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REVIEW PAPER

Sperm DNA Damages and Damage Detection Methods: Current Approachs

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Abstract

There are many factors affecting male fertility, whose causes are still largely unknown. DNA damage in spermatozoa, in particular, contributes significantly to infertility. Maintaining DNA integrity in sperm is essential for successful fertilization and embryo development. Sources of DNA damage in sperm include errors during chromatin packaging, DNA breaks caused by defective apoptosis, and oxidative stress. These DNA damages are critical for male fertility and lead to issues such as reduced fertilization rates, poor embryo quality, and lower pregnancy rates. While routine examination methods provide a general overview of male fertility, they are often insufficient for a definitive diagnosis of infertility and sterility. For instance, DNA damage has been detected in 15% of spermatozoa with normal values in standard sperm analyses. Additionally, assessing DNA damage in sperm along with functional parameters provides insight into fertilization ability and embryonic development. The goal here is to emphasize the importance of examining sperm DNA to assess male fertility and identify DNA damage and its sources. Common tests used to detect DNA damage include Aniline Blue, Toluidine Blue, Chromomycin A3 (CMA3), Sperm Chromatin Dispersion Test (SCD), TUNEL, Single Cell Gel Electrophoresis (COMET), Sperm Chromatin Structure Assay (SCSA), and Acridine

Introduction

Infertility refers to reduced reproductive capacity in living organisms. Over 15% of infertility cases worldwide are female-related, while 50% are caused solely by male factors or a combination of male and female factors (Choy and Eisenberg, 2018). For infertile men, sperm analysis is still based on traditional techniques (motility, viability, morphology, density, etc.). However, these techniques alone do not accurately reflect male fertility or the success of assisted reproductive technologies (such as In Vitro Fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI), etc.), as 15% of infertile men show normal sperm analysis results (Varghese et al., 2011). In addition to routine examinations, sperm DNA damage detection methods should also be used for a definitive diagnosis of male infertility.

DNA Structure in Spermatozoa

Sperm DNA integrity is essential for embryo development (Fatehi et al., 2006). Spermatozoa with impaired DNA integrity can fertilize oocytes; however, healthy embryo development may not occur (Aitken, 2017). A morphologically mature spermatozoon forms in the seminiferous tubules through molecular and chemical changes known as spermatogenesis. This process, which includes meiotic and mitotic divisions, occurs in three main stages: (1) spermatocytogenesis, where spermatogonia undergo mitotic divisions to form primary spermatocytes; (2) meiosis, where primary spermatocytes (2n) undergo meiotic divisions to form spermatids (n); and finally, (3) spermiogenesis, where spermatids mature into spermatozoa, completing cytoplasmic and nuclear transformations (Zini and Agarwal, 2011). A major change in the sperm nucleus is the repackaging of chromatin. The nucleus of the spermatozoon has transcriptional activity containing condensed DNA in repressed chromatin. Sperm DNA is in a compact structure, which forms during sperm maturation as histones, the DNA-binding proteins in somatic cells, are replaced by transition proteins (TP1-TP2). TPs are crucial because they reduce DNA damage (Balhorn, 1982). At the end of spermiogenesis, TPs are replaced by protamines

specific to spermatozoa, further compacting the structure. Sperm DNA is divided into three regions as illustrated in Figure 1: toroid loops formed by tightly bound protamines, promoter regions containing histones that cover 12-20% of the DNA, and MAR (Matrix Attachment Regions) responsible for DNA replication and gene expression (Vilfan et al., 2004; Simon et al., 2017). Protamines are half the size of histones, compacting sperm DNA six times more than somatic cell DNA. The arginine-binding regions in protamines neutralize the phosphodiester bonds of DNA. Cysteine within protamines forms disulfide bonds that stabilize the chromatin structure, providing maximum protection to sperm DNA against damage (Ward, 2009). In the histone-containing regions of sperm DNA, genes essential for spermiogenesis and post-fertilization development are located; these areas remain unchanged by protamination. During spermatogenesis, DNA topoisomerase physiologically breaks and re-ligates DNA, facilitating compaction. Physiological DNA breaks in sperm DNA reduce chromatin torsion, supporting the protamination process. These endogenous DNA breaks should not persist in mature spermatozoa (McPherson and Longo, 1993) and must be repaired by the cell before protamination is complete (Andrabi, 2007; Balhorn, 2007). Since mature spermatozoa lack a DNA repair mechanism, cells with DNA damage may still enter the ejaculate (Lewis and Agbaje, 2008). In the early stages of fertilization, oocytes and embryos can repair sperm DNA damage, but this capacity is limited and cannot fully repair double-strand breaks or breaks in histone-bound regions (Menezo et al., 2010). These specific histone-containing and MAR regions in sperm are crucial for embryonic development after fertilization. Therefore, when evaluating sperm quality parameters, it is essential to use sperm DNA damage detection methods alongside conventional methods to ensure a comprehensive approach (Ahmadi and Ng, 1999).

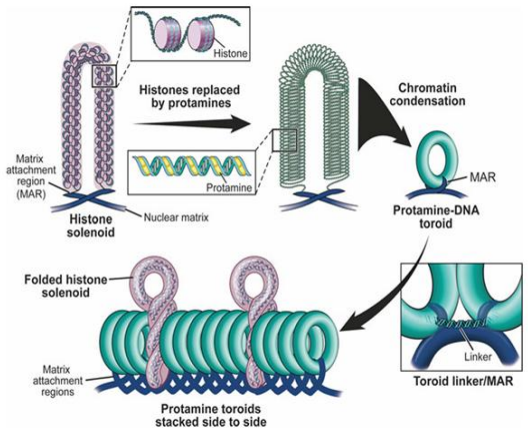

Figure 1. Parts of spermatozoon DNA (Agarwal and Singh, 2012)

DNA Damage Formation in Spermatozoa

There are three possible causes of damage to sperm DNA. These are: (1) abnormal or irregular chromatin packaging due to protamination errors, (2) abnormal apoptosis (programmed cell death), and (3) oxidative stress caused by various ROS/RON sources (Sotolongo et al., 2005). DNA damage resulting from these factors includes base mismatches, base loss (abasic sites), base modifications, DNA insertions and cross-links, pyrimidine dimers, single-strand breaks (SSBs), and double-strand breaks (DSBs) in the sperm nucleus. The increase in DNA damage can be induced by various factors such as lifestyle, diseases, medications, aging, infections, and exposure to chemicals (Figure 2) (Chatterjee and Walker, 2017).

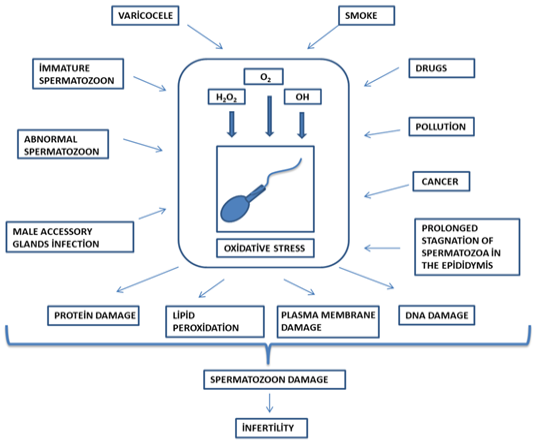

Figure 2. Factors causing damage to spermatozoa

Abnormal or Disordered Chromatin Packaging

The replacement of histones with sperm DNA proteins called protamines leads to the reorganization of sperm DNA and the tight binding of chromatin structures, making spermatozoa more resistant to potential damages. In other words, during spermatogenesis, chromatin is compacted through the exchange of histones with transition proteins and protamines (O'Donnell, 2014). The DNA topoisomerase II enzyme is responsible for repairing DNA breaks during the protamination process. When this process fails, spermatozoa with DNA breaks are produced. In a study conducted on infertile men, damage to protamines and sperm protamination was found to be associated with translational damage (Aoki et al., 2006). The amount and ratio of sperm-specific proteins Protamine 1 (P1) and Protamine 2 (P2) (in humans and mice) present in the sperm nucleus are crucial. The P1/P2 ratio should be around 1 on average. If this ratio is too high or too low, it is associated with an increase in sperm DNA damage, a decrease in fertilization rates, a decline in embryo quality, and ultimately lower pregnancy rates. Additionally, an increase in the histone protein ratio

(by 15%) causes abnormal DNA packaging, making the DNA more vulnerable to damage (Boekelheide, 2005).

Apoptosis (programmed cell death)

Apoptosis is the term used for programmed cell death, which normally occurs in many physiological processes. There are two apoptotic functions in spermatogenesis (Shukla et al., 2012). The first apoptotic function is to eliminate abnormal spermatozoa. The second is to limit the number of germ cells supported by Sertoli cells (Simon et al., 2013). In this way, apoptosis in the testis balances the ratio between germ and Sertoli cells by removing defective germ cells and controlling sperm production (abortive apoptotic process) (Singh and Stephens, 1998). The normal apoptosis of sperm cells plays a critical role in regulating sperm count, rapidly removing sperm with chromosomal abnormalities from the body, and maintaining sperm quality. During spermatogenesis, 25% to 75% of sperm cells are eliminated by apoptosis. Excessive formation of the apoptosis mechanism reduces sperm count in the ejaculate, while insufficient formation increases the number of defective spermatozoa (Agarwal and Singh, 2012).

Oxidative Stress

Oxidative stress (OS) in the testes results from an imbalance between reactive oxygen species (ROS), such as hydroxyl ions (OH), superoxide radicals (O2-), and hydrogen peroxide (H2O2), and antioxidant defense systems. This imbalance occurs due to a decrease in antioxidant defense or an increase in oxidant levels (Newsholme et al., 2016). In 1979, Jones et al. first proposed that human sperm is highly sensitive to OS and reported that it has a significant impact on male infertility (Jones et al., 1979). The primary sources of ROS in the ejaculate are immature spermatozoa and leukocytes. ROS are molecules required in reproductive processes, such as capacitation, hyperactivation, and the acrosome reaction, and are produced due to mitochondrial activation in normal testicular physiology. However, excessive production of ROS leads to sperm DNA breaks, lipid peroxidation, protein denaturation, and plasma membrane damage (Agarwal and Allameneni, 2005). Muratori et al. (2019) stated that sperm DNA fragmentation is induced by defective maturation and abortive apoptosis in the testis, or by ROS produced along the male reproductive system. ROS have been shown to cause various forms of DNA damage, including single and double-strand breaks, base modifications and deletions, cross-linking, and mutations (Agarwal and Allameneni, 2005).

DNA Damage in Spermatozoa

When sperm DNA integrity is studied by many researchers, it has been concluded that nuclear DNA damage negatively affects parameters commonly used, such as motility, viability, and morphology. A study has concluded that DNA damage in spermatozoa has a detrimental effect on reproduction (Saleh et al., 2002). Structural abnormalities in sperm DNA, such as chromatin anomalies, chromatin degradation, oxidation of DNA bases, inhibition of tubulin polymerization, DNA strand breaks, DNA-DNA and DNA-protein crosslinking, mispairing, and mutations, are significant factors affecting fertility (Türk et al., 2006). There are three possible outcomes for spermatozoa with DNA damage. The first is the activation of the apoptotic pathway, leading to the programmed death of the cell. The second is the tolerance of the damage, but this leads to mutations in future generations. Lastly, the repair mechanism of the cell can maintain genomic integrity, allowing the formation of healthy DNA-containing cells. Spermatozoa lack their own repair mechanisms. However, the necessary repair is provided by the oocyte after fertilization. If the damage is irreparable, the cell inevitably undergoes apoptosis and dies (Çevik, 2019).

Spermatozoa DNA Damage Detection Methods

Sperm DNA fragmentation (SDF) can result from failures in protamination, apoptosis, and oxidative stress. Therefore, when selecting the most suitable test to assess SDF, the underlying cause of the SDF should be taken into consideration (Jones et al., 1979).

Aniline Blue (AB)

Aniline blue is an acidic dye that has a high affinity for histones rich in lysine, which are not replaced by protamines during spermatogenesis. This dye is used to determine chromatin condensation. It does not affect protamines that are rich in cysteine/arginine. Therefore, it stains immature spermatozoa with histone-rich nuclei that have not completed protamination. The AB staining technique was first used by Terquem and Dadoune (1983). Spermatozoa with DNA damage are stained blue, while healthy cells do not take up the dye (Hammadeh et al., 2001).

Toluidine Blue (TB)

TB, a basic dye, tends to bind to phosphate residues found in the DNA of spermatozoa with immature or poorly packaged nuclei (Erenpreiss et al., 2001; Marchesi et al., 2010). Staining protocol: Sperm samples are spread on slides and air-dried. Cells are fixed on the slide using 96% ethanol-acetone (1:1) for 30 minutes at +4°C and hydrolyzed with 0.1N HCl for 5 minutes at +4°C. The samples on the slide are washed three times for 2 minutes each. Then, the sperm samples are stained and washed with 0.05% TB and 50% McIlvain buffer (pH 3.5) for 5 minutes. The samples are dried twice for 3 minutes in tertiary butanol and treated with xylene. Spermatozoa with

normal DNA integrity are stained light blue, while spermatozoa with damaged DNA are stained purple (Erenpreiss et al., 2001).

Chromomycin A3 (CMA3) test

CMA3 is an anthraquinone glycoside produced by the bacterium Streptomyces griseus that binds to DNA in the presence of magnesium and detects protamine deficiency (Lolis et al., 1996). Staining protocol: Sperm samples are fixed on slides with a methanol-glacial acetic acid (3:1, v/v) mixture at +4 °C for 20 minutes. Then, they are treated with CMA3 solution (10 mmol/L $MgCl_2$ in McIlvaine buffer) for 20 minutes, washed, and the cells are fixed with PBS-glycerol (1:1, v/v). CMA3-positive sperm cells (bright yellow or bright green staining) indicate insufficient DNA protamination, whereas CMA3-negative sperm cells (pale yellow or dull green staining) show high DNA protamination (Kazerooni, 2009; Marchiani et al., 2021).

Spermatozoon Chromatin Dissociation Test (SCD) / HALO Test

Sperm Chromatin Dispersion (SCD) was proposed by Fernández in 2003. This test, which is used to evaluate sperm DNA breaks, is based on the principle that when sperm samples are treated with an acid solution before the lysis buffer, nuclear proteins are removed, and DNA fragments in the sperm nucleus are separated. These fragments either form halos or do not. While little or no halos are observed in sperm cells with DNA breaks, large halos are formed in spermatozoa with intact DNA (Fernández et al., 2003). For examination, sperm samples are diluted with PBS to a concentration of 5–10 million/ml. The samples are mixed with a low- density liquid agarose gel (0.65% standard agarose dissolved in PBS at 80°C) and smeared onto a 50 µL slide. A coverslip is then placed on top. The slide is kept in a horizontal position at +4°C for 4 minutes to allow the gel to solidify. The samples are then immersed in 0.08N HCl in the dark at 22°C for 7 minutes for denaturation. Next, to neutralize and lyse the sperm samples, they are soaked in a solution containing 0.4 mol/L Tris, 0.8 mol/L DTT, 1% SDS, and 50 mmol/L EDTA (pH 7.5) for 10 minutes. The samples are then transferred to another neutralizing solution containing 0.4 mol/L Tris, 2 mol/L NaCl, and 1% SDS (pH 7.5) for 5 minutes. Afterward, the slides are carefully washed with Tris-borate EDTA buffer (0.09 mol/L Tris-borate and 0.002 mol/L EDTA, pH 7.5) for 2 minutes to remove ethanol, and the slides are air-dried. If evaluation is to be done using a light microscope, the samples are stained with Wright's stain; if a fluorescence microscope is used, they are stained with DAPI (4',6-diamidino-2-phenylindole). Additionally, Haloperm kits have been developed for evaluation using the tail (Fernández et al., 2003; Chohan et al., 2006). While the protocol is standardized, the analysis has some disadvantages due to its lack of full standardization. Moreover, it has been reported that this method, which can analyze even a low number of sperm cells, cannot detect DNA breaks related to the sperm nuclear matrix (MAR region) (Ribas-Maynou and Benet, 2019).

TUNEL [The Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine (TdT) Triphosphate (dUTP) Nick End Labeling Assay]

The TUNEL method can directly measure both single- and double-stranded DNA breaks. The principle of the test is based on the detection of open 3’-OH ends in the broken DNA. These ends are first catalyzed by Terminal deoxynucleotidyl transferase (TdT) and then labeled with the biomarker deoxyuridine triphosphate (dUTP). In other words, the more open 3’-OH ends (nicks) present in the DNA, the more FITC-dUTP will bind, resulting in stronger fluorescence signals in more cells [44]. This method directly measures the unevenly broken DNA ends (open 3’-OH) without the need for denaturation, using light microscopy, fluorescence microscopy, or flow cytometry (Simon et al., 2017; Javed et al., 2019). While the TUNEL method is more sensitive and reliable compared to other techniques, its procedure is more complex and expensive.

Single Cell Gel Electrophoresis (COMET)

The comet assay, also known as single-cell gel electrophoresis, was first proposed by Ostling and Johanson in 1984. This technique is based on the principle that fragmented DNA of different sizes exhibits varying levels of permeability within an electrophoretic field. Fragmented DNA strands are separated from the nucleus in the electrophoretic field according to their size. Single- and double-strand DNA breaks produce a characteristic comet appearance, with the size of the tail depending on the amount of DNA damage. In contrast, intact DNA remains confined within the nucleus. The displacement between the nuclear genetic material (comet head) and the migrated, unwound DNA tail (i.e., the length of the tail) serves as an index of sperm DNA damage (Shukla et al., 2012). The comet assay is a sensitive and simple method for evaluating DNA damage, requiring only a small number of spermatozoa (Sharma, 2013).

Sperm Chromatin Structure Assay (SCSA)

SCSA (Sperm Chromatin Structure Assay) is based on the metachromatic properties of acridine orange and is a flow cytometric adaptation of the acridine orange test. Unlike the tight binding of normal double-stranded DNA, which provides stability and acid resistance, the chromatin structure of damaged sperm DNA is relatively loose and can be easily denatured into a single strand by the action of an acidic substance (Evenson and Wixon, 2006). Due to the metachromatic staining property, damaged DNA is separated into single strands and appears red,

while intact double-stranded DNA strands appear green. Additionally, DNA fragmentation levels are measured as the DNA Fragmentation Index (DFI). Since SCSA is a standardized test with a fixed protocol and provides consistent results over a long period, it is widely used in the andrological evaluation of male infertility by many reproductive medicine units (Evenson et al., 1980; Evenson and Wixon, 2006).

Acridine Orange Test (AOT)

The Acridine Orange Test was first conducted by Evenson et al. in 1980. The Acridine Orange Test (AOT) is a simple microscopic procedure based on the treatment of DNA with acid, followed by staining with acridine orange. AOT evaluates the degree of DNA denaturation by the metachromatic shift of AO fluorescence from green (intact DNA) to red (denatured DNA), which is similar to the method used in SCSA (Wang and Swerdloff, 2014).

Conclusion

In the evolving scientific world, spermatozoa are continuously exposed to internal and external harmful factors due to faulty manipulations during assisted reproductive techniques and throughout spermatogenesis. Maintaining the compact structure of sperm DNA is crucial for the proper transmission of male genetic information to future generations. Additionally, the resulting damage adversely affects normal fertilization, embryo development, and the success of assisted reproductive techniques. In other words, changes in sperm DNA structure can be responsible for abnormal embryo development and, consequently, the abnormal development of the offspring. Therefore, methods for detecting sperm DNA damage are important and should be used in conjunction with conventional methods for evaluating male infertility and predicting the development of a healthy embryo.

Conflicts of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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