Review Article

Etiology and Evaluation of Sperm Chromatin Anomalies

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Abstract

Evidence suggests that human sperm chromatin anomalies adversely affect reproductive outcomes and infertile men possess substantially amount of sperm with chromatin anomalies than fertile men.

Routine semen analysis evaluates parameters such as sperm motility and morphology, but does not examine the nuclear DNA integrity of spermatozoa. It has been suggested that altered nuclear chromatin structure or damaged DNA in spermatozoa could modify the special cellular functions of human spermatozoa, and thereby affect the fertility potential. Intra-cytoplasmic sperm injection (ICSI) bypass the barriers to fertilization for such a sperm, then the effect of chromatin anomalies on the development remains a concern. Therefore, it is essential to develop and use accurate diagnostic tests, which may provide better prognostic capabilities than the standard sperm assessments. This review discusses our current understanding of the structure and organization of sperm DNA, the different procedures for assessment of sperm chromatin anomalies including comet assay, Chromomycin A3 (CMA3), sperm chromatin structure assay (SCSA), acridine orange test (AOT), terminal TdT-mediated dUTP-nick-end labelling (TUNEL) assay, aniline blue and sperm chromatin dispersion (SCD) test and the impact of chromatin anomalies on reproductive outcome.

Keywords: Sperm Chromatin Anomalies, Protamine Deficiency, DNA Damage

Introduction

Male factor infertility remains a significant problem contributing to 50% of the cases attending infertility clinics (1). Routine clinical evaluation of the contribution of the man towards the infertility of the couple is usually confined to measure of total sperm count, concentration, morphology, motility and seminal factors such as pH and antisperm antibodies(1). In general, although the threshold values for each parameter are characterized qualitatively as well as quantitatively, nevertheless approximately 15 % of the sterile men present normal semen parameters (2). Thus, assessment of sperm functional parameters, such as vitality, enzymatic activities, integrity of acrosome, and chromatin is recommended and no parameter on its own, as such, can be considered as an absolute diagnostic value for male infertility (2-5).

Many of these analyses may describe some aspects of the testis and sperm function. Hence, they may not attend to the integrity of the male genome contained in the head of sperm. Abnormalities in the male genome are a clear potential reason for post-fertilization failure (1). It was well-known that the presence of DNA damage, such as anomalies in the chromatin structure, sperm maturation, DNA integrity and breakage of both double-stranded and simple-strand DNA, and/or the presence of chromosomal anomalies like aneuploidy or structural genomic reordering have a close relation with infertility (6). Although, the tests for DNA integrity have been developed and applied in research practice, these are not being considered as a routine category of semen analysis by clinical andrologists (7).

Semen samples containing the high levels of DNA damage are often associated with decreased fertilization rates or embryo cleavage after IVF and intracytoplasmic sperm injection (ICSI) and may be linked to death of the early embryo (8). Although the most normal appearing and motile spermatozoa are selected, but there is always a chance for the spermatozoa containing varying degrees of DNA damage which may be selected. Thus, one of the main disadvantages of assisted reproduction technology (ART) is the bypass of the natural barriers throughout the female reproductive tract which would omit infertile sperm naturally (8). Hence, the spermatozoa with abnormal genome

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can reach the genetic material of the oocyte with minimal (IVF) or no effort (ICSI) at all. The miscarriage rate is higher upon ICSI, which possibly reflects the fact that genomically compromised spermatozoa are sometimes used and lead to severe DNA damage in the embryo (9, 10).
In this review, we will outline our current understanding of how sperm DNA is organized, mechanism undergoing sperm DNA to damage, how this damage may affect reproductive capacity and also evaluation of sperm chromatin/ DNA damage.

Sperm nuclear DNA damage and its origins

Spermatogenesis is a complex process by which male germ cell proliferates and matures through meiosis from diploid spermatogonia to haploid spermatozoa. Even though a small percentage of spermatozoa from fertile men possess detectable levels of DNA damage which can occur at any step of spermatogenesis (9). This anomaly is clearly associated with male infertility (10) and many factors including intra or extra testicular factors may be involved in these process. DNA damage may result from aberrant chromatin packaging during spermiogenesis (11), defective apoptosis before ejaculation (12) or excessive production of reactive oxygen species (ROS) in the ejaculate (13). In addition, extra testicular factors such as age (14), drugs (15), cigarette smoking (16), genital tract inflammation (10), hormonal factors (10), varicocele and testicular hyperthermia (10, 17) are main reasons of DNA damage which would not be covered during this review.

Alterations in sperm chromatin packaging

Sperm chromatin is a highly organized, compact structure, consisting of DNA and heterogeneous proteins. The condensed and insoluble nature of the sperm chromatin structure protects the genetic integrity during transport of the paternal genome through the male and female reproductive tracts. In somatic cell DNA is normally wrapped around an octamer of histones to form nucleosomes that eventually give rise to a solenoid DNA structure , unlike somatic cell, mammalian sperm DNA forms neucleoprotamine structure following replacement of histones by protamines during spermiogenesis. This structure is organized into loop domains attached to specific sites of the structural component of the nucleus called the sperm nuclear matrix (18). The DNA loop domain organization has been shown to be involved in both replication and transcriptional regulation and contains three types of proteins: protamine 1 (P1), which forms the major component of sperm DNA-binding proteins; protamine 2 (P2), that is expressed only in certain species, and transitions proteins (TP), which are intermediate in the process of histone/ protamine replacement (18-20).
Variable chromatin structures in spermatozoa have been attributed to the protamine content. For example, the chromatin of human spermatozoa has a lower level of free-sulphydryl groups available for disulphide bonding and is therefore potentially less stable than the chromatin in animal species that contains P1 alone. In addition, the retention of 15% nucleohistone structure in human spermatozoa lead to the formation or the retention of less-compact nucleosome structures (19-21). Following testicular replacement of nuclear his-tones with arginine/cysteine -rich protamines, in the epididymis, the thiol (SH) groups of the cysteine residues in the protamines are progressively oxidized and disulphide bonds (S-S) are formed, making the sperm nuclei tight or compact, and more resistant to detergents (7). On the other hand, Zinc secreted from prostate may also contribute to the formation of non covalent S--Zn--S bonds, which additionally stabilize the sperm chromatin structure.
It has been shown that infertile men have an increased sperm histone: protamine ratio than fertile counterparts (7). This alteration of histone: protamine ratio, that is also called abnormal packing, increases susceptibility of sperm DNA to external stresses due to poorer chromatin compaction. Recent studies also underlined the link between protamine deficiency and sperm DNA damage that resulted in poor fertilizing capacity (9,11). However, there is a non-random selection of these histone bound DNA sequences or genes in normal sperm DNA and these are mainly presumed to be involved in fertilization, early embryo development and possibly imprinting (10).
In fact, abnormal sperm protamine levels are a common defect in male infertility, indicating that the proper functioning of protamines is essential for successful egg-sperm fertilization process (22, 23). In addition, Sakkas et al reported that a high percentage of injected or penetrated oocyte that fails to progress to pronuclear stage contains condense sperm or sperm with premature chromosomal condensation (PCC). The latter phenomenon has been related to the effect of maturation promoting factor (MPF) in the metaphase II oocyte on the protamine deficient sperm or sperm with presence of excessive histones (24).

The role of apoptosis of human spermatozoa in DNA damage

Germ cell death occurring during normal spermatogenesis in mammals has been identified for more

than a century and estimated to be responsible for the loss of up to 75% of the potential spermatozoa number. In fact, one part of A2-A4 spermatogonia is eliminated by apoptosis and only 25% of the theoretically expected number of spermatocytes I is produced from the original population of spermatogonia A1. Moreover, selective death of spermatocytes and spermatids frequently occurs resulting in the elimination of 20% of these cells. Thus, it seems a natural conclusion that programmed cell death must play an important role in spermatogenesis (25, 26). It is also believed that apoptosis is possibility involved selective depletion of abnormal spermatozoa.

During apoptosis the cells shrink and exhibit several typical features, including cell membrane disruption, cytoskeletal rearrangement, nuclear condensation, and intra nucleosomal DNA fragmentation. Apoptotic cells are usually rapidly taken up and degraded by neighbouring cells before their intracellular contents leak into the extracellular space. (10, 18, 27).

Cell death takes place mainly by apoptosis, a physiological and continuous process characterized by a complex cascade of events. A number of studies carried out mainly in mice have shown that pro- and anti-apoptotic factors may play a critical role in spermatogenesis, maintaining male germ cell homeostasis. It has been shown that Sertoli cells can start and regulate germ cell apoptosis via the apoptosis stimulating fragment (Fas) system that is characterized by the interaction between Fas and Fas ligand (10). The complex cascade of events involved in apoptosis also includes the activation of endonuclease, which induce DNA strand breaks (10). The degradation of DNA into fragments approximately 185 bp and its multiples in size is one of the best characterized biochemical features of apoptotic cell death and is used as the basis for the commonly used labeling techniques for detecting apoptotic cells (10).

Some specific proteinases, called caspases (cysteinyl aspartate-specific proteinases), also have been claimed to play a major role in the regulation of apoptosis. More than a dozen of these specific proteinases have been reported to be related to apoptosis in the human seminiferous epithelium that are expressed as inactive proenzymes and participate in a cascade triggered in response to pro-apoptotic signals. Cell-surface death receptors such as Fas or Tumor Necrosis Factor-α receptor (TNF-α) are activated by ligand binding, resulting in the proteolytic activation of caspases, in the destruction of vital proteins and finally in the death of the cell (10, 18).

Different mechanisms have been attributed for presence abnormal ejaculated spermatozoa, one of which is “abortive apoptosis” that sperm have escaped programmed cell death and express various apoptotic markers (28-30).

Reactive oxygen species

Reactive oxygen species (ROS) include free radicals, which are active oxidizing agents. The primary source of ROS in seminal plasma is derived from spermatozoa themselves and from polymorphonuclear leukocytes (30-35). The production of ROS by spermatozoa is normal physiological process required for the occurrence of capacitation and the acrosome reaction (35).

The analyses of markers for oxidative stress and apoptosis showed a significantly positive correlation between ROS production, oxidative stress and DNA damage (35-37). Mitochondria, as the basic source of ROS, are involved in the activation of pro-apoptotic molecules, DNA fragmentation and apoptosis. (27). Over the past decade, studies have demonstrated that peroxidative damage to the sperm plasma membrane by ROS may impair sperm function, leading to the onset of male infertility (31, 32) because, spermatozoa are highly susceptible to oxidative damage due to an abundance of polyunsaturated fatty acids within the plasma membrane and a low concentration of scavenging enzymes within the cytoplasm.

On the other hand, high levels of ROS present in seminal plasma have been associated with poor morphology, motility and low sperm count (36). Lopes et al. have shown that ROS can be the cause of increased DNA fragmentation and poor-quality semen samples with a higher percentage of spermatozoa with fragmented DNA compared to normal fertile samples and pre-treatment with antioxidants can reduce this DNA damage (35). It is not clear why defective human spermatozoa generate high levels of ROS, although an extra-mitochondrial activity, emanating, probably, from the exposure to the unsaturated fatty acids cannot be excluded (35).

The link between poor sperm quality and increased ROS generation also lies in the retention of excess residual cytoplasm (cytoplasmic droplets) in abnormal spermatozoa. When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective. Spermatozoa released from the germinal epithelium carrying surplus residual cytoplasm are thought to be immature and functionally defective (38). Retention of residual cytoplasm by spermatozoa is positively correlated with ROS generation via mechanisms that may be mediated

by the cytosolic enzyme glucose-6-phosphate-dehydrogenase (G6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which, in turn, controls the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH). The latter is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH-oxidase (39).

Methods for detection of human sperm DNA damage

A variety of tests have been described to analyze the integrity of the paternal genome, including tests for the assessment of sperm chromatin (DNA dependent proteins) and for detecting DNA breaks. Chromomycin A3 (CMA3) and Aniline blue are used for evaluation of proteins that presents in the chromatin. DNA breaks are assessed directly or indirectly. Direct methods include the deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and the single-cell gel electrophoresis assay (Comet assay) whereas the indirect methods include Sperm Chromatin Dispersion (SCD), Acridine Orange Technique (AOT) and Sperm Chromatin Structure Assay (SCSA) (Fig 1, 2). Table 1 shows advantages and disadvantages' of each test.

Table 1: Tests used to analyse the integrity of the paternal genome:description of the assay principles, the detection methods, and their advantages and disadvantages

Assay	Assay principle	Detection method	Main advantages	Main disadvantages
TUNEL	Single and double strand DNA breaks	Fluorescence microscopy/Flow cytometry	Clinically significant, high sensitivity and specificity, large number of spermatozoa counted by flow cytometry	Special equipment; more expensive
COMET	Single and double strand DNA breaks or only double strand DNA breaks	Fluorescence microscopy	Related to TUNEL assay: cheap; high sensitivity; quantification of DNA damage in individual cells; evaluation of different types of DNA damage	Special equipment; experienced observer
SCD	Evaluation of DNA decondensation halo	Fluorescence microscopy/ Optical microscopy	Related to SCSA; cheap; easy to perform	Clinical relevance not yet proven
CMA3	Evaluation of underprotaminated chromatin	Fluorescence microscopy	Clinically significant; high sensitivity and specificity	Special equipment; distinction between positive and negative spermatozoa not always easy
Aniline blue	Evaluation of lysine residues of remaining histones	Optical microscopy	Clinically significant; high sensitivity and specificity: cheap, easy to perform	Clinical relevance not yet proven; inconsistencies due to subjective evaluation
Acridine orange	Differentiates between single and double stranded DNA	Fluorescence microscopy	Easy to perform; cheap	Special equipment; distinction between differently labeled spermatozoa not always easy
SCSA	Susceptibility of DNA to acid denaturation	Flow cytometry	Clinically significant; high sensitivity and specificity: large number of spermatozoa counted by flow cytometry; unbiased quantitative assessment of DNA-bound Acridine orange	Special equipment; more expensive

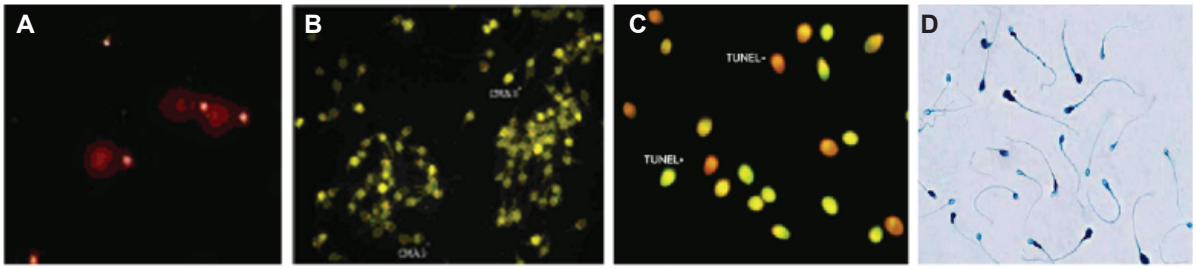

Fig 1: Evaluation of sperm chromatin by A) COMET assay, B) CMA3 Staining, C) TUNEL assay and D) Aniline blue

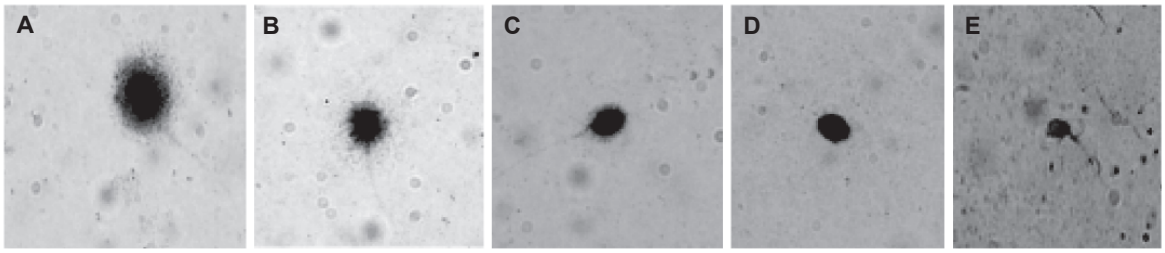

Fig 2: Sperm chromatin dispersion test: sperm with different halos size. The nuclei with large- to medium-size halos represent sperm with non-fragmented DNA (A&B), whereas nuclei with no or small halo represent sperm with fragmented DNA (C, D, E).

TUNEL (Terminal dUTP Nick-End Labeling) assay

This technique was first described by Gorczyca et al and used to detect DNA strand breaks in mammalian spermatozoa (40). In this procedure, the ends of fragmented DNA, either simple or double-stranded, are tagged to labeled nucleotides. The reaction is catalyzed, in situ, by a terminal transferase. This enzyme incorporates deoxyuridine modified with biotin, at the 3 '-OH end of the affected chain. Later, modified nucleotides are detected by a fluorochromed antibody. The nucleotide can also be directly marked by the fluorochrome. Theoretically, the signal obtained by every sperm would increase in agreement with the number of DNA breaks (7). The percentage of spermatozoa with fragmented DNA is determined by direct observation using an epifluorescence microscope or by flow cytometry. Hence, the TUNEL technique has been used in numerous studies (40). Tarozzi et al showed that infertile patients had a higher mean level of DNA fragmentation compared to men of proven fertility (18).

The comet assay

The COMET assay or single-cell gel electrophoresis for analyze DNA fragmentation in individual cells was first introduced in 1984 by Ostling and Johanson (see Olive and Banath, 1995) who used neutral buffer conditions to study double-stranded DNA breaks. It was later modified using alkaline electrophoresis buffers to increase the sensitivity to both single and double stranded DNA breaks (41,42). By this technique kinds of DNA fragmentation such as necrotic or apoptotic can also be recognized. Apoptotic cells produce tear-drop shaped comets due to the migration and accumulation of the short DNA fragments, and the intensity of the tail represents the amount of DNA fragments present (43). Apoptotic DNA fragmentation is characterized by double-stranded DNA breaks. Using the COMET assay, Tomsu et al. (44) noted that the COMET head and tail DNA parameters could be considered potentially useful predictors of embryo quality and IVF outcomes, especially in couples with unexplained infertility. It has also been shown that high loads of DNA damage were predictive of embryo development failure after ICSI. On the other hand, Nasr-Esfahani et al demonstrated that DNA damage is more frequent in protamine deficient spermatozoa. Unlike protamine deficiency, sperm DNA damage does not prevent fertilization. Nonetheless, embryos derived from spermatozoa with high DNA damage have a lower potential to reach blastocyst stage (8, 45, 46).

Sperm Chromatin Dispersion Test

Sperm DNA is packed in order to arrange the chromatin six times more compact than mitotic chromosomes. This DNA is organized in loops of minor size than those of somatic cells, anchored to the nuclear counterfoil. The DNA strands are tightly wrapped around the protamine molecules, forming tight loops, and disulfide cross-links between protamines are responsible for the compaction and stabilization of the sperm nucleus. If disulfide cross-links break and a specific lysis solution is used to extract proteins, DNA's loops relax constituting haloes around the residual nuclear central structure. It has been verified that following acid treatment, unlike the non-fragmented DNA, sperm with fragmented DNA showed an evidence of restricted DNA loop dispersions with very limited haloes or absence of haloes (47). Simply, valuing the size of the loop dispersion haloes by microscopy, both of bright field and of fluorescence, it is possible to recognize the presence of DNA's fragmentation in human sperm (47-48). Since DNA fragmentation assessment is carried out following acid treatments, therefore, this procedure assesses susceptibility of sperm to DNA fragmentation.

Chromomycin A3

CMA3 is a flurochrome which has been shown to compete with the protamines for binding to the minor groove of DNA (49-51). Therefore, CMA3

represents a useful tool for assessing the packaging quality of the sperm chromatin and may allow indirect visualization of protamine deficiency. There are some reports on CMA3 and its relation to sperm fertilization ability in IVF (In vitro fertilization) and ICSI (Intracytoplasmic Sperm Injection) (52-56). Previous studies have shown that sperm protamine deficiency is associated with fertilization failure. This phenomenon may have been induced by premature chromosomal condensation (PCC) or associated with other late spermiogenic defects such as post acrosomal defects or absence of SAOAF (Sperm Associated Oocyte Activating Factor) (9, 22, 57, 58).

Sperm Chromatin Structure Assay

The SCSA is a fluorescence-activated cell sorter test that measures the susceptibility of sperm DNA to heat or acid induced DNA denaturation in situ, followed by staining with acridine orange (18). This test is similar to acridine orange test. However, in this procedure flow cytometry is used instead of epifluorescence microscopy. Thus, it is possible to measure large number of spermatozoa per sample and the technique is therefore simple and highly reproducible (18). Acridine orange is a metachromatic dye that fluoresences red when associated with denatured DNA and green when associated with normal, double-stranded DNA (59). The SCSA measures several parameters:

DNA fragmentation index (DFI) represents the sperm fraction with detectable denaturable single-stranded DNA mainly due to DNA breaks and the highly DNA stainable cells (HDS) describes the sperm fraction with increased double-stranded DNA accessibility to the metachromatic dye, mainly due to altered replacement of histones with protamines. In clinical applications the DFI not only distinguished fertile men from those who were infertile, but also identified samples that were compatible with pregnancies conceived (<28-30%) (60).

The categories proposed by Evenson et al. for individual fertility potential according to DFI fraction are: excellent <15%, good 15–24%, fair 25–30% and poor >30% DFI, and if DNA staining is >15% the fertility potential is downgraded at least one category (61). Two large independent studies on the relationship between SCSA results and sperm fertilization capacity have been carried out in the USA and Europe (62-63). Both these studies demonstrated that when >30% of spermatozoa have abnormal chromatin as evaluated by SCSA, human male infertility is hampered independent of sperm number, morphology and motility. Aravindan et al established a significant relationship between the SCSA, COMET and TUNEL assays for human spermatozoa (64). In addition, several studies performed AO test with fluorescence staining, however, it has been indicated that the SCSA is a more accurate test (50, 59).

Aniline blue

The degree of sperm chromatin condensation (nuclear stability) can be evaluated by toluidine or aniline blue staining (65). Terquem and Dadoune demonstrated that the acidic aniline blue staining technique selectively stains persisting lysine-rich histones and thus allows the visualization of spermatozoa with defective chromatin condensation (66). Due to the shift from somatic histones to specific protamines during spermatid development (67) and nuclear condensation, the elongated spermatids lose their ability to be stained with acidic aniline blue dye. While normal mature spermatozoa appear unstained, remaining histones in spermatozoa with insufficient chromatin condensation and in immature elongated spermatids are stained (68). Dadoune et al. observed pathological aniline blue staining in 20% of morphologically-normal spermatozoa in normozoospermia specimens; these findings were confirmed by Hofmann et al. (69, 70). In addition, several studies have suggested that percentage of aniline blue is higher in infertile men. Nasr et al bserved a significant correlation between aniline blue staining and in vitro fertilization rate (50, 66).

Conclusion

Evidence now exists in the literature to show that sperm DNA damage influences the fertility outcome after ART procedures. Even if spermatozoa with abnormal DNA fertilize an oocyte and live birth occurs, there is a possibility of congenital abnormalities in the offspring. Therefore, it is prudent to check for DNA damage in infertile patients undergoing ARTs. The protocols used to detect chromosomal abnormalities and DNA damage should be standardized to allow their routine use in clinical laboratories. The results of the sperm DNA damage evaluation may help the physicians to better counsel infertile couples referred for assisted conception about their chances of having a live birth of a healthy baby.

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