Sperm DNA Damage: Causes and Laboratory Detection

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Satish Kumar Adiga and Guruprasad Kalthur

10.1 Introduction

Infertility is a major health issue globally which is defined as “the inability of a sexually active couple having unprotected sexual intercourse to achieve pregnancy within one year.” Prevalence of infertility in the general population is estimated to be around 15–20 %, and in India alone there are more than 20 million infertile couples (Poongothai et al. 2009). Approximately 50 % of the infertility is due to male factor, which makes up 15 % of all couples. Decrease in semen quality and concomitant increase in the incidence of male infertility (Adiga et al. 2008; Feki et al. 2009; Joffe 2010) all over the world have drawn attention to the medical and scientific communities to focus on understanding the pathophysiology as well as on correcting the problem. Though the exact cause for the decline in semen quality is unclear, environmental factors, change in lifestyle, and pathological conditions like testicular cancer, cryptorchidism, varicocele, etc. show a strong correlation (Carlsen et al. 1992, 1993; Li et al. 2011; Jørgensen et al. 2012).

Semen analysis is the cornerstone of male infertility evaluation. Though it is an excellent tool to assess the testicular function, its ability to predict the fertilizing ability of the spermatozoa is not clear. Though the conventional parameters such as sperm concentration, motility, and morphology are higher in normal fertile men, there exists some degree of overlap between fertile and infertile men with respect to semen parameters, especially in the idiopathic group (Aitken et al. 1984). Approximately 15 % of the infertile men are said to have normal spermiogram (Guzick et al. 1998). Therefore, more emphasis is given on correlating the genetic integrity of the spermatozoa with fertility potential.

S.K. Adiga, PhD (✉) • G. Kalthur, PhD
Division of Clinical Embryology, Kasturba Medical College,
Manipal University, Manipal 576 104, India
e-mail: satish.adiga@manipal.edu

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10.2 Structure of Sperm DNA

A human spermatozoon has unique characteristics with respect to its structure and function. Specifically, the nucleus has a stable, highly condensed, and compact organization of chromatin which probably ensures that the genetic material of the paternal origin is protected from the harsh environments. High degree of compaction observed in sperm chromatin is provided by the presence of protamines which contributes to approximately 85–90 % of the nuclear protein in spermatozoa. Protamines replace the histones during spermiogenesis process which are highly basic due to the presence of basic amino acids such as arginine in high percentage. This confers a very strong affinity toward the nucleic acids compared to the histones. In addition, the disulfhydryl linkage between SH groups of two cysteine residues which are placed far apart in their native conformation further helps in compaction of the nuclear material. Due to these reasons, it is quite resistant to nucleases unlike in somatic cells (Sotolongo et al. 2003).

10.3 Etiology of Sperm DNA Damage

The origin of sperm DNA damage is complex and a diverse phenomenon. Various possible causes of sperm DNA damage are listed below:

(a) *Chromatin remodeling*: The sperm chromatin is highly packed compared to somatic cell nucleus. McPherson and Longo (1993) postulated that the extensive modification of chromatin during spermiogenesis requires temporary nicks in sperm DNA. Endogenous nuclease such as topoisomerase II may help in generating and then ligating nicks to facilitate protamination by relieving torsional stress (McPherson and Longo, 1993). However, these endogenous nicks may persist in mature spermatozoa suggesting an incomplete maturation process and abnormal chromatin organization (Manicardi et al. 1995).

(b) *Membrane organization*: The membrane of the human spermatozoa is rich in polyunsaturated fatty acids (PUFAs) which are the major sites of lipid peroxidation which generates reactive oxygen species (ROS). In addition, spermatozoa have minimal cytoplasm and hence lack the support of cytoplasmic antioxidant enzymes as well as molecules to tackle the free radicals. Therefore, although spermatozoa have a very compact chromatin organization, they are highly susceptible to free radical-induced DNA damage.

(c) *Poor antioxidant defense*: Seminal plasma is rich in enzymatic and nonenzymatic antioxidants which work together to counteract with ROS. The major antioxidant enzymes present in seminal plasma are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). Besides these enzymes, the seminal plasma is rich in low-molecular-weight compounds such as glutathione, pantothenic acid, carnitine, coenzyme Q, vitamin E, vitamin C, selenium, and zinc (Walczak-Jedrzejowska et al. 2013). Decrease in the level of either of them can decrease the total antioxidant capacity of the seminal plasma. A large number of studies have observed that the infertile men have low level of

antioxidants in their seminal plasma (Smith et al. 1996; Sanocka et al. 1996; Lewis et al. 1997) which also correlates well with high level of oxidative stress (Agarwal et al. 2004; Zini et al. 2009; Aitken et al. 2010) and DNA damage (Cohen-Bacrie et al. 2009).

(d) *Apoptosis*: Spermatogenesis is a complex process which involves mitotic and meiotic events in highly proliferating germ cell compartment after attaining puberty. An intricate relation with Sertoli cell during the entire process is very crucial for the production of genetically normal spermatozoa. The abnormal germ cells, similar to all other histone-containing cells, are eliminated by apoptosis. However, it is not clear whether the protamine-containing spermatozoa are eliminated by apoptosis or not (Aitken et al. 2013). Once the spermatozoon leaves the lumen of seminiferous tubule, it is not in contact with Sertoli cells. The harsh conditions to which spermatozoa are exposed during their transport and storage in male reproductive tract may induce sperm DNA damage without apoptosis.

(e) *Infections and pathological conditions*: Various pathological conditions and genital tract infections in men have been associated with sperm DNA damage. Men with recurrent genitourinary infection such as paraplegics are noted to have high level of DNA damage (Brackett et al. 2008). Increased leukocyte concentration and DNA damage are observed in the ejaculates of men with genital tract infection or inflammation. Systemic infections with HIV, hepatitis B and C, leprosy, malaria, etc. have been associated with increased DNA damage which is thought to be due to elevated oxidative stress or the chromatin modification induced by proinflammatory cytokines during spermiogenesis process. Similarly, conditions like cryptorchidism, varicocele, and testicular cancer are usually associated with high percentage of spermatozoa with DNA damage in the ejaculate. Commonly used chemotherapeutic agents like cyclophosphamide and cis-platinum are known to induce DNA damage in spermatozoa (Das et al. 2002).

(f) *In vitro conditions*: Spermatozoa experience various types of stress during their manipulation in vitro which culminates in loss of DNA integrity. Rigorous pipetting, centrifugation, exposure to light, and cryopreservation process can lead to oxidative stress-induced DNA damage (Iwasaki and Gagnon 1992; Shekarriz et al. 1995; Watson 2000). In addition, the removal of seminal plasma rich in antioxidants during processing of ejaculate for assisted reproductive technologies (ARTs) can further increase the oxidative stress (Potts et al. 2000). Presence of leukocytes, round cells, and immature spermatozoa in the sperm pellet are the possible source of ROS during the in vitro incubation of spermatozoa.

10.4 Types of DNA Damage in Spermatozoa

Usually spermatozoa carry both single- and double-strand DNA breaks. In addition, damaged sperm chromatin is known to contain base adducts like 8-OHdG and ethenonucleosides such as 1,N6-ethenoadenosine and 1,N6-ethenoguanosine (Badouard et al. 2008). Recent evidences suggest that spermatozoa contain

numerous cross-linking with DNA–DNA or DNA–protein cross-linking which are significantly greater than in the dispersed interphase nuclei of somatic cells (Qiu et al. 1995).

10.4.1 Methods to Assess Sperm DNA Damage

Comet Assay Also known as single-cell gel electrophoresis (SCGE), it is a popular electrophoretic method used to assess the DNA integrity. Under electrophoretic conditions, the broken DNA strands migrate to anode. The broken DNA strands are visualized using fluorescent dye which binds to DNA. The spermatozoa with DNA damage will have a head composed of intact DNA and tail containing broken DNA fragments, attaining typical comet shape. The amount of DNA present in the tail and tail length indicates the extent of DNA damage (Fig. 10.1). The DNA damage assessment can be made by using software or by manual scoring. Even though with this test both single- and double-strand breaks can be assessed, it is a time-consuming method, and the threshold level is not defined for clinical application.

Sperm Chromatin Structure Assay (SCSA) This is a flow-cytometric method which is based on the susceptibility of the sperm chromatin to undergo partial denaturation in situ under acidic conditions. Spermatozoa with damaged chromatin denature when exposed to acidic conditions, while those with normal chromatin remain stable (Evenson et al. 1980). After exposure of spermatozoa to acidic conditions, spermatozoa are stained with acridine orange (AO), a metachromatic fluorescent dye.

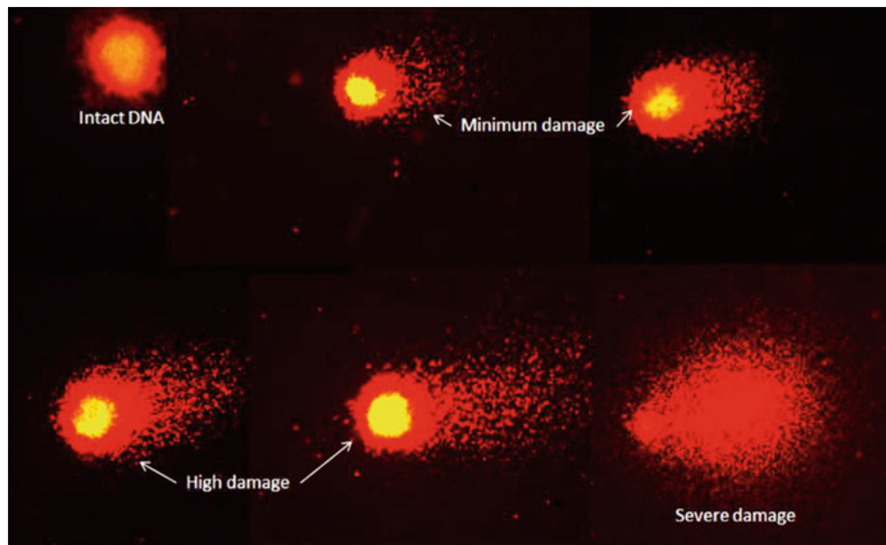

Fig 10.1 Comet assay: human spermatozoa stained with ethidium bromide and observed under fluorescent microscope (400× magnification)

When it intercalates with double-stranded DNA, it fluoresces green and fluoresces red when bound to single-stranded DNA. The extent of DNA denaturation following acid treatment is determined by measuring the metachromatic shift from green fluorescence to red fluorescence. Usually five to ten thousand cells are analyzed and assessed using a specific software. Though the method is highly reproducible and quite rapid, it is an expensive method.

Acridine Orange Test (AOT) This test is the simplified version of SCSA which uses fluorescent microscope to assess the metachromatic shift in the fluorescence by acridine orange (Tejada et al. 1984). It fluoresces green when bound to double-stranded DNA (excitation maximum at 502 nm and an emission maximum at 525 nm) and fluoresces red when bound to single-stranded DNA or RNA (excitation maximum 460 nm and an emission maximum 650 nm). For the assay, spermatozoa are exposed to mild acid treatment and stained with AO. Spermatozoa with intact DNA fluoresce green, and sperm with DNA damage fluoresces red or orange (Fig. 10.2). It is relatively a simple, rapid, and cheap method. The major disadvantages of this method are heterogeneous staining of the slide, rapid quenching, development of series of intermediate colors depending on the extent of chromatin denaturation, and large degree of inter- and intra-observer variations. Therefore, this method is considered to have low sensitivity and specificity to detect sperm DNA damage.

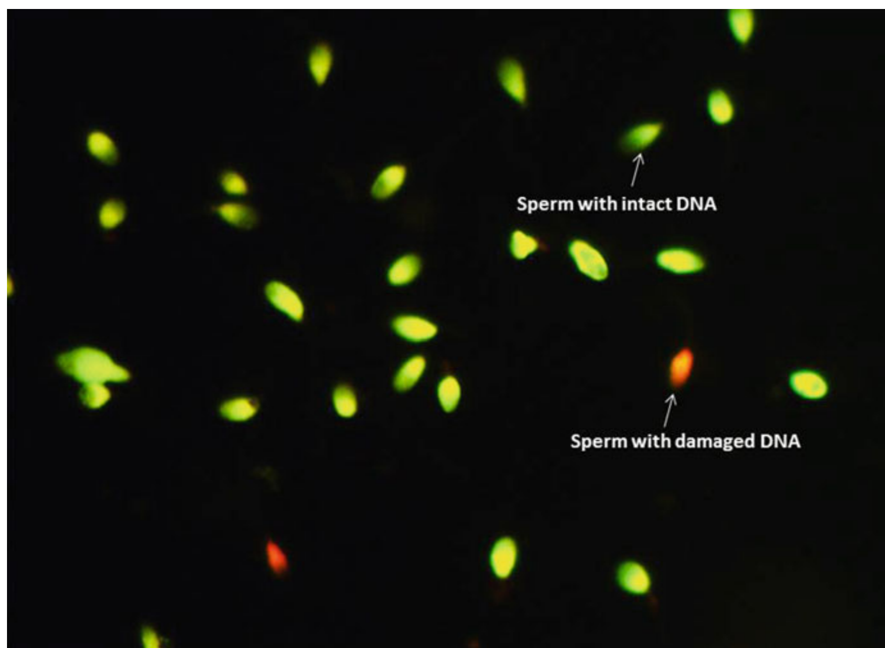

Fig. 10.2 Acridine orange test: human spermatozoa stained with acridine orange and observed under fluorescent microscope (400× magnification)

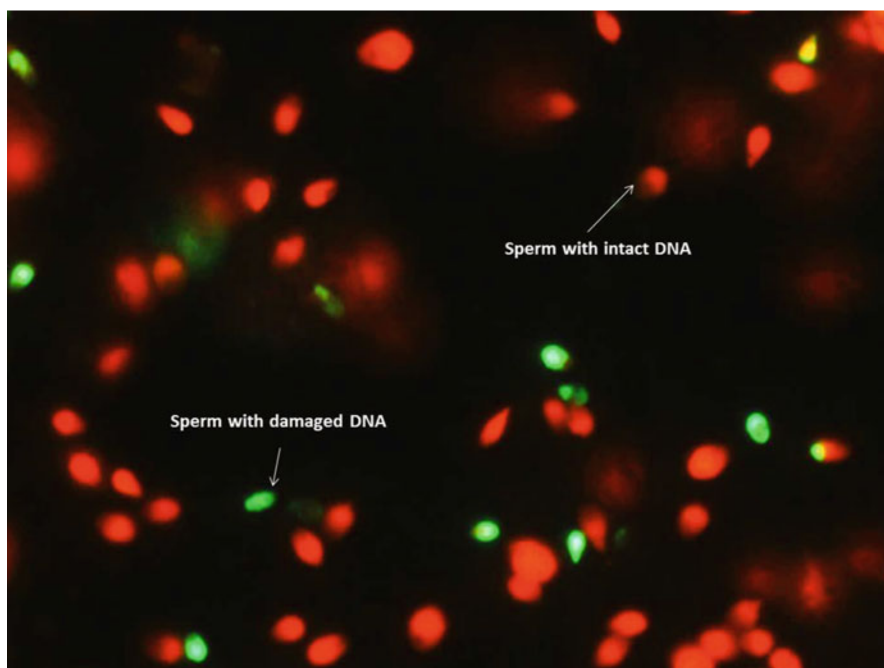

Fig. 10.3 TUNEL assay: human spermatozoa counterstained with propidium iodide (PI) and observed under fluorescent microscope using FITC filter (400× magnification)

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated 2′-Deoxyuridine 5′-Triphosphate Nick-End Labeling (TUNEL) Assay This assay detects the DNA breaks directly without changing the chromatin structure unlike in comet assay or sperm chromatin structure assay. The assay is based on the ability of the terminal deoxynucleotidyl transferase (TdT) to label the 3′-OH end of broken DNA strand with nucleotides (5-bromo-2′-deoxyuridine 5′-triphosphate nucleotide) usually tagged with a fluorochrome (Fig. 10.3) or a chromogen which can be visualized by observing under fluorescent or light microscope, respectively. The advantage of light microscopic method is that the signal obtained is permanent unlike in fluorescent microscopic method. Since it is a kit-based method, the results are reproducible. However, the kits are usually expensive with differences in working protocol, and the threshold level is not defined for clinical application.

Sperm Chromatin Dispersion (SCD) Test This test is based on the principle that mild acidic denaturation of sperm DNA and lysis of protamines will create a halo of chromatin loops around the sperm head when DNA is intact and small or no halo around the sperm head when DNA is fragmented (Fig. 10.4). It is a relatively simple and inexpensive method to detect sperm DNA integrity which was first described by Fernández et al. (2003). However, it does not give information on the extent of DNA damage in spermatozoa, and there are a limited number of studies to support its clinical application.

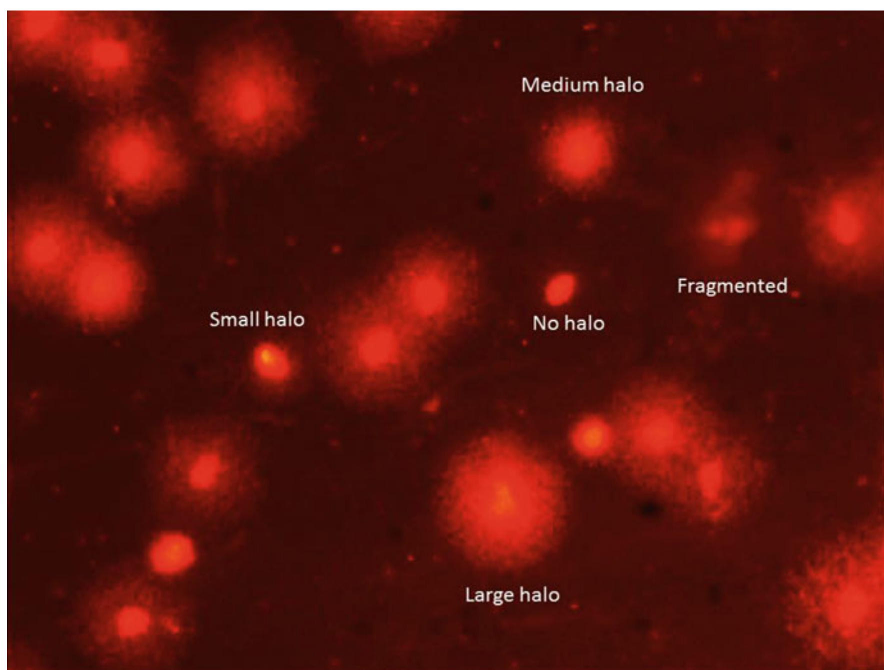

Fig. 10.4 Sperm chromatin dispersion test: human spermatozoa stained with ethidium bromide and observed under fluorescent microscope (400× magnification)

Aniline Blue Staining This is a very simple light microscopic method which is based on the difference in chromatin packing of spermatozoa. It gives an indirect measure of protamine–histone ratio in spermatozoa. Aniline blue is an acidic dye which has a greater permeability/affinity for histones in the sperm nucleus. Increased aniline blue staining of sperm indicates loose chromatin packing. The blue-stained spermatozoa are considered as immature, and pinkish sperm are mature (Fig. 10.5). Even though it is a rapid and cheap method, heterogeneous staining pattern limits its clinical application.

Toluidine Blue Staining Toluidine blue is a nuclear dye used for metachromatic and orthochromatic staining of chromatin that stains phosphate residues of the sperm DNA with loosely packed chromatin and fragmented ends. When the stain attaches with lysine-rich regions of histone, it produces a violet-bluish intense coloration, whereas a pale-blue color is produced by interactions with protamines in the chromatin. The sample can be analyzed using an ordinary microscope, but intermediate coloration increases inter-user variability.

Chromomycin A3 (CMA3) Staining This method is a simple fluorescence microscopic method which gives information on the degree of protamination and chromatin packing in spermatozoa. CMA3 specifically binds to GC (guanine–cytosine)-rich sequences in DNA. GC-rich region is also the site where protamines bind with DNA. The higher the CMA3 staining, the lower will be the protamine level and the

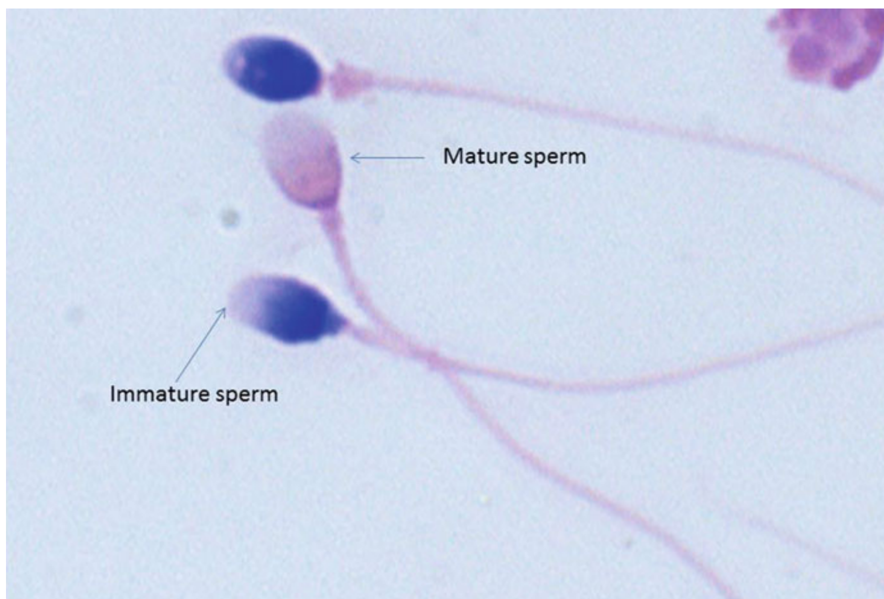

Fig. 10.5 Aniline blue staining: human spermatozoa stained with aniline blue dye and observed under light microscope (1000× magnification)

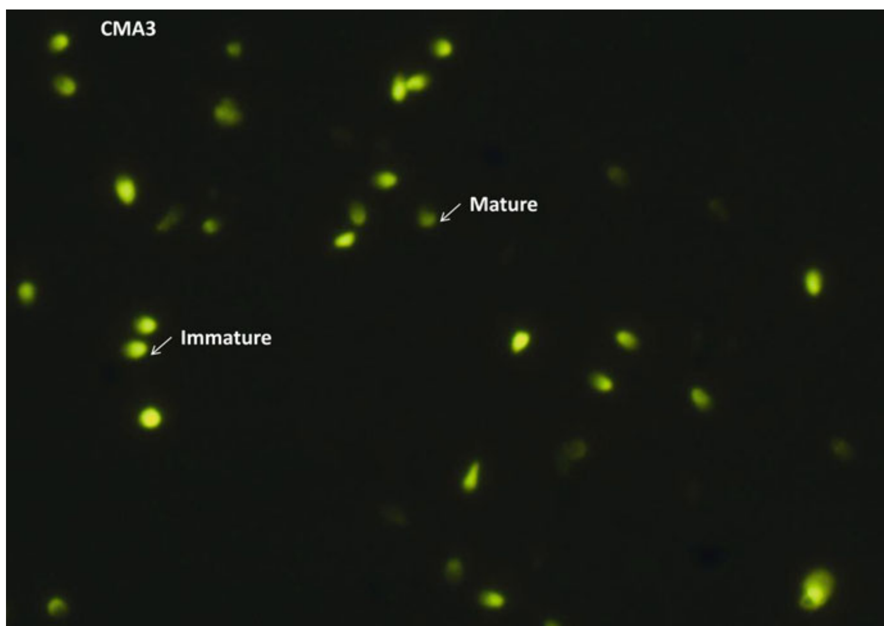

Fig. 10.6 Chromomycin A3 staining: human spermatozoa stained with chromomycin A3 and observed under fluorescent microscope (400× magnification)

poorer will be the chromatin packing in spermatozoa (Fig. 10.6). The spermatozoa with bright yellow are considered as immature, while with dull yellow are considered as mature. Due to rapid quenching of fluorescence and high inter- and intra-observer variation, this method has low clinical application.

8-OH Deoxyguanosine (8-OHdG) Estimation This is a chromatographic method which detects the level of ROS-induced by-product, 8-OHdG level in sperm DNA. The assay requires HPLC (high-performance liquid chromatography) which involves DNA extraction, enzymatic digestion of DNA, elution through HPLC column, and finally analysis of 8-OHdG. The method is very expensive and depends upon the DNA yield.

10.5 Clinical Management of Sperm DNA Damage

With considerable evidence of association of sperm DNA damage and male infertility, more research emphasis is given on enhancing the DNA integrity both in vivo and ex vivo. Treating of male infertility patients with antioxidant molecules to reduce the oxidative stress has become the most sensible approach (Fraga et al. 1991; Zini et al. 2005). Similarly, supplementation of culture medium with antioxidants, vitamins, and metals during in vitro processing of semen sample has shown drastic improvement in sperm functional competence (Kalthur et al. 2012; Talevi et al. 2013; Fanaei et al. 2014) suggesting its promising role during the preparation of spermatozoa for ART which could improve the outcome.

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