Role of sperm chromatin abnormalities and DNA damage in male infertility

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Sperm DNA integrity is essential for the accurate transmission of genetic information. It has a highly compact and complex structure and is capable of decondensation—features that must be present in order for a spermatozoon to be considered fertile. Any form of sperm chromatin abnormalities or DNA damage may result in male infertility. In support of this conclusion, it was reported that in-vivo fecundity decreases progressively when >30% of the spermatozoa are identified as having DNA damage. Several methods are used to assess sperm chromatin/DNA, which is considered an independent measure of sperm quality that may yield better diagnostic and prognostic approaches than standard sperm parameters (concentration, motility and morphology). The clinical significance of this assessment lies in its association not only with natural conception rates, but also with assisted reproduction success rates. Also, it has a serious impact on the offspring and is highly prognostic in the assessment of fertility in cancer patients. Therefore, screening for sperm DNA damage may provide useful information in cases of male idiopathic infertility and in those men pursuing assisted reproduction. Treatment should include methods for prevention of sperm DNA damage.

Key words: apoptosis/DNA damage/infertility/oxidative stress/sperm

Introduction

Male factor infertility plays a role in approximately 50% of infertile couples (World Health Organization, 1999). A number of aetiologies have been identified as potential causes of male infertility, which include gene mutations, aneuploidies, infectious diseases, ejaculatory duct occlusion, varicocle, radiation, chemotherapy and erectile dysfunction (Ollero et al., 2001). One area of research that has been studied intensely during the past decade as a cause for male infertility is the integrity of DNA in the nucleus of mature ejaculated spermatozoa (Sakkas et al., 1999a). This focus on the genomic integrity of the male gamete has been further intensified by the growing concern about transmission of genetic diseases through ICSI (Barroso et al., 2000). Normally, the sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins. It is condensed and insoluble in nature—features that protect genetic integrity and facilitate transport of the paternal genome through the male and female reproductive tracts (Manicardi et al., 1998). For a spermatozoon to be fertile, it must be capable of undergoing decondensation at an appropriate time in the fertilization process (Amann, 1989). Infertile men manifest various nuclear alterations, including an abnormal chromatin structure, chromosomes with microdeletions, aneuploidies and DNA strand breaks (Hofman and Hilscher, 1991).

Accumulating evidence suggests that disturbances in the organization of the genomic material in sperm nuclei are negatively correlated with the fertility potential of spermatozoa, either in vivo or in vitro (Sun et al., 1997; Spano et al., 2000). Some recent reports have indicated that when >30% of sperm DNA is damaged, natural pregnancy is not possible (Evenson et al., 1999, 2002). Also, it has been suggested that sperm DNA integrity may be a more objective marker of sperm function as opposed to the standard semen analysis. This was attributed to the fact that conventional semen analysis does not adequately represent the diverse array of biological properties that the spermatozoon, as a highly specialized cell, expresses (Zini et al., 2001a; Evenson et al., 2002). In addition, the results of semen analyses can be very subjective and prone to both intra- and inter-observer variability (Keel and Webster, 1990). In this review, the different aspects related to sperm DNA damage will be highlighted in an attempt to clarify its role in male infertility. A thorough review of the scientific literature was conducted by Medline search (via PubMed and OvidWeb) using the keywords 'sperm DNA/chromatin...
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damage’. The abstracts of all English language articles listed in Medline from 1966 onwards were checked.

Human sperm chromatin structure

The formation of mature spermatozoa is a unique process involving a series of meiotic and mitotic changes in cytoplasmic architecture, replacement of somatic cell-like histones with transition proteins, and the final addition of protamines leading to a highly packaged chromatin (Pocchia, 1986). Sperm DNA is organized in a specific manner that keeps the chromatin in the nucleus compact and stable. It is packed with a special type of small, basic protein into a tight, almost crystalline status that is at least six times more condensed than in mitotic chromosomes (Fuentes-Mascorro et al., 2000). It occupies almost the entire nucleus volume, whereas somatic cell DNA only partly fills the nucleus.

The DNA in somatic cell nuclei is first packaged into nucleosomes (Pienta and Coffey, 1984). These structures consist of a protein core formed by an octamer of histones with two laps of wrapped DNA (around base pairs). The nucleosomes are then further coiled into regular helices also called solenoids (Finch and Klug, 1976). These two types of DNA packaging increase the volume of the chromatin (Ward and Coffey, 1991). Sperm nuclei, however, do not have the volume required for this type of packaging, since packing the DNA in even a single, closely packed nucleosome would require 9.9 μm³, which is more than twice the volume of an average sperm nucleus (Van Holde and Zlatanova, 1996). Thus, a completely different type of DNA packaging must be present in mammalian sperm nuclei.

In 1991, Ward and Coffey proposed four levels of organization for packaging in the spermatozoon: (i) chromosomal anchoring, which refers to the attachment of the DNA to the nuclear annulus; (ii) formation of DNA loop domains as the DNA attaches to the newly added nuclear matrix; (iii) replacement of histones by protamines, which condenses the DNA into compact doughnuts; and (iv) chromosomal positioning (Ward and Coffey, 1991). In order for the sperm nucleus to evolve and become highly condensed with a species-specific shape, it undergoes a complicated series of reactions through which somatic histones and non-histone chromatin proteins are replaced during a variable period of time by one or more protamine types (Loir and Laneau, 1984). Protamines are highly basic proteins about half the size of a typical histone (5–8 kDa) (Fuentes-Mascorro et al., 2000). Arginines form from 55 to 79% of the amino acid residues of protamines, permitting a strong DNA binding. Sperm epididymal maturation involves a final stage of chromatin organization in which protamine cross-linking by disulphide bond formation occurs—a step that is supported by the fact that protamines contain a significant number of cysteine residues that participate in sperm chromatin compaction by forming multiple inter- and intraprotamine disulphide cross-links. All these interactions make mammalian DNA the most condensed eukaryotic DNA (Ward and Coffey, 1990).

The sperm’s entire haploid genome is organized into DNA loop domains that have an average length of 27 kilobytes. These loops, which can be visualized by using fluorescent in-situ hybridization (FISH), are attached at their bases to a structural element within the sperm nucleus known as the nuclear matrix. When the human sperm undergoes decondensation, the DNA remains anchored to the base of the tail. This fact suggests the presence of a nuclear annulus-like structure in human sperm (Barone et al., 1994). This DNA organization not only permits the very tightly packaged genetic information to be transferred to the egg, but also ensures that the DNA is delivered in a physical and chemical form that allows the developing embryo to access the genetic information (Sakkas et al., 1999a).

Origin of sperm DNA damage

The population of spermatozoa in an ejaculate can be highly heterogeneous. This appears to be even more evident in patients whose sperm parameters fall below normal values. The positive relationship between poor sperm parameters and DNA damage in spermatozoa points to inherent problems in spermatogenesis in specific patients (Lopes et al., 1998a).

Environmental stress, gene mutations and chromosomal abnormalities can disturb the highly refined biochemical events that occur during spermatogenesis, and this can ultimately lead to an abnormal chromatin structure that is incompatible with fertility (Evenson et al., 2002). Sperm nuclear chromatin abnormalities/DNA damage could occur at the time of, or result from, DNA packing at spermiogenesis (Sailler et al., 1995). Alternatively, it could be the result of free radical-induced damage (Aitken et al., 1998) or a consequence of apoptosis (Gorczyza et al., 1993a). The exact mechanism(s) by which chromatin abnormalities/DNA damage arise in human spermatozoa is not precisely understood, but three main theories have been proposed, namely defective sperm chromatin packaging, apoptosis and oxidative stress.

Defective sperm chromatin packaging

This theory arises from studies performed in animal models, and is linked to the unique manner in which mammalian sperm chromatin is packaged. It has been postulated that chromatin packaging may necessitate endogenous nuclease (topoisomerase II) activity to create and ligate nicks that facilitate protamination during spermiogenesis (McPherson and Longo, 1993). These nicks are thought to relieve torsional stress and aid chromatin rearrangement during the displacement of histones by protamines (McPherson and Longo, 1992). Therefore, the presence of endogenous nicks in spermatozoa may indicate anomalies during spermiogenesis and an incomplete maturation process (Manicardi et al., 1995).

Apoptosis

Apoptosis is a mode of cellular death based on a genetic mechanism that induces a series of cellular, morphological and biochemical alterations, leading the cell to suicide (Nagata, 1997). This process usually takes place at specific moments in normal embryonic development to allow the definitive form of tissues and in adult life to discard cells that no longer have a function, or have an altered function (Vaux and Korsemeyer, 1999).

In mammalian testes, germ cells expand clonally through many rounds of mitoses before undergoing the differentiation steps that result in mature spermatozoa. This clonal expansion
is excessive and thus requires a mechanism such as apoptosis to match the number of germ cells with the supportive capacity of Sertoli cells (Sinha Hikim and Swerdloff, 1999). Therefore, in the context of male reproduction, apoptosis controls the overproduction of male gametes and restricts the normal proliferation levels during conditions unsuitable for sperm development. Methods such as the flow cytometric terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling (TUNEL) assay, which detects apoptosis-specific DNA fragmentation, annexin-V binding, detecting apoptosis-related translocation of plasma membrane phosphatidylserine and immunohistochemistry have been employed to identify germ cell apoptosis (Tesarik et al., 1998; Berensztein et al., 2002). The identification was successful in spermatogonia, spermatocytes and spermatids in the testis of men with normal spermatogenesis, and even in patients with non-obstructive azoospermia (Jurisicova et al., 1999).

Pathways involving the cell-surface protein Fas may mediate apoptosis in sperm (Lee et al., 1997). Binding of Fas ligand (FasL) or agonistic anti-Fas antibody to Fas kills cells by apoptosis (Suda et al., 1993). In normal mice and rat testes, Sertoli cells express FasL and signal the killing of Fas-positive germ cells, thus limiting the size of the germ cell population to numbers that they can support (Rodriguez et al., 1997). In men with normal semen characteristics, the percentage of Fas-positive spermatozoa is small. However, in men with abnormal semen parameters, the percentage of Fas-positive spermatozoa can be as high as 50%. This observation indicates that the correct clearance of spermatozoa via apoptosis is not occurring in infertile men. Therefore, the presence of spermatozoa that possess apoptotic markers, such as Fas positivity and DNA damage, indicates that in men with abnormal semen parameters, an ‘abortive apoptosis’ has taken place (Huszar et al., 1997).

Failure to clear Fas-positive spermatozoa may be due to dysfunction at one or more levels. Because Sertoli cells can limit this proliferation by producing FasL, it has been postulated that oligozoospermic men with reduced spermatogenesis may not produce enough spermatozoa to trigger this action (Francavilla et al., 2000). In these men, Fas-positive spermatozoa may escape the signal to undergo apoptosis (Sakkas et al., 1999b). Fas-positive spermatozoa may also exist because of problems in activating Fas-mediated apoptosis. This hypothesis may explain why patients with abnormal semen characteristics also possess a higher percentage of spermatozoa containing DNA damage and abnormal spermatozoa that display markers of apoptosis (Sakkas et al., 2002).

Another major component of apoptotic machinery that contributes to sperm DNA damage involves members of a family of aspartic acid-directed cysteine proteases called caspases (Thornberry and Lazebnik, 1998). The FasL/Fas ligation in the inner mitochondrial membrane leads to activation of caspases 8 and 9. Once activated, these caspases transduce a signal to effector caspases, including caspase 3, which in turn appears to induce activation of caspase-activated deoxyribonuclease (CAD; also called DNA fragmentation factor-40 or caspase-activated nuclease) leading to DNA degradation (Kim et al., 2001).

**Oxidative stress**

In recent years, concern has been expressed about the generation of reactive oxygen species (ROS) in the male reproductive tract. This is because ROS, at high levels, are potentially toxic to sperm quality and function (Saleh and Agarwal, 2002). ROS are highly reactive oxidizing agents, among which are included hydrogen peroxide, superoxide and free radicals, the latter being defined as ‘any atom or molecule that possesses one or more unpaired electrons’ (Warren et al., 1987). The presence of high ROS levels has been reported in the semen of between 25 and 40% of infertile men (Padron et al., 1997).

Two factors protect the sperm DNA from oxidative insult: the characteristic tight packaging of the DNA; and the antioxidants present in seminal plasma (Twigg et al., 1998a). However, oxidative stress (OS) may develop as a result of an imbalance between ROS generation and antioxidant scavenging activities (Sikka, 2001). In general, DNA bases and phosphodiester backbones are very susceptible to peroxidation. In addition, spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes (Sharma and Agarwal, 1996).

Strong evidence suggests that high levels of ROS mediate the occurrence of high frequencies of single- and double-strand DNA breaks commonly observed in the spermatozoa of infertile men (Fraga et al., 1996; Kodama et al., 1997; Sun et al., 1997; Aitken and Krausz, 2001). The formation of 8-hydroxy-2-deoxyguanosine (8-OhdG) has been considered as a key biomarker for this oxidative DNA damage (Ames et al., 1993). Recently, a significant positive correlation between ROS and DNA fragmentation (r = 0.4; P = 0.02) was reported (Barroso et al., 2000). Furthermore, studies in which the sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links and chromosomal rearrangements (Twigg et al., 1998b; Duru et al., 2000).

**Evaluation of sperm nuclear DNA damage**

Based on the critical importance of accurate transmission of genetic information to the offspring, several assays have been developed to evaluate sperm chromatin/DNA integrity. These assays have been also used in an attempt to establish a significant correlation with male infertility.

**Comet assay**

The comet assay measures DNA damage by quantifying the single- and double-stranded breaks associated with DNA damage (McKelvey-Martin et al., 1997). In this assay, spermatozoa are stained with a fluorescent DNA-binding dye.
The resulting images, which resemble ‘comets’, are measured after staining to determine the extent of DNA damage (Ostling and Johanson, 1984). The characteristics that have been used for analysis include the diameter of the nucleus and the comet length (Singh et al., 1988). One of the principles of the comet assay is that nicked double-stranded DNA tends to remain in the comet head, whereas short fragments of nicked double- and single-stranded DNA migrate into the tail area (Klaude et al., 1996). Thus, spermatozoa with high levels of DNA strand breaks would show increased comet tail fluorescent intensity (Hughes et al., 1996) and comet tail length (Singh and Stephens, 1998). However, useful thresholds have not been established for the comet assay.

\textit{In-situ nick translation (NT) assay}

The NT assay quantifies the incorporation of biotinylated-deoxyuridine triphosphate (dUTP) at single- and double-stranded DNA breaks in a reaction that is catalysed by the template-dependent enzyme, DNA polymerase I. The NT assay identifies spermatozoa that contain appreciable and variable levels of endogenous DNA damage (Manicardi et al., 1995).

The clinical value of the NT assay is severely limited because no correlation has been proven with fertilization during in-vivo studies (Irvine et al., 2000), and because of its lack of sensitivity compared with other assays (Twigg et al., 1998a).

\textit{TUNEL assay}

The TUNEL assay quantifies the incorporation of deoxyuridine triphosphate (dUTP) at single- and double-stranded DNA breaks in a reaction catalysed by the template-independent enzyme, terminal deoxynucleotidyl transferase (TdT) (Gorczyza et al., 1993b). Incorporated dUTP is labelled such that breaks can be quantified by flow cytometry, fluorescent microscopy or light microscopy. The TUNEL assay cannot be employed for routine clinical use due to a lack of useful thresholds.

\textit{Sperm chromatin structure assay (SCSA)}

The SCSA is a flow cytometric assay that relies on the fact abnormal sperm chromatin are highly susceptible to physical induction of partial DNA denaturation \textit{in situ} (Drazynkiewicz et al., 1975; Evenson et al., 1980). The extent of DNA denaturation following heat or acid treatment is determined by measuring the metachromatic shift from green fluorescence (acridine orange intercalated into double-stranded nucleic acid) to red fluorescence (acridine orange associated with single-stranded DNA) (Drazynkiewicz et al., 1976). The most important parameter of the SCSA is the DNA fragmentation index (%DFI), which represents the population of cells with DNA damage (Evenson et al., 2002).

\textit{Acridine orange test}

The acridine orange test (AOT) was introduced as a simplified microscopic method of the SCSA that does not require expensive flow cytometry equipment and a SCSA-trained technician (Tejada et al., 1984). It relies on visual interpretation of fluorescing spermatozoa and debris that fall into a broad range of colours under microscopic examination. Indistinct colour, rapidly fading fluorescence and heterogeneous slide staining exacerbate problems with interpretation (Duran et al., 1998). Such conditions preclude using the AOT for critical clinical diagnosis and prognosis of a semen sample (Drazynkiewicz and Kapucinski, 1990), since the AOT may introduce many sources of variation.

Although some laboratories have used the AOT in an attempt to improve male fertility evaluations (Hoshi et al., 1996), the predictive value of the test for human fertility remains controversial. However, in relation to the clinical significance of this assay, a strong positive correlation exists between the AOT and TUNEL assays. In addition, the AOT correlates negatively with sperm motility (Zini et al., 2001b).

\textit{Sperm chromatin dispersion (SCD) test}

This assay has been recently described as a simple and inexpensive method for the analysis of sperm DNA fragmentation. The SCD test is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo when mixed with a aqueous agarose following acid denaturation and removal of nuclear proteins (Fernandez et al., 2003).

\textit{Other methods}

Other methods may be used to detect DNA damage in human spermatozoa, such as electron microscopy (Zamboni, 1992), enzyme-linked immunosorbent assay (ELISA) (Van Loon et al., 1991), FISH (Fernandez and Gozalvez, 2002), and high-performance liquid chromatography, which is used to measure the level of 8-OhdG (Floyd et al., 1986).

\textit{Aetiology of DNA damage}

A variety of causes have been correlated with increased levels of sperm DNA damage, and in turn, detrimentally affect the status of male fertility.

\textit{Leukocytospermia}

Leukocytes in general are present in most ejaculates and are thought to play an important role in immunosurveillance and phagocytic clearance of abnormal sperm (Tomlinson et al., 1992). However, increased concentrations of leukocytes in semen indicate the presence of a genital tract infection or inflammation and have been reported to be associated with an increase in immature germ cell concentration (Sigman and Lopes, 1993).

In a study conducted by the present authors’ group (Alvarez et al., 2002), higher amounts of DNA-damaged cells were reported in the raw semen samples of leukocytospermic patients compared with normal donors (39 ± 10.9 versus 24.9 ± 10.2%; P < 0.01). Following the fractionation of semen samples into different portions according to their stage of maturation, it was also reported that chromatin alterations were highest in the immature fraction (Figure 1) (Alvarez et al., 2002).
One potential hypothesis for these findings is that leukocytospermia could be a marker for an inflammatory process in the testis. The presence of pro-inflammatory mediators such as cytokines could lead to alterations in the regulation of spermiogenesis and subsequently to DNA aberration (Cohen and Pollard, 1995). Another possible explanation would be the ROS-induced cross-damage of sperm by leukocytes (Comhaire et al., 1999; Reichart et al., 2000).

Finally, it is important to note that even systemic infection may affect sperm integrity. This was suggested by the fact that during an episode of influenza, there is an increase in DNA damage in the sperm produced during that particular spermatogenic cycle (Evenson et al., 2000).

Cigarette smoking

Cigarette smoke has mutagenic properties, having been associated with an overall reduction in semen quality, and specifically a reduction in sperm count and motility and an increase in number of abnormal cells (Sofikitis et al., 1995; Kunzle et al., 2003). Similarly, it was reported that cigarette smoking affects sperm DNA integrity. Using both the SCsA and TUNEL assays, sperm DNA damage was significantly higher in smokers than non-smokers ($P < 0.02$ and $P < 0.05$ respectively) (Potts et al., 1999a). Also, in another study using only the SCsA, it was reported that the %DFI was significantly higher in infertile men who smoked ($P = 0.02$) (Saleh et al., 2002a). This observation was first described in 35 smokers included in an IVF programme; these subjects had a significantly higher percentage of spermatozoa with DNA damage than did non-smokers ($4.7 \pm 1.2$ versus $1.1 \pm 0.2%; P = 0.02$) (Sun et al., 1997).

A possible explanation for these findings could be the increased leukocyte-induced OS on developing or mature sperm (Potts et al., 1999a). The exact mechanism(s) of increased seminal leukocyte infiltration into the semen of infertile smokers is not clear and warrants further research. Metabolites of cigarette smoke components may induce an inflammatory reaction in the male genital tract, with subsequent release of chemical mediators of inflammation. These inflammatory mediators such as interleukin (IL)-6 and IL-8 can recruit and activate leukocytes (Comhaire et al., 1999). In turn, activated leukocytes can generate high levels of ROS in semen, which may overwhelm the antioxidant strategies and result in OS (Aitken et al., 1995). Another causative factor would be the fact that the seminal plasma in smokers contains lower levels of antioxidants than that of non-smokers (Fraga et al., 1996).

Iatrogenic sperm DNA damage

A wide variety of sperm preparation protocols are currently available for use in assisted conception therapy. However, these strategies involve repeated high-speed centrifugation and the isolation of spermatozoa from the protective antioxidant

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**Figure 1.** Correlation between leukocyte concentration in semen and % DNA fragmentation index (DFI) values in sperm from the different fractions resulting from 47, 70 and 90% density gradient centrifugation. (A) Fraction 1 ($r^2 = 0.71; P = 0.009$). (B) Fraction 2 ($r^2 = 0.79; P < 0.0001$). (C) Fraction 3 ($r^2 = 0.73; P = 0.007$). (D) Fraction 4 ($r^2 = 0.82; P = 0.002$)
environment provided by seminal plasma, which have been shown to damage sperm DNA via mechanisms that are mediated by the enhanced generation of ROS (Zalata et al., 1995).

Normally, seminal plasma contains high- and low-molecular-weight factors that protect spermatozoa against free radical toxicity. They include enzymatic ROS scavengers such as Cu, Zn superoxide dismutase (SOD) and catalase (Siciliano et al., 2001). Also, seminal plasma contains chain-breaking antioxidants such as ascorbate, urate, albumin, glutathione and taurine (Holmes et al., 1992; Thiele et al., 1995). Thus, the seminal plasma plays a crucial protective role against ROS, and its removal during sperm preparation may be hazardous to sperm DNA integrity. The use of testicular sperm extraction (TESE) for ICSI will carry the same hazardous factor of excluding the protective roles of seminal plasma (Potts et al., 1999b).

Another form of iatrogenic interference that might lead to DNA damage is that of cryopreservation, which is used extensively in artificial insemination and assisted reproduction technique (ART) programmes. Although it was once proved that the cryopreservation of testicular sperm does not increase baseline levels of DNA damage (Steele et al., 2000), most other studies indicate that the freeze–thaw process significantly damages spermatozoal DNA (Donnelly et al., 2001a; b; Labbe et al., 2001). Furthermore, results from experiments in which spermatozoa were frozen in the absence of cryoprotectants revealed that significantly more cells with fragmented DNA occurred among those exposed to one, three and five freeze–thaw cycles (65 ± 6, 76 ± 11 and 92 ± 6% respectively) compared with fresh sperm (19 ± 16%; P < 0.05) (Linfor and Meyers, 2002). In a recent study, flash-freezing in liquid nitrogen without cryopreservative represented the most appropriate method for human sperm cryopreservation (Duty et al., 2002).

The sperm DNA damage in these situations may be attributed to the fact that spermatozoa from infertile men have a greater incidence of irregular chromatin organization and thus show a significant decrease in chromatin resistance to thermal denaturation. Another factor would be the lack of protective constituents in seminal plasma (Donnelly et al., 2001a).

Sperm DNA damage in cancer patients

Testicular cancer, Hodgkin’s disease and leukaemia are among the most common malignancies affecting men of reproductive age (Agarwal et al., 1996). In particular, the incidence of testicular cancer has increased during the past 5 to 6 years (Hallak et al., 1999). As the treatment modalities for malignant diseases are improving, the effects of aggressive therapy are becoming more apparent, and infertility has become a major sequela of cancer treatment (Richter et al., 1984).

Referral to sperm banks for cancer patients is often provided before initiating chemotherapy, radiation therapy or surgery. However, cancers adversely affect sperm count and motility in pre-freeze and post-thaw specimens (Agarwal and Newton, 1991). Reports indicate that infertility is associated with testicular cancer even before any therapy is given. Untreated cancer patients have significantly higher DNA damage than healthy fertile men; indeed, the %DFI in these patients was reported to be significantly higher than that in fertile controls (21.9 ± 2.0 versus 10.7 ± 3.5; P = 0.007) (Kobayashi et al., 2001).

In patients with testicular cancer, a germ cell defect is thought to be responsible for poor semen quality (Snager et al., 1992). In about 52% of patients with testicular cancer and in 40% with other types of cancer, the total sperm count is reduced at diagnosis and at treatment (Chapman et al., 1979). Although pregnancies and births have been reported using cryopreserved sperm from cancer patients, these semen samples have decreased fertilization potential (Khalifa et al., 1992), mainly because poor semen quality before freezing has been associated with poor post-thaw outcome (Snager et al., 1992). The extent of DNA damage may help determine how semen should be cryopreserved before therapy begins. Specimens with high sperm concentration and motility and low levels of DNA damage could be preserved in relatively large aliquots suitable for intrauterine insemination (IUI). If a single specimen of good quality is available, then it should be preserved in multiple small aliquots suitable for IVF or ICSI (Kobayashi et al., 2001).

Drugs and irradiation

Chemotherapeutic drugs such as fludarabine, cyclophosphamide and busulphan can cause testicular damage as manifested by reduced testicular volume, oligozoospermia, elevated FSH and LH and lower testosterone concentrations (Chatterjee et al., 2000). High levels of sperm DNA damage can be seen following even a single dose of these drugs, which may persist for several months after cessation of their use (Bucci and Meistrich, 1987; Cai et al., 1997). Cocaine has also been proven to affect sperm DNA; in a recent study, cocaine exposure led to an increase in sperm DNA strand breaks—an interesting finding that has been attributed to an increase in apoptosis (Li et al., 1999).

Male germ cells are sensitive to the mutagenic effects of irradiation. Although sperm DNA damage exists following radiotherapy, no increase in genetic defects or congenital malformations was detected among children conceived by parents who had previously undergone treatment (Arnon et al., 2001).

Finally, one type of alternative medicine—herbal therapy—has recently become popular despite a lack of scientific experimentation to assess its effectiveness and safety. In a study performed to evaluate the effect of some these widely used herbs on sperm DNA, high concentrations of St. John’s wort, gingko biloba and *Echinacea purpurea* were found to damage the reproductive cells and were even mutagenic to sperm cells (Ondrizek et al., 1999). Hence, the hazardous sequelae following intake of these compounds is emphasized.

The role of other factors such as sexually transmitted *Ureaplasma urealyticum* and environmental and occupational
exposures in increasing the incidence of sperm DNA damage remains unclear (Golden et al., 1999; Reichart et al., 2000).

Clinical significance of sperm DNA damage

Male infertility

Accumulating evidence suggests that disturbances in the organization of the genomic material into sperm nuclei are negatively correlated with the fertility potential of the spermatozoa (Table I). At the present time, it is clear that a sperm chromatin structure of poor quality may be indicative of male subfertility regardless of the number, motility and morphology of spermatozoa. In a prospective study involving 165 American (presumably fertile) couples desiring to achieve pregnancy, DNA integrity assessment using SCSA values proved to be the best predictor of the couples’ inability to become pregnant (Evenson et al., 1999). Many other studies established the fact that infertile patients have higher levels of DNA strand breaks than fertile subjects, thereby confirming the diagnostic value of sperm DNA damage parameters in evaluating sperm function and male infertility (Host et al., 1999a, b). A semen sample is considered fertile if the maximum proportion of cells revealing evidence of DNA damage (%DFI) does not exceed approximately 30% (Evenson et al., 1999).

Sperm chromatin anomalies may also play a role in the pathogenesis of spermatogenic disorders such as maturation arrest. In an attempt to verify this role, round spermatids (Sa stage) and late elongated spermatids (Sd stage) were extracted from testicular biopsy samples from infertile patients with non-obstructive azoospermia. There were higher frequencies of DNA damage in round spermatids from patients with complete spermiogenesis failure compared with elongated spermatids from patients with incomplete spermiogenesis failure (Tesarik et al., 1998).

Finally, it is interesting to note that spermatozoa from infertile patients are generally more susceptible to the effects of DNA-damaging agents such as H2O2 and radiograph exposure (McKelvey-Martin et al., 1997). Some antioxidants such as ascorbate and alpha-tocopherol were able to provide significant protection against such damage (Donnelly et al., 1999).

Relationship of DNA damage to other semen parameters

Reports attempting to relate sperm chromatin/DNA damage with conventional semen analysis parameters are summarized in Table II. These studies indicate that spermatozoa from patients with abnormal sperm count, motility and morphology have increased levels of DNA damage.

In a recent report, sperm DNA denaturation had the lowest average coefficient of variation (CV), followed by motility and

Table I. Correlation between sperm DNA damage and in-vivo male fertility potential

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study population</th>
<th>Technique</th>
<th>Parameter</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hughes et al. (1996)</td>
<td>Donors (20) Infertile patients (n)</td>
<td>Comet</td>
<td>DF</td>
<td>81.7 ± 24.5</td>
</tr>
<tr>
<td>Hughes et al. (1996)</td>
<td>20 Norm (20)</td>
<td>Oligo (20)</td>
<td>Comet; after H2O2 exposure</td>
<td>81.7 ± 24.5</td>
</tr>
<tr>
<td>Kodama et al. (1997)</td>
<td>17 Norm (20)</td>
<td>Oligo (20)</td>
<td>HPLC 8-OhdG</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Shen et al. (1999)</td>
<td>19 Infertile patients (60)</td>
<td>HPLC</td>
<td>8-OhdG</td>
<td>10.03</td>
</tr>
<tr>
<td>Spano et al. (2000)</td>
<td>215 males with no previous knowledge of fertility status</td>
<td>SCFA</td>
<td>8-OhdG</td>
<td>4.79</td>
</tr>
</tbody>
</table>

*Compared with donors.

DD = DNA denaturation; DF = DNA fragmentation; DFI = DNA fragmentation index; HPLC = high-performance liquid chromatography; Non-azo = non-azoospermic; Norm = normozoospermic; NS = not significant; OAT = oligoasthenoteratozoospermia; 8-OhdG = 8-hydroxy-2-deoxyguanosine; oligo = oligozoospermic; TC = testicular cancer.

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Table II. Correlation between sperm DNA damage and sperm characteristics

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study population</th>
<th>Technique</th>
<th>Parameter</th>
<th>Resultsa</th>
<th>Concentration</th>
<th>Morphology</th>
<th>Motility</th>
<th>Fertilization capacityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kodama et al (1997)</td>
<td>17 donors (n) 19 patients (n)</td>
<td>HPLC</td>
<td>8-OhdG</td>
<td>-0.49 (0.001)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Shen et al. (1999)</td>
<td>54 donors (n) 60 patients (n)</td>
<td>HPLC</td>
<td>8-OhdG</td>
<td>-0.42 (&lt; 0.001)</td>
<td>0.38 (&lt; 0.001)</td>
<td>-0.24 (&lt; 0.01)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Irvine et al. (2000)</td>
<td>12 donors (n) 29 patients (n)</td>
<td>Comet</td>
<td>DF</td>
<td>-0.54 (0.001)</td>
<td>-0.37 (0.026)</td>
<td>-0.37 (0.026)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Chan et al. (2001)</td>
<td>39 donors (n) 40 patients (n)</td>
<td>Comet</td>
<td>DF</td>
<td>-0.66 (&lt; 0.0001)</td>
<td>-0.38 (0.016)</td>
<td>-0.38 (0.016)</td>
<td>0.493 (&lt; 0.05)</td>
<td></td>
</tr>
<tr>
<td>Tomlinson et al. (2001)</td>
<td>140 donors (n) 140 patients (n)</td>
<td>NT</td>
<td>DF</td>
<td>-0.24 (0.01)</td>
<td>-</td>
<td>-0.20 (0.004)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Saleh et al. (2003)</td>
<td>16 donors (n) 92 patients (n)</td>
<td>SCSA</td>
<td>DFI</td>
<td>-0.31 (0.001)</td>
<td>-0.040 (&lt; 0.0001)</td>
<td>-0.47 (&lt; 0.0001)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

aResults expressed as r = correlation coefficient, with P-value in parentheses.
bMeasured by the zona-free hamster oocyte penetration assay.

Concentration in two consecutive samples from infertile men (CV = 21, 24 and 35% respectively) (Zini et al., 2001b). These data are in keeping with the similarly low (~20%) value reported by another study conducted using a group of unselected semen donors (Evenson et al., 1991).

The fact that sperm DNA integrity is an objective marker of sperm function may help provide a diagnosis for cases of unexplained male infertility. In a recent study conducted at the Cleveland Clinic (Saleh et al., 2002b), standard semen parameters and SCSA parameters were compared in fertile donors and in infertile men with normal and abnormal semen parameters. The only significant difference between these groups was that the %DFI was significantly higher in the infertile patients (P = 0.02). On the other hand, no significant difference in %DFI was observed between the infertile men with normal and abnormal semen parameters (P = 0.27). Based on these results, it was possible to conclude that sperm DNA damage analysis usually reveals hidden abnormalities in men with infertility that is classified as idiopathic based on apparently normal standard semen parameters (Saleh et al., 2002b).

**Fertilization and ART**

The upsurge in the use of ART has increased the emphasis on the sperm chromatin quality. A summary of published reports correlating the outcome of ART with sperm DNA damage is shown in Table III.

**IUI**

IUI is one of the most widely used modalities for the treatment of infertility, and has a variety of indications including non-severe male factor infertility, unexplained infertility, cervical mucus hostility and ovulatory disturbances. With overall success rates varying widely at between 5% and 66% per cycle (Allen et al., 1985), the degree of DNA fragmentation as a predictor of IUI success was investigated using the TUNEL assay. One study found the degree of DNA fragmentation to be significantly lower in samples that resulted in pregnancy than in those that did not (7.3 ± 3.5 versus 13.9 ± 10.8, P < 0.044). In addition, no woman inseminated with a sample having >12% sperm with fragmented DNA achieved a pregnancy. Furthermore, patients who were inseminated with samples containing the highest degrees of DNA damage (10–12%) experienced miscarriages (Duran et al., 2002).

**IVF**

Traditionally, in IVF treatment cycles, poor embryo quality is regarded as an oocyte-related problem. In contrast to embryo quality, which is a good indicator of successful pregnancy outcome, standard semen parameters have proved disappointing at predicting the outcome of IVF treatment cycles (Tomlinson et al., 2002). In this respect, any additional sperm parameter would be of great importance, since the influence of the paternal gametes affects embryo development until the blastocyst stage (Shoukir et al., 1998).

Sperm DNA damage was reported to have a significant negative correlation with embryo quality and hence successful establishment of pregnancy following IVF cycles. This can be potentially useful as a prognostic test in couples about to embark on IVF treatment (Tomlinson et al., 2001).

**ICSI**

During the course of ICSI, the sperm cell is injected directly into the cytoplasm of the mature oocyte. The classical sperm parameters or the sperm membrane–oocyte interaction are no longer relevant and therefore, an evaluation of sperm DNA integrity is most important in these cases. Even though during ICSI, damage to sperm DNA does not preclude fertilization and pronucleus formation (Twigg et al., 1998c), several authors have reported significant correlations between sperm DNA damage and fertilization as well as pregnancy rates following ICSI (Hammadeh et al., 1996; Lopes et al., 1998b; Larson et al., 2000). Many studies indicate that correct chromatin packaging around the protamine core seems to be a necessary condition for optimal expression of the male gamete fertility potential. However, this condition does not seem mandatory for a successful fertilization as demonstrated.
By ICSI, where normal fertilization and pregnancy rates can be achieved with cells that have not completed spermiogenesis, such as epididymal and testicular spermatozoa (Silber et al., 1995).

Although the ICSI procedure uses the most normal-appearing and motile spermatozoa, the quality of the semen sample from which the sperm is chosen must be taken into consideration. In general, the fertilization rate in ICSI does not exceed 65% in most clinics, despite the mechanical injection of one spermatozoon into a mature oocyte (Palermo et al., 1995). A possible explanation for this lower than expected fertilization rate could be that sperm selected from semen of patients with male factor infertility may have defects in their DNA. Such abnormalities as loosely packaged chromatin and damaged DNA have already been observed in poor-quality semen samples (Foresta et al., 1992). However, it should be pointed out that studies performed to evaluate the influence of chromatin structure defects on the sperm-fertilizing capabilities and/or embryo development have been tested in the context of ARTs and, therefore, evaluated mainly in subjects with serious infertility problems. Thus, when poor-quality semen samples are used for ICSI, there is a greater likelihood that some sperm selected for injection, despite appearing normal, contain fragmented DNA (Esterhuizen et al., 2002).

SCSA parameters predicted a zero implantation rate and no pregnancy (confirmed by ultrasound) following ART in a group of 89 couples undergoing conventional IVF or ICSI. No patients achieved a clinical pregnancy confirmed by ultrasound if SCSA values exceeded the total DFI (27%, \( P < 0.01 \)), moderate DFI (15%, \( P < 0.01 \)) or high DFI (15%, \( P < 0.05 \)) thresholds. Total, moderate and high DFI thresholds had 100% specificity and 100% positive predictive values for failure to initiate an ongoing pregnancy (unpublished observation).

**TESE**

Sperm which are surgically extracted from the epididymis or testicular tissue, and are frequently used in ICSI trials in cases of obstructive azoospermia, usually reveal a significantly high percentage of DNA breaks. The breaks may be a result of the prolonged stay of the spermatozoa in the obstructed genital tract. It is also possible that DNA decondensation of the chromatin may be incomplete in non-ejaculated spermatozoa, implying that these cells are sensitive to damaging or toxic agents (Ramos et al., 2002). In an attempt to detect the effects of obstruction and stasis on the DNA of these spermatozoa using the comet assay, it was reported that the percentage of undamaged DNA in testicular spermatozoa of men with obstructive azoospermia was significantly better than in proximal epididymal spermatozoa. Thus, the use of testicular spermatozoa should be preferred for ICSI to treat men with obstructive azoospermia (Steele et al., 1999).

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### Table III. Correlation between sperm DNA damage and the outcome of various assisted reproductive techniques

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study population (n)a</th>
<th>ART procedure</th>
<th>Technique</th>
<th>Parameter</th>
<th>Outcomeb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fertilization rate</td>
</tr>
<tr>
<td>Sun et al. (1997)</td>
<td>Semen samples (298)</td>
<td>IVF</td>
<td>TUNEL</td>
<td>DF</td>
<td>–0.16 (0.05)</td>
</tr>
<tr>
<td>Lopes et al. (1998b)</td>
<td>150</td>
<td>ICSI</td>
<td>TUNEL</td>
<td>DF</td>
<td>–0.23 (0.0117)</td>
</tr>
<tr>
<td>Larson et al. (2000)</td>
<td>24</td>
<td>ICSI</td>
<td>SCSA</td>
<td>DFI</td>
<td>15.4 ± 4.6 vs. 31.3 ± 3.2; ( P = 0.001^c ); no pregnancies if &gt;27%</td>
</tr>
<tr>
<td>Host et al. (2000)</td>
<td>Oligo (50)</td>
<td>IVF</td>
<td>TUNEL</td>
<td>DF</td>
<td>–0.61 (&lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td>Oligo (50)</td>
<td>ICSI</td>
<td>SCSA</td>
<td>DFI</td>
<td>2.0 ± 0.3 vs. 4.0 ± 0.7b; ( P = 0.02 )</td>
</tr>
<tr>
<td>Tomlinson et al. (2001)</td>
<td>140</td>
<td>IVF</td>
<td>NT</td>
<td>DF</td>
<td>0.567 (&lt; 0.05)</td>
</tr>
<tr>
<td>Raman et al. (2001)</td>
<td>15</td>
<td>IVF</td>
<td>Comet</td>
<td>DF</td>
<td>7.3 ± 3.5 vs. 13.9 ± 10.8b; ( P = 0.044 ), no pregnancies if &gt;12%</td>
</tr>
<tr>
<td>Duran et al. (2002)</td>
<td>119</td>
<td>IUI</td>
<td>TUNEL</td>
<td>DF</td>
<td>–</td>
</tr>
<tr>
<td>Tomsu et al. (2002)</td>
<td>40</td>
<td>IVF</td>
<td>Comet</td>
<td>DF</td>
<td>–</td>
</tr>
<tr>
<td>Morris et al. (2002)</td>
<td>60</td>
<td>ICSI</td>
<td>Comet</td>
<td>DF</td>
<td>–</td>
</tr>
<tr>
<td>Saleh et al. (2003)</td>
<td>33</td>
<td>IUI (19); IVF (10); ICSI (4)</td>
<td>SCSA</td>
<td>DFI</td>
<td>–0.70 (0.03)</td>
</tr>
</tbody>
</table>

aUnless stated otherwise, indicates number of male patients included in the ART programme.
bUnless stated otherwise, results are expressed as \( r \) = correlation coefficient, \( P \)-values in parentheses.
cPregnant group compared with non-pregnant group.

ART = assisted reproduction technique; DF = DNA fragmentation; DFI = DNA fragmentation index; ICSI = intracytoplasmic injection; IUI = intrauterine insemination.
Embryo quality

Associations between increased DNA fragmentation and decreased embryo cleavage have been reported after IVF and ICSI (Sakkas et al., 1998). The histone-associated DNA in the male pronucleus is active even during early embryonic development (Gardiner-Garden et al., 1998). Several studies have attempted to establish a correlation between sperm DNA integrity and cleavage rates and embryo quality (Morris et al., 2002; Tomsu et al., 2002). The results of a recent study showed a significant increase in the levels of sperm DNA damage in infertile men who failed to initiate a clinical pregnancy with ART compared with those who succeeded; the median and interquartile ranges (25%, 75%) were 38 (28, 43) and 21 (13, 25) respectively; \( P = 0.001 \). Also, sperm DNA damage showed a negative correlation with embryo quality following IVF and ICSI \( (r = -0.70; P = 0.03) \) (Saleh et al., 2003). However, whether DNA-damaged spermatozoa can impair the process of embryo development remains unclear. Nonetheless, it has been reported that damage to sperm DNA may be linked to an increase in early embryo death (Sakkas et al., 1999a).

Effect of DNA-damaged spermatozoa on the offspring

The variations in the highly defined nuclear architecture of sperm chromatin might influence the initiation and regulation of paternal gene activity in embryo development (Haaf and Ward, 1995).

Infertility

One possible consequence of OS-mediated sperm DNA damage is infertility in the offspring (Aitken, 1999). This possibility relates specifically to forms of male infertility involving deletions on the long arm \( (q) \) of the Y chromosome, and is based on the fact that the Y chromosome contains a high number of repetitive DNAs which are the targets for homologous intrachromosomal recombination. This mechanism can lead to sequence deletions (Kuroda-Kawaguchi et al., 2001).

In the non-recombining area of the Y chromosome (NYR), three regions have been identified that contain genes of importance to spermatogenesis; these loci have been designated as azoospermia factors (AZF) a, b, and c (Roberts, 1998). Deletions in each of these areas produce a particular testicular phenotype: deletions in AZFa produce Sertoli cell-only syndrome; AZFb deletions are associated with germ cell arrest at the pachytene stage; and deletions in AZFc cause arrest at the spermatid stage of development (Vogt et al., 1992). These deletions are not observed in fertile men or in most fathers of affected patients; therefore, the Y-chromosome deletions leading to male infertility arise de novo in the germ line of the patient’s father (Cooke, 1999).

Paternal smoking

Because cigarette smoke causes oxidative DNA damage in sperm due to its high content of oxidants and its depletion of plasma and tissue antioxidants, tobacco smoking may lead to mutations in sperm and subsequently cancer, birth defects and genetic diseases in the offspring (Fraga et al., 1996).

Repair and prevention of sperm DNA damage

Oocyte remodelling of sperm chromatin structure

Within the fertilized oocyte, sperm DNA damage can be repaired during the period between sperm entry into the cytoplasm and the beginning of the next S phase, by virtue of pre- and post-replication mechanisms (Matsuda and Tobari, 1989; Genesca et al., 1992). Consequently, the biological impact of abnormal sperm chromatin structure depends on the combined effects of the level of chromatin damage in the spermatozoa and the capacity of the oocyte to repair that pre-existing damage. However, if spermatozoa are selected from samples with extensively damaged DNA and are used in ARTs such as ICSI or IVF, the oocyte’s repair capacities might be inadequate, leading to fragmentation and a low rate of embryonic development that results in a high rate of early pregnancy loss (Ahmadi and Ng, 1999a; b).

Transition proteins

The mammalian transition proteins (TPs) are expressed at a high level at mid-spermiogenesis steps coinciding with chromatin remodelling, and are involved in the repair of DNA single-strand breaks (SSB). TP1 can stimulate the repair of SSB in vitro, as well as the in-vivo repair of UV-induced DNA lesions. It has been suggested that the TP1 proteins can participate in the repair process following genotoxic insults, and therefore they may play an active role in maintenance of the integrity of the male haploid genome during spermiogenesis (Caron et al., 2002). In order to investigate the role of different genes controlling the repair mechanisms, knockout mouse models for TPs and the protamines were used. This has confirmed the contribution of these basic proteins to the DNA repair mechanisms (Boissonneault, 2002).

DNA repair enzymes also play a crucial repairing role during meiotic recombination. Defects in two members of the MutS family, namely Pms2 and Msh2, led to the failure of this repair mechanism. The patterns of expression for these genes encoding mismatch repair enzymes are consistent with the proposed roles of the gene products in mismatch repair during both DNA replication and recombination (Richardson et al., 2000).

Sperm preparation techniques

Ejaculation in sperm wash medium has been used to increase the proportion of antibody-free spermatozoa in semen samples containing anti-sperm antibodies and thereby enhance the fertilization rate in vitro (Elder et al., 1990). This method has also proven beneficial in semen samples with an increased percentage of DNA-damaged spermatozoa. Hence, it was postulated that the addition of medium before liquefaction could inhibit the binding of bacteria and detritus to the sperm surface and subsequently diminish DNA damage caused by
ROS, allowing improved fertilization efficiency (Zollner et al., 2001).

Sperm preparation techniques may positively affect the recovery of a selected healthy population of cells. The DNA integrity of prepared spermatozoa is always significantly higher than that of raw semen (Donnelly et al., 2000). Semen samples after simple preparation techniques such as density gradient centrifugation can be enriched with morphologically normal spermatozoa and spermatozoa with improved nuclear integrity (Colleu et al., 1996; Golan et al., 1997). This normalizing effect of density gradient may be the reason why sperm parameters prior to processing have little prognostic value in terms of fertilization and pregnancy using IVF (Tomlinson et al., 2001). This beneficial effect coincides with the relatively high post-IVF fertilization rate (76 ± 5.3%) after use of a simple swim-up technique (Younglai et al., 2001).

Several sperm preparation techniques have been subjected to an evaluation regarding their ability to improve the DNA integrity of a sperm population. In one study using discontinuous Percoll density gradient (95.0 and 47.5%), the DNA integrity of a sperm population. In one study using discontinuous Percoll density gradient (95.0 and 47.5%), the DNA integrity of prepared spermatozoa was significantly higher than that of raw semen (P < 0.005) (Donnelly et al., 1999). Glass wool filtration also significantly decreased the %DFI when compared with raw semen samples (12.7 ± 9.5 and 19.1 ± 10.4 respectively, P < 0.01) (Larson et al., 1999). Similarly, sperm specimens prepared by Percoll or PureSperm density gradients protocols resulted in a significant decrease in the percentage of sperm DNA fragmentation as shown by the by TUNEL assay (P < 0.001) (Sakkas et al., 2000).

When the effects of two methods of sperm preparation—density gradient centrifugation and swim-up technique—on sperm DNA integrity were compared, the results showed the latter approach to result in better DNA integrity (Zini et al., 2000). These data, however, urge re-examination of the different types of sperm-processing techniques in order to minimize sperm DNA damage.

**In-vitro culture**

In-vitro culture for 48–72 h at 37°C has been reported to improve the motility and post-thaw recovery rate of testicular spermatozoa (Molina et al., 1995; Emiliani et al., 2000). However, there are many sources of ROS that may lead to DNA damage when spermatozoa are cultured in vitro, including leukocytes, abnormal spermatozoa, transition metals present in the culture medium and the preparation technique itself. Nevertheless, evidence was presented that the in-vitro culture of testicular spermatozoa does not increase their susceptibility to DNA damage. For patients with obstructive azoospermia, the proportion of spermatozoa containing single-stranded DNA damage decreased significantly after 3 days of culture (P = 0.005) (Emiliani et al., 2001). The disintegration of single-strand DNA-damaged spermatozoa and the parallel development of immature double-strand DNA spermatozids may provide an explanation for this phenomenon. Similarly, another study revealed that testicular immature germ cell culture for 48 h facilitated the selection of TUNEL-negative spermatids (Tesarik et al., 1999).

**Antioxidants**

Because ROS generation is a major source of sperm DNA damage, antioxidants may protect sperm DNA. When added in vitro, ascorbic acid (600 μmol/l), alpha-tocopherol (30 and 60 μmol/l) and urate (400 μmol/l) have each been reported to provide significant protection (P < 0.001) from subsequent DNA damage by X-irradiation. Thus, supplementation in vitro with these antioxidants separately can beneficially affect the sperm DNA integrity (Hughes et al., 1998).

Isoflavones (genistein and equol) are plant compounds that supposedly have health benefits in a variety of human diseases, including coronary heart disease and endocrine-responsive cancers. Their physiological effects include antioxidant activity, and therefore a role is suggested for them in the prevention of male infertility. Compared with ascorbic acid and alpha-tocopherol, genistein was the most potent antioxidant, followed by equol, ascorbic acid and alpha-tocopherol when added at physiological concentrations. Genistein and equol, when added in combination, were more protective than when added singly. Based on these preliminary data, these compounds may play a role in antioxidant protection against sperm DNA damage (Sierens et al., 2002).

**Conclusions**

In summary, sperm chromatin is a very complex structure, and its capability to decondense is one of the essential criteria for considering a spermatozoon to be fertile. DNA integrity in sperm is essential for the accurate transmission of genetic information and, in turn, the maintenance of good health in future generations. It is an independent measure of sperm quality that provides better diagnostic and prognostic capabilities than standard sperm parameters for male fertility potential. Numerous studies have reported a negative correlation of in-vivo and in-vitro fecundity with the percentage of DNA-damaged spermatozoa in semen samples. Several methods are currently used to assess DNA damage. However, the establishment of a cut-off point between normal levels in the average fertile population and the minimal levels of sperm DNA integrity required to achieve pregnancy using these different assays is still lacking, except for SCSA.

**References**


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