EVALUATION OF SPERM DAMAGE: BEYOND THE WHO CRITERIA

Nabil Aziz, Ashok Agarwal

INTRODUCTION

For over twenty-five years, the World Health Organization (WHO) has served to provide a standardized approach to the assessment of the fertility potential of semen sample. These standards are concerned with measurable parameters such as the physical properties of an ejaculate, estimating the count of its cellular content be it sperm or leukocytes, grading sperm morphology and motility, and examining a possible immune interaction between sperm and the content of seminal plasma or the preovulatory mucus produced by the uterine cervix. The adoption of these standards worldwide has been enhanced through local schemes of quality control measures leading to andrology laboratory accreditation and certification. The first andrology laboratory manual published by the WHO in 1981 was the culmination of clinical experience and research in the previous eighty years (1). In its successive editions, the WHO manual portrayed stricter criteria in assessing parameters of interest and as a result values that were thought to be compatible with normal male fertility were modified (2,3). The resonating success of the WHO criteria is met by call for further scrutiny of sperm quality to address numerous concerns born from clinical and research work carried out in more recent years. First, the results of semen analyses can be very subjective and prone to intra- and interobserver variability (4). Second, although the traditional, manual-visual light microscopic methods for evaluating semen quality maintain their central role in assessment of male fertility potential, often a definitive diagnosis of male fertility cannot be made as a result of basic semen analysis (5). Conventional semen analysis per se cannot cover the diverse array of biological properties that the spermatozoon expresses as a highly specialized cell (6,7). Third, there has been growing awareness that the predictive power of the cutoff values of different sperm parameters is not absolute and that there is some degree of overlap between fertile and infertile male populations. As a result, many infertile couples with no detectable abnormalities are labeled with the clinically vague diagnosis of idiopathic infertility. On a different front, further modification of the cutoff points compatible with fertility has been advocated. Fourth, we now have a better understanding of the impact of processes such as the sperm capacitation and the acrosome reaction, sperm oxidative stress and apoptosis on sperm-egg interaction, and the fertilizing ability of sperm, both in vivo and in vitro. The assessment of these aspects of sperm function and physiology are beyond the current remit of the WHO manual. Last but not least, numerous studies in the literature have demonstrated that semen quality is declining and that the incidence of testicular cancers is increasing (8). These observations have been attributed to damage in sperm chromatin. During in vivo reproduction, the natural selection process ensures that only spermatozoon with normal genomic material fertilizes an oocyte. However, some assisted reproduction technologies (ART) bypass this natural selection process, leading to the possibility that an abnormal spermatozoon is selected to fertilize the oocyte.

This chapter reviews the clinical significance of sperm chromatin abnormalities, oxidative stress (OS), apoptosis, and microwave hazards for male gametes highlighting the laboratory methods available to assess these aspects of sperm structure and function.

HUMAN SPERM CHROMATIN

Human Sperm Chromatin Structure and Packaging

Chromatin packaging refers to the highly complex and specific structure into which ejaculated sperm DNA is folded in order to properly deliver the genetic information to the egg. Unlike the relatively loose structure of chromatin (DNA and nuclear proteins) in somatic cells, sperm chromatin is tightly compacted because of the unique associations between the DNA and sperm nuclear proteins (predominantly highly basic proteins known as protamines) (9,10). In the later stages of spermatogenesis, the spermatid nucleus is remodeled and condensed, a process that involves among other things the displacement of histones by transition proteins and then by protamines that have half the size of histones molecule (11). The DNA strands are tightly wrapped around the protamine molecules (about 50 kb of DNA per protamine), forming tight and highly organized loops (toroids) (10). Inter- and intramolecular disulfide cross-links between the cysteine-rich protamines are responsible for the compaction and stabilization of the sperm nucleus. It is thought that this nuclear compaction is important to protect the sperm genome from external stresses such as oxidation or temperature elevation that may be encountered in the sperm trajectory in the male and female genital tracts (12).

It is estimated that 85 percent of sperm chromatin is tightly packaged by protamines, but up to 15 percent of the DNA remains packaged by histones at specific DNA sequences associated with the nuclear periphery and with telomeres (13,14).
The histone-bound DNA sequences that are less tightly compacted and placed at the periphery suggest that these DNA sequences or genes may be involved in fertilization and early embryo development (13). An excess of nuclear histones (>15 percent) results in poorer chromatin compaction and a subsequent increased susceptibility to external stresses (e.g., oxidation or temperature elevation in the female reproductive tract) (12).

In comparison with other species (15), human sperm chromatin packaging is exceptionally variable, both within and between men. This variability has been mostly attributed to its basic protein component. Infertile men, as compared with fertile controls, have an increased sperm histone:protamine ratio (11,16,17). Moreover, in contrast to mammals whose spermatozoa contain only one type of protamine (P1), human spermatozoa contain a second type of protamine (P2), which is deficient in cysteine residues (18). Consequently, the disulfide cross-linking responsible for more stable packaging is diminished in human sperm as compared to species containing P1 alone (19). Aberrant P1/P2 ratios arise from an abnormal concentration of P1 and/or P2, either of which is associated with male infertility (20–28). Prior to this chromatin rearrangement, recombination is essential for spermatogenesis to occur; as seen in studies using animal knockout models, decreased recombination is associated with diminished spermatogenesis (29).

The main bulk of the sperm DNA is in the nucleus and only a small fraction is of mitochondrial origin within the sperm midpiece. The sperm mitochondrial DNA is a small, circular DNA that is not bound to proteins (30), which exhibits a high rate of mutation (31). Sperm motility is related to the mitochondrial volume within the sperm midpiece, and mutations or deletions in the mitochondrial DNA have been associated with reduced sperm motility (31). Although inheritance of mitochondrial DNA is primarily maternal, paternal transmission of mitochondrial DNA mutations has been reported (32). The examination of mitochondrial DNA may gain some importance in the evaluation of male infertility, particularly in relation to assisted reproductive technologies.

Types and Mechanisms of DNA Damage

Defects in the genomic material in mature sperm may take the form of packaging or nuclear maturity defects, DNA fragmentation (single-strand nicks or double-strand breaks), DNA integrity defects, or sperm chromosomal aneuploidies (33). These sperm chromatin defects have been associated with a diversity of disease conditions, environmental stress factors, and life-style issues. These include cancer, drug use, high fever, and infections, elevated testicular temperature (e.g., use of hot baths, saunas, down-filled blankets, laptop computers, and prolonged periods of driving), varicocele, air pollution, cigarette smoking, alcohol, and advanced age (34–37). These conditions exert their effect through recognized molecular mechanisms of DNA damage. Scientists agree on four distinct mechanisms by which DNA can be compromised or damaged, although there may be others: defective sperm chromatin condensation, apoptosis (38,39), oxidative stress (40,41), and genetic lesions (42–44). It is likely that multiple of these mechanisms are involved in causing DNA damage in any one disease (45).

The two main components of abnormal chromatin packaging are defective histone-protamine replacement (discussed above) and chromatin fragmentation. DNA fragmentation is particularly frequent in the ejaculates of subfertile men (46). Physiological and environmental stress, as well as gene mutations and chromosomal abnormalities, can all disturb the highly refined biochemical events that occur during spermatogenesis. This disruption can ultimately lead to abnormal chromatin structure that is incompatible with fertility. Stress can also cause sperm chromatin fragmentation by inducing chromatin structural problems through apoptosis or necrosis (47). In addition, chromatin fragmentation can arise during spermiogenesis if the DNA nicking and ligating activities of the endogenous nuclease, DNA topoisomerase II (topo II), are abnormal. High levels of both topo II and DNA nicks are present in elongating spermatids (42,48). The presence of DNA nicks may reflect the need to relieve torsional strain resulting from negative supercoiling associated with the displacement of nucleosomal histones by protamines and modification of tertiary structure in elongating spermatids (42,49,50). Therefore, in elongating spermatids, the presence of nicks is likely a physiological necessity. These nicks are not deleterious when they are ligated by topo II prior to the completion of spermiogenesis and ejaculation (42). However, if topo II ligating activity is abnormal or blocked by exposure to topo II inhibitors (51), nicks may not be repaired properly, and they may remain in otherwise mature, morphologically normal, ejaculated spermatozoa. Oocytes and early embryos have been shown to repair sperm DNA damage (52). Consequently, the biological effect of abnormal sperm chromatin structure depends on the combined effects of sperm chromatin damage and the capacity of the oocyte to repair it.

Genetic lesions are another mechanism that create insults or gaps within the genome and may yield effects ranging from minimal to catastrophic (53). They can be divided into three classes, based on the type of impact they present (54). The first class consists of chromosomal aneuploidies and rearrangements, where batteries of genes on specific chromosomes have changes in expression dosages or changes in their normal genomic environments. The second class encompasses submicroscopic deletions (microdeletions), where deletions or rearrangements of multiple genes mapped in a molecular environment have changes in their expression patterns. The third class embodies single-gene defects where expression of a single gene (or key element) is changed or lost, causing male infertility. These lesions can affect all of the human chromosomes, including any of the 300 genes estimated to be involved in male fertility. They can occur within introns as well as exons, making their impact difficult to predict (53).

The Detection of Nuclear Chromatin Abnormalities

Detection of Chromosome Aneuploidy

During metaphase I or II of meiosis, nondisjunction can occur, resulting in sperm with an abnormal complement of chromosomes. Fluorescent in situ hybridization (FISH) in interphase sperm cells affords convenient evaluation of sperm chromosome ploidy (55) and has revealed that aneuploidy occurs in humans at a much higher rate than in other organisms (56–60). A typical probe is designed to recognize a relatively large section of a particular chromosome (usually 0.2–2 Mb) and then labeled with fluorochrome. After hybridization of the probe with a sample of the sperm, the labeled portion of the chromosome appears as a fluorescent domain within the sperm nucleus and can be identified using fluorescence microscopy. There are multiprobe assays for different chromosome combination to allow the distinction between isolated chromosome disomy and diplomy.
Due to the limitation of the eye for detecting color differences, assays are limited to three or four probes at a time. Men with severe oligozoospermia, asthenozoospermia, and/or teratozoospermia have been shown to have increased sperm aneuploidy rates, and this is the likely cause of their abnormal WHO sperm parameters (61–64). There is evidence that there is no selection against aneuploid sperm during fertilization or embryo development to the first cell division (65). However, it is still unknown to what extent increased aneuploidy in sperm contributes to adverse outcomes in assisted reproduction and natural conception (66). The risk includes the chance of spontaneous abortion or delivery of a child with a congenital abnormality. Thus, probes for chromosomes X, Y, 13, 18, and 21 allow us to detect sperm that may lead to various important aneuploidy syndromes: triple X, Klinefelter, Turner, XYY, Patau, Edwards, or Down/C213 syndrome. A screening program in which men with severe teratozoospermia and severe oligozoospermia undergoing assisted reproduction, in combination with an investigation of the chromosomal status of embryos, will help clarify the likelihood of paternal transmission of aneuploidies and its effect on embryogenesis (67).

Although the test has high specificity, it is labor intensive requiring close attention to strict scoring criteria to ensure precision and minimize intertechnician variability. So far, the lack of automation has limited the number of studies that employed this technique in reproductive medicine.

Detection of Sperm Nuclear DNA Damage

Several assays have been developed to evaluate sperm chromatin integrity, and their capability to assess male fertility potential has been under active scrutiny (Table 19.1). In general, all assays can be divided into three groups: 1) sperm chromatin structural probes, 2) tests for direct assessment of sperm DNA fragmentation, and 3) sperm nuclear matrix assays. Other methods less frequently used include high-performance liquid chromatography.

Chromatin Structural Probes Using Nuclear Dyes

Chromatin structural probes using nuclear dyes are both sensitive and simple to use and therefore attractive for clinical utilization. Their cytochemical bases, however, are rather complex because several factors may influence the staining of the chromatin: 1) secondary structure of DNA, 2) regularity and density of chromatin packaging, and 3) binding of DNA to chromatin proteins.

Detection of DNA Secondary Structure and Conformation Defects

Even a single DNA strand break causes conformational transition of the DNA loop domain from a supercoiled state to a relaxed state. Supercoiled DNA avidly takes up intercalating dyes [like acridine orange (AO)] because this reduces the free energy of torsion stress (orthochromasy: monomeric AO binds to DNA and fluoresces green). In contrast, the affinity for intercalation is low in relaxed DNA and is lost in fragmented or denatured DNA. In this case, an external mechanism of dye binding to DNA phosphate residues and dye polymerization is favored (metachromasy, aggregated AO binds to DNA, and fluoresces red) (80,81). Since the 1960s, it has been known that fragmented DNA is easily denatured (82,83). Tejada et al. (68). introduced the microscopic AO assay, a simplified fluorescent

---

**Table 19.1: Various Methods for Assessing Sperm Chromatin Abnormalities.**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Parameter measured</th>
<th>Method of analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine orange (68,69)</td>
<td>DNA denaturation (acid)</td>
<td>Fluorescent microscopy</td>
</tr>
<tr>
<td>Toluidine blue stain (70)</td>
<td>DNA fragmentation</td>
<td>Optical microscopy</td>
</tr>
<tr>
<td>Acidic aniline blue (71)</td>
<td>Nuclear maturity (DNA protein composition)</td>
<td>Optical microscopy</td>
</tr>
<tr>
<td>Chromomycin A3 (72)</td>
<td>Nuclear maturity (DNA protein composition)</td>
<td>Fluorescent microscopy</td>
</tr>
<tr>
<td>Sperm chromatin dispersion (73)</td>
<td>DNA fragmentation</td>
<td>Fluorescent microscopy</td>
</tr>
<tr>
<td>DNA breakage detection–fluorescent in situ hybridization (74)</td>
<td>DNA fragmentation (ssDNA)</td>
<td>Fluorescent microscopy</td>
</tr>
<tr>
<td>In situ nick translation (75)</td>
<td>DNA fragmentation (ssDNA)</td>
<td>Fluorescent microscopy</td>
</tr>
<tr>
<td>TUNEL (76)</td>
<td>DNA fragmentation</td>
<td>Optical microscopy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorescent microscopy</td>
</tr>
<tr>
<td>Solar (neutral) (77)</td>
<td>DNA fragmentation (dsDNA)</td>
<td>Fluorescent microscopy</td>
</tr>
<tr>
<td>Solar (alkaline) (78)</td>
<td>DNA fragmentation (ssDNA/dsDNA)</td>
<td>Fluorescent microscopy</td>
</tr>
<tr>
<td>Sperm chromatin structure assay (79)</td>
<td>DNA denaturation (acid/heat)</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>OHdG measurement (39)</td>
<td>8-OHdG</td>
<td>High-performance liquid chromatography</td>
</tr>
</tbody>
</table>

8-OHdG, 8-hydroxy-2-deoxyguanosine; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.
microscopic method using acid fixative that does not require flow cytometry equipment. The AO assay may be used in conjunction with flow cytometry. It has been demonstrated that staining with AO assay shows a significant difference between fertile and infertile males. The “cutoff” value for normal chromatin percentage that is compatible with natural fertility varies between 80 and 50 percent (green fluorescence) (84,85). It has been shown that sperm with excess single-stranded DNA (ssDNA) that is detected by a low-incidence green fluorescence (<50 percent) negatively affects the fertilization process in a standard IVF program. However, no correlation was found with pregnancy rate and live births achieved by ICSI, except in patients having 0 percent of spermatozoa with ssDNA, in whom the pregnancy rate was significantly high (86).

Toluidine blue is another basic nuclear dye used for metachromatic and orthochromatic staining of chromatin (70). It becomes heavily incorporated in the damaged dense chromatin. This stain is a sensitive structural probe for DNA structure as well as packaging. Sperm head with good chromatin integrity stain light blue. Abnormal nuclei (purple sperm heads) have been shown to be correlated with counts of red-orange sperm heads as, revealed by the AO method (87).

Chromatin Packaging Density

Chromatin proteins in sperm nuclei with impaired DNA packaging appear to be more accessible to binding with the acidic dye, as found by the aniline blue (AB) test (88). An increase in the ability to stain sperm by acid AB (blue color) indicates a looser chromatin packaging and increased accessibility of the basic groups of the nucleoprotein due to the presence of excess residual histones (89). This large, bulky dye is unable to bind to the densely packaged chromatin of normal sperm (with its full complement of protamines). Results of acid AB sperm assessment correlate well with the AO test (90) and have shown a clear association between abnormal sperm chromatin and male infertility (91). However, the correlation between the percentage of AB-stained spermatozoa and other sperm parameters remains controversial (92). Most important is the finding that chromatin condensation as visualized by aniline blue staining is a good predictor for IVF outcome, although it cannot determine the fertilization potential, cleavage, and pregnancy rate following ICSI (93).

Chromatin Proteins

Similar to the AO test, the Chromatin Structure Assay (SCSA) (94) measures the susceptibility of sperm nuclear DNA to acid-induced conformational transition in situ by quantifying the metachromatic shift of AO fluorescence from green (native DNA) to red (denatured or relaxed DNA or RNA). It is an indirect indicator of DNA damage because it measures the amount of ssDNA after treatments that normally do not denature sperm DNA (heat or acid PH). SCSA uses flow cytometry to measure the relative amount of red versus green fluorescence on a per sperm basis, in large number of sperm (typically 5,000 to 10,000 per sample) in only a few minutes. Each sperm is classified as normal or abnormal based on the amount of ssDNA it contains, and the percentage of abnormal cells is calculated for each semen sample. A threshold was established that identifies samples compatible with pregnancy (<30 percent sperm cell damage). This cutoff point has been shown to have a predictive value for both in vivo and in vitro fertilization (95–97). A recent review indicated that in a meta-analyses, SCSA infertility test was significantly predictive for reduced pregnancy success using in vivo, IUI, and routine IVF and to a lesser extent ICSI fertilization (98). It is claimed that because the SCSA is more constant over prolonged periods of time than routine WHO semen parameters, it may be used effectively in epidemiological studies of male infertility (99).

The prohibitive cost of running flow cytometry assay to many laboratories and the stringent quality control required meant that this test is available through central laboratories to which semen samples should be sent for testing. The SCSA is less specific relative to the DNA damage tests described below in that it may detect alterations in protamine content and disulfide cross-linkage within the protamine, as well as sperm DNA damage. Indeed, SCSA data include 1) the percentage of sperm with undetectable, medium, and high levels of DNA fragmentation, 2) the percentage of sperm with a high level of DNA staining (immature chromatin with less protamines); and 3) relative amounts of seminal debris, bacteria, and broken cells since native semen is used for the analysis (100). This lower specificity can be an advantage when predicting infertility since sperm that are defective in one or more ways will be detected (100).

Chromomycin-A3 (CMA3) is another slide-staining technique, which has been used as a measure of sperm chromatin condensation anomalies. CMA3 is a fluorochrome specific for GC-rich sequences and is believed to compete with protamines for association with DNA. The extent of staining is, therefore, related to the degree of protamination of mature spermatozoa (72,101). On balance, the most widely used techniques for sperm chromatin structure assessment are the SCSA, AO, and TB tests. The later two are simple to perform but are labor intensive and subject to inter- and intraobserver variability compared to the former.

Tests for Direct Assessment of Sperm DNA Fragmentation

The most widely used of these tests are in situ nick translation assays, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay (TUNEL), and single-cell gel electrophoresis assay (COMET). Their basic principles are summarized in Table 19.1. Nick translation (75) is a relatively simple assay for fluorescence microscopy that quantifies the incorporation of biotinylated dUTP at SSDNA breaks in a reaction catalyzed by the template-dependent enzyme, DNA polymerase I. The TUNEL assay quantifies the same incorporation at breaks in double-stranded DNA using a reaction catalyzed by terminal deoxynucleotidyl transferase. TUNEL can be assessed using bright-field, fluorescence microscopy, or flow cytometry (Figure 19.1). Several reports have demonstrated that an increased fraction of human spermatozoa showing DNA strand breaks has a negative impact on the success rate of assisted fertilization techniques (102,103). These assays are specific in that they detect DNA strand breaks, but the origin of the breaks is not always clear. In somatic cells, TUNEL has been reported to be more selective in detecting DNA degradation typical of apoptosis, whereas nick translation is thought to be indicative of necrosis (104).

The COMET assay quantifies ssDNA and/or double-stranded DNA breaks (dependant on the pH conditions, see
Table 19.1), using single-cell electrophoresis of spermatozoa stained with a fluorescent DNA-binding dye (Figure 19.2). It is, therefore, suggested as a very sensitive assay for DNA damage evaluation. The COMET assay has been shown to correlate significantly with TUNEL and SCSA assays (105). It is simple to perform, has a low intraassay coefficient of variation, and a low performance cost (106). Because it is based on fluorescent microscopy, the assay requires an experienced observer to analyze the slides and interpret the results. Similar to SCSA test, the COMET assay has been successfully used in the evaluation of DNA damage after cryopreservation (107). It may also predict embryo development after IVF and ICSI, especially in couples with unexplained infertility (108,109).

Although the assay seems simple in principle, applying it to spermatozoa is not straightforward mainly because it is first necessary to open up the highly compact sperm nucleus so that the DNA is releasable during electrophoresis (100). This requires harsh chemical treatment with detergents, enzymes (RNase and/or proteinase K), and/or disulfide-reducing agent that may induce DNA breaks. Furthermore, the stainability of sperm DNA is dynamic, increasing as the sperm DNA opens up and its associated proteins (protamine) are degraded and removed. Therefore, direct comparisons between the amounts of DNA remaining in the sperm (comet) head with that moving into the comet tails are difficult to make. Additionally, many protocol variations have been reported, with differences not only in pH but also in composition of the cell lysis solution, the timing and conditions of the electrophoresis, and the introduction of new chromatin dyes. Once comets are generated, they have been measured in different ways and with different software programs. Finally, the outcome(s) has been reported differently: some labs calculate the percentage of sperm-forming comets, and others report average measures of the extent of DNA migration for a given sperm population (8,46,110). Thus, the method is still evolving, and standardization is lacking causing difficulty in comparing results across laboratories.

Sperm Nuclear Matrix Assays

Two similar assays have been described that can be allocated to this group. The sperm nuclear matrix stability assay and the sperm chromatin dispersion test are based on the ability of intact DNA deprived of chromatin proteins to loop around the sperm nucleus carcass (73,111,112). Published data show that germ-line mutations in the nuclear matrix protein may lead to deficient DNA repair and chromatin organization (113), so matrix pathologies can impair fertility and should be considered in future.
OXIDATIVE STRESS

Reactive oxygen species (ROS) in low, controlled levels in the extracellular space play an important physiological role, modulating gene and protein activities vital for sperm proliferation, differentiation, and function. OS is defined as a cellular condition associated with an imbalance between the production of free radicals, mainly ROS, and their scavenging capacity by antioxidants. When the production of ROS exceeds the available antioxidant defense, significant oxidative damage occurs to many cellular organelles by damaging lipids, proteins, DNA, and carbohydrates, thus ultimately leading to cell death.

It is reported that up to 40 percent of infertile men have high seminal ROS levels (114,115). Moreover, high ROS production has been found to be inversely correlated with the outcome of IVF (116). Infertile males that produce high levels of ROS have a fivefold less chance of initiating a pregnancy than infertile males that produce low levels of ROS (117). High levels of seminal ROS have also been correlated with poor sperm morphology and high sperm deformity index (41).

ROS represent a broad category of molecules that includes a collection of radical and nonradical oxygen derivatives (Table 19.2) (118). In addition, there are other classes of free radicals that are nitrogen derived called reactive nitrogen species and lipid derived called reactive lipid species (Table 19.2) (119,120).

### Origin of ROS in Male Reproductive System

#### Sperm-Produced ROS

Following spermiation, spermatozoa extrude cytoplasm. Since cytoplasm is the major source of antioxidants, lack of cytoplasm causes a deficiency in antioxidant defense (Figure 19.3).

![Figure 19.3. Etiology and management of oxidative stress. Many factors, including primary pathological condition of male reproductive system, systemic disorders, and environmental factors, increase oxidative stress status, which causes spermatozoa dysfunction leading to infertility.](image-url)
Ironically, when this process is hindered, residual cytoplasm forms a cytoplasmic droplet in the sperm midregion, exhibiting high rates of ROS generation (121–123), which may be related to the enhanced presence of glucose-6-phosphate dehydrogenase. This enzyme fuels the generation of NADPH that, in turn, stimulates the production of ROS (122,124). Independent reports have also demonstrated that biochemical markers of the cytoplasmic space, such as creatine kinase, are positively correlated with the induction of peroxidative damage (121,125). Spermatozoa with cytoplasmic droplet, usually referred to as immature spermatozoa, appear more frequently in human semen compared with other animals. This has been attributed to inefficient human spermigenesis that involves fewer steps, leading to less rigorous quality control (126). The increased presence of residual cytoplasm in infertile males suggests the control of spermigenesis is even less efficient than that observed under normal conditions. This results in the release of significantly higher numbers of immature spermatozoa with cytoplasmic retention into the seminiferous tubules.

In addition to this major source of ROS production, there are three other possible sources of excess ROS generation from within the human sperm itself (53). The first is through leakage of electrons from the mitochondrial transport chain (127). This was proposed because of tests performed on rat spermatozoa, indicating increased translocation of mitochondrial free radicals into the sperm genome. However, this was not demonstrated in human spermatozoa (36). The second proposed source is through NADPH-oxidase in sperm. This theoretical oxidase would serve to transfer electrons from NAD(P)H to ground-state oxygen to create the superoxide anion. It is known oxidase would serve to transfer electrons from NAD(P)H to ground-state oxygen to create the superoxide anion. It is known that NAD(P)H in leukocytes helps to contribute to ROS production in rat spermatozoa, but it has yet to be demonstrated in humans (127,128). The third proposed intracellular source of ROS production is through the generation of nitric oxide (NO) in the postacrosomal and equatorial regions of the sperm (129–131).

**External Sources**

1) Leukocytes, particularly neutrophils and macrophages, have been associated with excessive ROS production, and they ultimately cause sperm dysfunction (132–136). ROS produced by leukocytes forms the first line of defense in any infectious process. DNA and structural damage can be found in spermatozoa from leukocytospermic patients (41,137,138). Leukocytes act either directly by synthesizing ROS or indirectly by inducing other neighboring white cells via soluble factors as cytokines (139). The scavenging effect of antioxidants is greatly diminished under such infectious conditions (140).

2) Female genital tract tissues or fluids may be the source of ROS including NO (141).

3) Environmental and lifestyle factors: this source of OS lies outside of the host’s body and includes xenobiotic agents such as organophosphorous pesticides that disrupt the endocrine system (Figure 19.3). These agents possess estrogenic properties, capable of inducing ROS production by male germ cells (128,142). Cigarette smoking is also known to increase ROS levels through increased leukocyte generation and increased seminal leukocytes (143). Infertile smokers are known to harbor increased levels of seminal oxidative stress compared to infertile nonsmokers (144). Finally, scrotal heat stress was demonstrated in stallions and mouse model to damage sperm chromatin structure, possibly by oxidative stressors (145,146). Raised testicular heat may explain raised seminal ROS in infertile patients with varicocele (147). Increase in scrotal temperature in laptop computer users has been reported (148).

**ROS and Sperm Physiological Functions**

It is now recognized that low, controlled levels of extracellular ROS produced by spermatozoa are involved in sperm capacitation and acrosome reaction (149,150). The mechanism by which ROS regulates these processes is unclear but may involve tyrosine phosphorylation of sperm proteins (124). Low concentrations of NO, a free radical with a relatively long half-life (7 s), promotes capacitation (151) and zona pellucida binding (152) by regulating cyclic adenosine monophosphate concentration and adenyl cyclase activity.

**Pathological Effects of ROS on Sperm Function**

Due to the unique structural composition, high ROS levels in seminal plasma have been associated with inhibition of sperm function and viability due to the peroxidation of membrane polysaturated fatty acids (153). This leads to loss of sperm membrane fluidity required for sperm adhesion and oocyte fusion. High levels of ROS including NO have detrimental effect on sperm kinetics through effecting a reduction in adenosine triphosphate (154).

DNA bases pyrimidines and purines and deoxyribose sugar are most susceptible to OS. Oxidation of the sugar by the hydroxyl radical is the main cause for DNA strand breaks. Oxidative damage can cause base degradation, DNA fragmentation, and cross-linking to protein (155). In addition, incorporation of oxidized deoxyribonucleoside triphosphate causes gene mutation or altered gene expression (156). The rate of DNA fragmentation is increased in the ejaculate of infertile men (157–161) as indicated by the high level of 8-OHdG, which is a product of DNA oxidation (Table 19.1). Sperm DNA is normally protected from oxidative insult by two factors: the antioxidants present in seminal plasma and the characteristic tight packaging of the DNA. ROS-induced oxidative damage also plays an important role in initiating programmed cell death, apoptosis.

**Assessment of OS**

To accurately quantify OS, levels of ROS and antioxidants should be measured in fresh samples. Direct methods such as pulse radiolysis and electron-spin resonance spectroscopy have been useful for other systems of the body. However, the relatively low volume of the seminal plasma, short life span of ROS, and need to evaluate in fresh samples have led to nonusage of direct methods for the male reproductive system (162).

One of the most widespread methods of measuring ROS is the chemiluminescence assay, which uses sensitive probes such as luminol and lucigenin for quantification of redox activities of the spermatozoa (163). Although the sensitivity of these probes is high, they are susceptible to interference. Leukocyte contamination is a major confounder. Also, the time of analysis after collection (less than one hour) and the high sperm count requirement (>1 × 106/ml) are some of the drawbacks to this
Antioxidant Measurement

The presence of low antioxidant in the seminal plasma is another important reason for increased OS, leading to male infertility. Hence, it is important to measure the total antioxidant capacity (TAC) of the semen. Different methods such as oxygen radical absorption capacity (166), ferric reducing ability of plasma (167), and phycoerythrin fluorescence–based assay are available for measuring TAC. However, the most widely used method for measuring TAC in semen is enhanced chemiluminescence. This method requires expensive instrumentation and is cumbersome and time consuming. Another emerging method for measurement of TAC is the colorimetric assay. First described by Miller et al. in 1993, this method gained its popularity as simple, rapid, and inexpensive alternative to enhanced chemiluminescence method (168).

To accommodate for the variations in ROS and TAC values, the concept of combined ROS-TAC score was proposed (169). ROS-TAC score was computed using principal component analysis. ROS-TAC scores were calculated from proven fertile men with low levels of ROS. The composite ROS-TAC scores from these men were representative of the fertile group and any score less than thirty was considered infertile.

APOPTOSIS

Apoptosis is a mode of programmed cellular death based on a genetic mechanism that induces a series of cellular, morphological, and biochemical alterations, leading the cell to suicide without eliciting an inflammatory response, pain, or scarring distinguishing apoptosis from necrosis (170). Apoptosis is required for normal spermatogenesis in mammals and is believed to ensure cellular homeostasis and maintain the delicate balance between germ cells and Sertoli cells. Its second role is for the depletion of abnormal spermatozoa (43,171).

Features and Mechanisms of Apoptosis

Morphologically, apoptosis is characterized by chromatin aggregation, cytoplasmic condensation, and indentation of nuclear and cytoplasmic membranes in apoptotic cells. Finally, the nucleus undergoes fragmentation and the whole cell blebs and fragments into apoptotic bodies (172).

In general, somatic cell apoptosis can be induced through extrinsic mechanisms acting at the plasma membrane, mitochondrial, or nuclear level (Figure 19.4) (173). The plasma membrane–dependent mechanism is typified by the interaction of the Fas receptor (CD95) and a Fas ligand that can proceed through two pathways (174). The type I pathway is mitochondria independent, involving an adaptor protein to recruit caspase-8 to the cytoplasmic domain of the Fas receptor to form a death-induced signaling complex and activation of caspase-8. The type II pathway is mitochondria dependent and involves the release of cytochrome c from the mitochondria, inducing the activation of caspase-9 and/or -7 (Figure 19.4) (175).

At the cytoplasmic level, several stimuli, including the activation of the mitochondrial membrane Bax (a member of the pro-apoptotic Bcl-2 family of proteins) lead to the release of cytochrome c (173). In the cytosol, cytochrome c stimulates a cascade of events leading to activation of caspase-3. The outer mitochondrial membrane is permeabilized releasing apoptosis-inducing factor (AIF) and/or cytochrome c. AIF directly translocates to the nucleus where it provokes large-scale DNA fragmentation and initial chromatin condensation. At the nuclear level, the genome contains genes that are transcribed as a response to apoptotic stimuli. For example, p53 functions normally as a regulator of the cell cycle and a tumor suppressor in vivo. Following DNA damage, p53 induces apoptosis by upregulation of the expression of the pro-apoptotic Bax gene and simultaneous downregulation of Bcl-2 expression, a sensitive regulator-inhibitor of apoptosis (176). During apoptosis, phosphatidylserine, normally present on the cytoplasmic face of the plasma membrane, is allowed to migrate to the outer leaflet, thus marking the cells for destruction by phagocytes (177,178).

Apoptosis may be initiated by ROS-induced oxidative damage of mitochondria membrane resulting in the release of cytochrome c that activates the caspases family. Too much OS can terminate apoptosis by inactivating the caspase enzyme cascade (181,182). Antioxidants can either suppress or facilitate
Table 19.3: Reported Studies That Correlated the Presence of Apoptotic Markers in Semen and the WHO Semen Parameters Including the Sperm Deformity Index (SDI)

<table>
<thead>
<tr>
<th>WHO sperm parameter</th>
<th>Relationship with sperm apoptotic markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm viability and motility</td>
<td>Negative correlation (39,164,191,198–200)</td>
</tr>
<tr>
<td>Sperm normal morphology</td>
<td>No correlation (203)</td>
</tr>
<tr>
<td>SDI</td>
<td>Positive correlation (204)</td>
</tr>
</tbody>
</table>

Apoptosis (182). Infertile men have been shown to have high levels of cytochrome c in the seminal plasma indirectly reflecting significant mitochondrial damage caused by high levels of ROS. Levels of ROS in infertile men are correlated positively with apoptosis, which in turn is negatively correlated with conventional semen parameters (118,162).

Apoptosis and Male Infertility

Mature sperm cells have been reported to express distinct markers of terminal apoptosis-related cell damage (38,183–186), although they lack transcriptional activity and have a very small amount of cytoplasm (187,188). Externalization of PS activated caspase-3, loss of the integrity of the mitochondrial membrane potential (MMP), and DNA fragmentation and membrane-bound death receptor Fas are markers of terminal apoptosis expressed by varying proportions of ejaculated sperm (189).

There is an established consensus on the implication of apoptosis in male infertility (184,190–192) and poor WHO semen parameters (Table 19.3); however, the exact mechanisms of its involvement remains to be elucidated (143). Relatively high rates of apoptosis have been reported in testicular biopsies from infertile men with different degrees of testicular insufficiency (194). The proportions of apoptotic sperm is reported to be higher in ejaculated semen samples from infertile men compared to healthy men (191). Moreover, sperm caspases become more activated in patients with infertility than in healthy donors during cryopreservation (189,195). Although apoptosis is considered a mechanism to ensure selection of sperm cells with undamaged DNA, sperm with DNA damage that are not eliminated by apoptosis may fertilize an ovum (108,186). Poor chromatin packaging and/or damaged DNA have been implicated in the failure of sperm decondensation after intracytoplasmic sperm injection, resulting in fertilization failure (196,197).

To date, it is not clear whether the apoptotic markers detected in spermatozoa are residues of an abortive apoptotic process started before ejaculation (38,192,205–207) or whether they result from apoptosis initiated in the postejaculation period (189). During faulty sperm development, the elimination of aberrant germ cells by apoptosis may be deranged leading to the release of surviving immature cells with activated caspase-3 in the cytoplasmic droplet (204,208). It is suggested that the presence of antiapoptotic Bcl-XL protein provides protection against activated caspase-3 (208). It is also proposed that apoptosis may be reflective of mechanisms related to endocrinopathies, varicoceles, and inflammation/infection and involves OS as initiator of apoptosis (118,190). Also, because spermatozoa are terminally differentiated cells, like neutrophils, they may exhibit a defined ex vivo lifetime that could be inherent to mature spermatozoa or could be related to anoikis, which is programmed cell death when cells are not anchored to an extracellular matrix (189,209).

Magnetic-activated cell sorting (MACS) using annexin V–conjugated superparamagnetic microbeads can effectively separate nonapoptotic spermatozoa from those with deteriorated plasma membranes based on the externalization of PS. MACS separation of sperm yields two fractions: annexin V negative (intact membranes and nonapoptotic) and annexin V positive (externalized PS and apoptotic) (210,211). A sperm preparation protocol that combines MACS with double-density centrifugation has been described to provide spermatozoa of higher quality in terms of motility, viability, and apoptosis indices compared with other conventional sperm preparation methods (203). It has been suggested that the protocol can also be used to improve cryosurvival rates following freezing and thawing and to enhance ART outcome (212,213).

Assessment of Apoptosis in Ejaculated Sperm

Sperm expression of apoptotic markers both in semen and sperm preparation has been examined applying a diversity of techniques including:

- PS externalization using a monoclonal mouse anti-human PS antibody (39,41,203,204,214).
- Caspase-3 activation using fluorescein-labeled inhibitor of caspase, which is cell permeable and noncytotoxic and which binds covalently to active caspase-3 (41,203,204,215–219).
- MMP integrity using a lipophilic cationic dye (5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolyl carbocyanine chloride) to detect intact MMP in spermatozoa (41,203,204,216).
- Chromatin fragmentation (201,202,207,220).
- Membrane-bound death receptor Fas and p53 (205,207).

MICROWAVE SPERM DAMAGE

Microwaves can affect reproductive function via an electromagnetic wave (EMW)–specific effect, thermal molecular effect, or combination of the two (221). Increase in tissue or body temperature on exposure to EMW is known to cause reversible disruption of spermatogenesis (222,223). Various investigators have addressed the concern that use of devices emitting microwaves might have adverse impacts on sperm function.

Animal Studies

It was demonstrated that application of radiofrequency waves of 869–894 MHz (five days a week for four weeks) in a population of twenty rats resulted in a decrease in seminiferous tubular diameter and epithelial thickness (224). Similarly, a time-dependent rise in the germ cell apoptosis of the rat testis after exposure to high power microwave (HPM) radiation of 10 MW/cm² was reported (225). The same study found that only five minutes of
Microwave Sperm Damage

Human Studies

It was reported that in 371 men undergoing infertility evaluations, the duration of possession, and the daily transmission time of cell phones correlated negatively with the proportion of rapidly progressive motile sperms (228). This suggested that prolonged exposure to microwaves emitting devices might have negative effects on the sperm motility. In another prospective study involving thirteen men with normal semen analysis, it was found that using GSM phones for six hours a day for five days decreased the rapid progressive motility of spermatozoa (229). Similarly, a decrease in sperm motility in semen samples of twenty-seven men exposed to 900 MHz cell phone for five minutes was reported (230). A recent study from Cleveland Clinic involving 361 men attending an infertility clinic reported that the use of cell phones adversely affect the quality of semen by decreasing the sperm counts, motility, viability, and morphology in a use-dependent manner (231).

In spite of the these startling revelations, most of these studies had some limitations, such as the inability to analyze covariates such as life style issues, occupational history, and radiofrequency radiation (RFR) exposure from other sources like radio towers, PDA’s Bluetooth devices, and computers. However, despite the limitations of previous studies, they have revealed important findings that have started an intense debate on this topic, thus necessitating the need for further studies to determine whether spermato genesis, sperm function, sperm quality, and sperm fertilizing potential are affected by the exposure to devices emitting radiofrequency microwaves.

Association between DNA Damage, Apoptosis, OS, and Microwave Sperm Damage

Although microwaves have been suggested to cause sperm damage, the mode of action is still unclear. DNA damage and OS are among the most commonly studied mechanisms. Spermatozoa are extremely vulnerable to DNA damage as they lose their cytoplasm containing antioxidant enzymes and their capacity for DNA repair (232). Lai and Singh first reported DNA strand breaks from low-intensity microwave radiofrequency radiations in rat brain cells. In their study, two hours exposure to 2,450 MHz continuous and pulsed RFR produced a dose-dependent increase in ssDNA and double-strand DNA breaks (233). More recently, Aitken et al. found significant damage to mitochondrial and nuclear genome in epididymal spermatozoa of rats with radio frequency EMW exposure of 900 MHz twelve hours per day for seven days (234). Although there is no evidence of the adverse affects of RFR on human sperm DNA, investigators have found evidence of EMW-induced DNA damage in other human tissues. In vitro exposure of human cultured diploid fibroblasts to 1,800 MHz RFR for sixteen hours induced ssDNA and double-stranded DNA breaks (235).

However, whether RFR is capable of inducing oxidative stress, which would lead to sperm cell damage, is still debatable. Musaev et al. found that high-intensity microwave exposure stimulated basal lipid peroxidation levels in rat hypothalamus (236). However, Hook et al. did not find any alteration in the level of intracellular oxidants and antioxidant defenses in mouse macrophage cells on exposure to RFR fields (237). Conflicting studies have also been published regarding the effect of EMW exposure on the secretion of an antioxidant melatonin (18–20,238–240).

Studies analyzing the effects of radiofrequency radiation on apoptosis failed to find any significant effect. An exposure of 1,800 MHz signal for twelve hours failed to induce apoptosis in human Mono Mac 6 cells (241). Similarly, no evidence of apoptosis has been detected after exposing human leukemia cells in vitro to RFR waves twenty-five times higher than the reference levels set by the International Commission on Non-Ionizing Radiation Protection (242). The effects of RFR on human sperm cell apoptosis have not been evaluated yet.

Rizk and Abdalla (2008) highlighted that given the vulnerability of spermatozoa to genotoxic and oxidative damage, and the clinical significance of this damage in terms of fertility, pregnancy, and childhood health, studies are urgently needed on the impact of RF microwaves on OS and DNA damage in the male germ line (243).

CONCLUSIONS

OS, sperm DNA damage, and apoptosis are clearly implicated in the pathogenesis of male infertility. These interlinked molecular events are associated with various clinical and laboratory manifestations that may be present in infertile males. It has been suggested that EMW and RFR may induce OS and DNA damage in the male germ line. In view of this evolving understanding of sperm molecular structure and function, additional assessment of sperm damage beyond the WHO criteria may serve to provide a definitive diagnosis of the underlying causes of idiopathic male fertility. This may also identify the group of men and their offspring that through techniques such as intracytoplasmic sperm injection may perpetually propagate their genetic complement linked to male infertility. Strategies required to handle this risk should encompass the standardization of the laboratory techniques required to test for sperm damage. It is conceivable that the WHO next task is to provide the required standards to achieve this goal. In doing so, the WHO would provide standardized two-level approach for male-fertility assessment. Level one should be adequately served by the criteria and standards included in its current manual, with the objective of offering initial screening for men presenting within an infertile relationship. Level two testing has the objective of offering definitive diagnosis for men with abnormal findings in level one assessment and for those who are offered intracytoplasmic sperm injection.

KEY POINTS

- OS, sperm DNA damage, and apoptosis are clearly implicated in the pathogenesis of male infertility.
Although standardized assays for diagnosing these conditions need to be improved, assessment is advised in selected cases where the exact diagnosis is suspected.

Identifying the exact nature of the defect will help in selecting proper management, which in turn will improve natural and assisted reproduction success rates and help to ensure healthy offspring.

We have to continually be aware of the possible deleterious impact of new technological advances such as those using EMW and RFR on human fertility.

REFERENCES


10. Brewer LR, Corzett M, Balhorn R. Protamine induced conden-


53. Marchesi DE, Feng HL. Sperm DNA integrity from sperm to egg. *J Androl*. Published ahead of print.


58. Marchesi DE, Feng HL. Sperm DNA integrity from sperm to egg. *J Androl*. Published ahead of print.


