higher risk of producing unbalanced sperm, such as infertile men, carriers of karyotype anomalies, men with advanced age, subjects treated with chemotherapy, and partners of women with repeated miscarriages and repeated failure during ARTs (61). A further assay that will probably become part of the male infertility diagnosis is quantitative PCR, which is used to evaluate telomere length. Telomeres are non-coding DNA sequences composed of highly conserved hexameric tandem nucleotide repeats (TTAGGG) located at the ends of chromosomes and confer chromosome stability and genome integrity. Telomere length is a complex

trait maintained by telomerase and determined by normal cell division, ROS, genotoxic insults, genetic predisposition, aging, lifestyle factors, psychological stress, and the age of the father at the time of conception (62). The few available studies do not fully explain the role of sperm telomeres, but interestingly, it appeared that although sperm and leukocyte telomere lengths tend to be strictly correlated in the same individual, leukocyte telomere length decreases and sperm telomere length increases with age (63). Moreover, one recent study analyzed sperm telomere length in small groups of fertile and infertile subjects with normal sperm counts and found a lower telomere length in the sperm of this latter group (64). Even if further studies are needed to clarify the pathophysiology link between sperm telomere length and male fertility, there is a good chance that this analysis will become a new potential biomarker of sperm.

Expert opinion

Male infertility is one of the clearest examples of a complex phenotype with substantial genetic and molecular basis. To date, there are various methods of semen analysis, but there is no gold standard. In fact, among

all these techniques, not one can give all the information about the quality and quantity of damage on the individual sperm. Furthermore, threshold values of normality and/or pathology have not yet been identified, and therefore, each laboratory should set a threshold by itself. An analysis of all the available methods may be the first step to identify those that provide the greatest number of information with an acceptable value. Furthermore, considering the growing number of techniques being developed, it is crucial to standardize and align them in all laboratories dealing with sperm analysis.

Outlook

Certainly, in the coming years, existing methods will be refined and many new techniques will be developed. This review considers biomolecular diagnostic tests for male infertility, reporting on the pros and cons of the most commonly used techniques in molecular and functional sperm evaluation. To date, it is not possible to predict which methods will take over, but this review could be a starting point to understand which techniques are worth developing and which are useful and beneficial for the patients and the health-care system.

Highlights

- Male infertility is attracting increasing interest, and a decline in semen quality worldwide is evident.
- Standard semen analysis alone cannot distinguish fertile and infertile subjects.
- The main molecular investigations concern DNA integrity, DNA packaging, sperm aneuploidies, and mitochondrial function
- To date, there is no gold standard, and in many cases, the threshold values of normality and/or pathology have not yet been identified.
- New methods that will probably be included in routine laboratory tests in the near future are Raman microspectroscopy, CGH array, and quantitative PCR, which is used to evaluate telomere length.
- Certainly, there will be many new methods that will be studied, developed, and proposed to the laboratories in the coming years.
- It is not yet clear which methods will take over and which methods will eventually be discarded.
- It is necessary to continuously update and test methods to identify the most effective.

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