Human Reproduction Update, Vol.9, No.4 pp. 331–345, 2003 DOI: 10.1093/humupd/dmg027

Role of sperm chromatin abnormalities and DNA damage in male infertility

A.Agarwal[1] and Tamer M.Said

Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Glickman Urological Institute, The Cleveland Clinic Foundation, Cleveland, Ohio, USA

[1]To whom correspondence should be addressed at: Center for Advanced Research in Human Reproduction, Infertility and Sexual Function, Glickman Urological Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Desk A19.1, Cleveland, OH 44195, USA. E-mail: agarwaa@ccf.org

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, varicocele, 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

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 (Poccia, 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 helixes 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 μm^3, 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 Lanneau, 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 intra-protamine 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 (Sailer *et al*., 1995). Alternatively, it could be the result of free radical-induced damage (Aitken *et al*., 1998) or a consequence of apoptosis (Gorcyza *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 Korsemyer, 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.

In-situ nick translation (NT) assay

The NT assay quantifies the incorporation of biotinylated-deoxyuridine triphosphate (dUTP) at single-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).

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) (Gorcyza *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.

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 *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).

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 Kapuscinski, 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).

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).

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).

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.

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 $\pm$ 10.9 versus 24.9 $\pm$ 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).

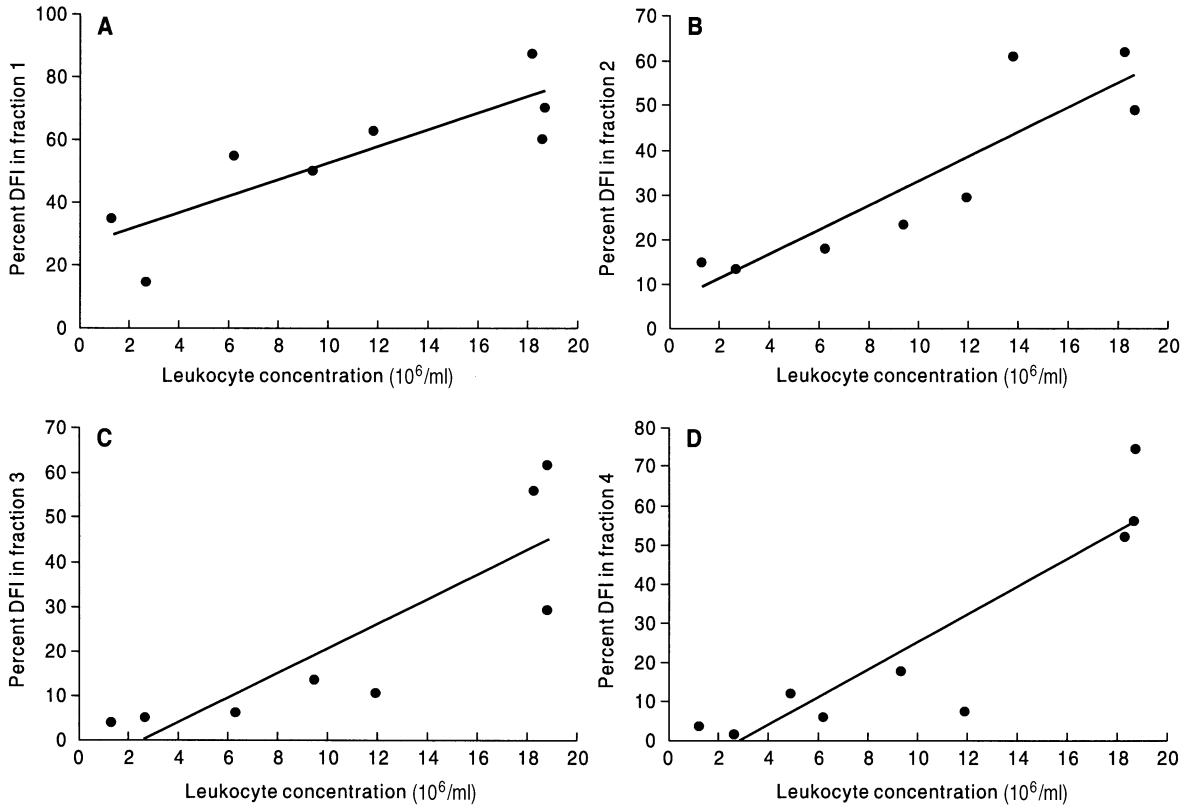

Figure 1. Correlation between leukocyte concentration in semen and % DNA fragmentation index (DFI) values in sperm from the different fractions resulting from 47, 70 and 90% density gradient centrifugation. (A) Fraction 1 ($r^2 = 0.71$; $P = 0.009$). (B) Fraction 2 ($r^2 = 0.79$; $P < 0.0001$). (C) Fraction 3 ($r^2 = 0.73$; $P = 0.007$). (D) Fraction 4 ($r^2 = 0.82$; $P = 0.002$)

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 ± 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

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 $\pm$ 6, 76 $\pm$ 11 and 92 $\pm$ 6% respectively) compared with fresh sperm (19 $\pm$ 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 $\pm$ 2.0 versus 10.7 $\pm$ 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 *Echinacae purpura* 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

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

Reference	Study population		Technique	Parameter	Results			
	Donors (*n*)	Infertile patients (*n*)			Donors	Infertile patients	*P*	
Hughes *et al*. (1996)	20	Norm (20) Oligo (20)	Comet	DF	81.7 ± 24.5	79.9 ± 42.3 85.1 ± 28.6	NS NS	
Hughes *et al*. (1996)	20	Norm (20) Oligo (20)	Comet; after H_2O_2 exposure	DF	81.7 ± 24.5	56.1 ± 68.3 45.0 ± 36.7	< 0.05 < 0.05	
Kodama *et al*. (1997)	17	19	HPLC	8-OhdG	1.5 ± 0.2	1.0 ± 0.1	< 0.05	
Shen *et al*. (1999)	54	60	HPLC	8-OhdG	10.03	4.79	< 0.001	
Spano *et al*. (2000)	215 males with no previous knowledge of fertility status		SCSA	DFI		Fecundability dropped if >20%; no pregnancy occurred when >40%		
Gandini *et al*. (2000)	23	OAT (29) Hodgkin's (28) TC (30)	TUNEL	DF	2.5 ± 1.2	11.0 ± 4.3 11.3 ± 4.9 11.3 ± 4.4	< 0.001	
Zini *et al*. (2001)	7	33	AO TUNEL	DD DF	25.4 ± 3.0 13.3 ± 2.5	10.2 ± 2.3 27.6 ± 2.5	0.028 0.016	DD and DF: (r = 0.71; P < 0.0001)
Zini *et al*. (2002)		Norm (13) Non-azo (75)	AO	DD		11.1 ± 3.7 23.1 ± 1.8	< 0.001	
Saleh *et al*. (2002b)	16	Norm (21) Non-azo (71)	SCSA	DFI	15 (11,20)	23 (15,32) 28 (18,41)	0.002[a] 0.27[a]	

[a]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.

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 H_2O_2 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 II. Correlation between sperm DNA damage and sperm characteristics

Reference	Study population		Technique	Parameter	Results[a]			
	Donors (*n*)	Patients (*n*)			Concentration	Morphology	Motility	Fertilization capacity[b]
Kodama *et al* (1997)	17	19	HPLC	8-OhdG	–0.49 (0.001)	–	–	–
Shen *et al*. (1999)	54	60	HPLC	8-OhdG	–0.42 (< 0.001)	0.38 (< 0.001)	–0.24 (< 0.01)	–
Irvine *et al*. (2000)	12	29	Comet	DF	–0.54 (0.001)	–0.37 (0.026)	–0.37 (0.026)	
			NT	DF	–0.66 (< 0.0001)	–0.38 (0.016)	–0.38 (0.016)	
Chan *et al*. (2001)		39	Comet	DF	–	–	–	0.493 (< 0.05)
Tomlinson *et al*. (2001)		140	NT	DF	–0.24 (0.01)	–	–0.20 (0.004)	
Saleh *et al*. (2003)	16	92	SCSA	DFI	–0.31 (0.001)	–0.040 (< 0.0001)	–0.47 (< 0.0001)	

[a]Results expressed as r = correlation coefficient, with P-value in parentheses.
[b]Measured by the zona-free hamster oocyte penetration assay.
DF = DNA fragmentation; HPLC = high-performance liquid chromatography; NT = in-situ nick translation; 8-OhdG = 8-hydroxy-2-deoxyguanosine.

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 (Tomsu *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

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

Reference	Study population (n)[a]	ART procedure	Technique	Parameter	Outcome[b]		
					Fertilization rate	Embryo cleavage rate	Pregnancy
Sun *et al*. (1997)	Semen samples (298)	IVF	TUNEL	DF	–0.16 (0.05)	–0.20 (0.02)	–
Lopes *et al*. (1998b)	150	ICSI	TUNEL	DF	–0.23 (0.0117)	No correlation	
Larson *et al*. (2000)	24	ICSI	SCSA	DFI	–	–	15.4 ± 4.6 vs. 31.3 ± 3.2; $P = 0.001$[c]; no pregnancies if >27%
Host *et al*. (2000)	Oligo (50)	IVF	TUNEL	DF	–0.61 (< 0.01)	–	–
	Oligo (50)	ICSI			0.06 (> 0.05)	–	–
Tomlinson *et al*. (2001)	140	IVF	NT	DF	–	–0.20 (0.004)	2.0 ± 0.3 vs. 4.0 ± 0.7[b]; $P = 0.02$
Raman *et al*. (2001)	15	IVF	Comet	DF	0.567 (< 0.05)	–	–
Duran *et al*. (2002)	119	IUI	TUNEL	DF			7.3 ± 3.5 vs. 13.9 ± 10.8[b]; P = 0.044, no pregnancies if >12%
Tomsu *et al*. (2002)	40	IVF	Comet	DF	–	–0.567 (<0.044)	–
Morris *et al*. (2002)	60	ICSI	Comet	DF	–	–12.77 (0.003) with 100% embryo cleavage rate	–
Saleh *et al*. (2003)	33	IUI (19); IVF (10); ICSI (4)	SCSA	DFI	–0.70 (0.03)	–0.70 (0.03)	$P < 0.0001$

[a]Unless stated otherwise, indicates number of male patients included in the ART programme.
[b]Unless stated otherwise, results are expressed as r = correlation coefficient, P-values in parentheses.
[c]Pregnant group compared with non-pregnant group.
ART = assisted reproduction technique; DF = DNA fragmentation; DFI = DNA fragmentation index; ICSI = intracytoplasmic injection; IUI = intrauterine insemination.

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).

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 (NRY), 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 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 spermatids 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

Agarwal, A. and Newton, R.A. (1991) The effect of cancer on semen quality after cryopreservation of sperm. *Andrologia*, **23**, 329–332.

Agarwal, A., Shekarriz, M., Sidhu, R.K. and Thomas, A.J., Jr (1996) Value of clinical diagnosis in predicting the quality of cryopreserved sperm from cancer patients. *J. Urol.*, **155**, 934–938.

Ahmadi, A. and Ng, S.C. (1999a) Developmental capacity of damaged spermatozoa. *Hum. Reprod.*, **14**, 2279–2285.

Ahmadi, A. and Ng, S.C. (1999b) Fertilizing ability of DNA-damaged spermatozoa. *J. Exp. Zool.*, **284**, 696–704.

Aitken, R.J. (1999) The Amoroso lecture. The human spermatozoa – a cell in crisis. *J. Reprod. Fertil.*, **115**, 1–7.

Aitken, R.J. and Krausz, C. (2001) Oxidative stress, DNA damage and the Y chromosome. *Reproduction*, **122**, 497–506.

Aitken, R.J., Buckingham, D., Brindle, J., Gomez, E., Baker, G. and Irvine, S. (1995) Analysis of sperm movement in relation to the oxidative stress

created by leukocytes in washed sperm preparations and seminal plasma. *Hum. Reprod.*, **10**, 2061–2071.

Aitken, R.J., Gordon, E. and Harkiss, D. (1998) Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol. Reprod.*, **59**, 1037–1046.

Allen, N.C., Herbert, C.M., III, Maxson, W.S, Rogers, B.J., Diamond, M.P. and Wentz, A.C. (1985) Intrauterine insemination: a critical review. *Fertil. Steril.*, **44**, 569–580.

Alvarez, J.G., Sharma, R.K., Ollero, M., Saleh, R., Lopez, M., Thomas, A.J., Jr, Evenson, D.P. and Agarwal, A. (2002) Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay. *Fertil. Steril.*, **78**, 319–329.

Amann, R.P. (1989) Can fertility potential of a seminal sample be predicted accurately? *J. Androl.*, **16**, 89–98.

Ames, B.N., Shigenaga, M.K. and Hagen, T.M. (1993) Oxidants, antioxidants and the degenerative disease and aging. *Proc. Natl Acad. Sci. USA*, **90**, 7915–7922.

Arnon, J., Meirow, D., Lewis-Roness, H. and Ornoy, A. (2001) Genetic and teratogenic effects of cancer treatments on gametes and embryos. *Hum. Reprod. Update*, **7**, 394–403.

Barone, J.G., De Lara, J., Cummings, K.B. and Ward, W.S. (1994) DNA organization in human spermatozoa. *J. Androl.*, **15**, 139–144.

Barroso, G., Morshedi, M. and Oehringer, S. (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum. Reprod.*, **15**, 1338–1344.

Berensztein, E.B., Sciara, M.I., Rivarola, M.A. and Belgorosky, A. (2002) Apoptosis and proliferation of human testicular somatic and germ cells during prepuberty: high rate of testicular growth in newborns mediated by decreased apoptosis. *Clin. Endocrinol. Metab.*, **87**, 5113–5118.

Boissonneault, G. (2002) Chromatin remodeling during spermiogenesis: a possible role for the transition proteins in DNA strand break repair. *FEBS*, **514**, 111–114.

Bucci, L.R. and Meistrich, M.L. (1987) Effects of busulfan on murine spermatogenesis: cytotoxicity, sterility, sperm abnormalities, and dominant lethal mutations. *Mutat. Res.*, **176**, 259–268.

Cai, L., Hales, B.F. and Robaire, B. (1997) Induction of apoptosis on the germ cells of adult male rats after exposure to cyclophosphamide. *Biol. Reprod.*, **56**, 1490–1497.

Caron, N., Veilleux, S. and Biossonneault, G. (2002) Stimulation of DNA repair by the spermatozoal TP1 protein. *Mol. Reprod. Dev.*, **58**, 437–443.

Chan, J.P., Corselli, J.U., Patton, W.C., Jacobson, J.D., Chan, S.R. and King, A. (2001) A simple comet assay for archived sperm correlates DNA fragmentation to reduced hyperactivation and penetration of zona-free hamster oocyte. *Fertil. Steril.*, **75**, 186–192.

Chapman, R.M., Sutcliffe, S.B., Rees, L.H., Edwards, C.R. and Malpas, J.S. (1979) Cyclical combination chemotherapy and gonadal function. Retrospective study in males. *Lancet*, **1**, 285–289.

Chatterjee, R., Haines, G.A., Perera, D.M., Goldstone, A. and Morris, I.D. (2000) Testicular and sperm DNA damage after treatment with fludarabine for chronic lymphocytic leukemia. *Hum. Reprod.*, **15**, 762–766.

Cohen, P.E. and Pollard, J.W. (1995) Cytokines and growth factors in reproduction. In Bronson, R. (ed.), *Reproductive Immunology*. Blackwell Science, Cambridge, MA.

Colleu, D., Lescoat, D. and Gouranton, J. (1996) Nuclear maturity of human spermatozoa selected by swim-up or Percoll gradient centrifugation procedures. *Fertil. Steril.*, **65**, 160–164.

Comhaire, F.H., Mahmoud, A.M., Depuydt, C.E., Zalata, A.A. and Christofe, A.B. (1999) Mechanism and effects of male genital tract infection on sperm quality and fertilizing potential: the andrologist's view point. *Hum. Reprod. Update*, **5**, 393–398.

Cooke, H.J. (1999) Y chromosome and male infertility. *Rev. Reprod.*, **4**, 5–10

Donnelly, E.T., McClure, N. and Lewis, S.E. (1999) The effects of ascorbate and alpha-tocopherol supplementation *in vitro* on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis*, **14**, 505–512.

Donnelly, E.T., O'Connell, M., McClure, N. and Lewis, S.E. (2000) Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. *Hum. Reprod.*, **15**, 1552–1561.

Donnelly, E.T., Steele, E.K., McClure, N. and Lewis, S.E. (2001a) Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. *Hum. Reprod.*, **16**, 1191–1199.

Donnelly, E.T., McClure, N. and Lewis, S.E. (2001b) Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. *Fertil. Steril.*, **76**, 892–900.

Drazynkiewicz, Z. and Kapuscinski, J. (1990) Acridine orange: a versatile probe of nucleic acids and other cell constituents. In Melamed, M.R., Lindmo, T. and Mendelsohn, M.L. (eds), *Flow Cytometry and Sorting*. 2nd edition, Wiley-Liss, New York, pp. 291–314.

Drazynkiewicz, Z., Traganos, F., Sharpless, T. and Melamed, M.R. (1975) Thermal denaturation of DNA *in situ* as studied by acridine orange staining and automated cytofluorometry. *Exp. Cell Res.*, **90**, 411–428.

Drazynkiewicz, Z., Traganos, F., Sharpless, T. and Melamed, M.R. (1976) Lymphocyte stimulation: a rapid multiparameter analysis. *Proc. Natl Acad. Sci. USA*, **73**, 2881–2884.

Duran, E.H., Gurgan, T., Gunalp, S., Enginsu, M.E., Yarali, H. and Ayhan, A. (1998) A logistic regression model including DNA status and morphology of spermatozoa for prediction of fertilization *in vitro*. *Hum. Reprod.*, **13**, 1235–1239.

Duran, E.H., Morshedi, M., Taylor, S. and Oehninger, S. (2002) Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. *Hum. Reprod.*, **12**, 3122–3128.

Duru, N.K., Morshedi, M. and Oehninger, S. (2000) Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. *Fertil. Steril.*,**74**, 1200–1207.

Duty, S.M., Singh, N.P., Ryan, L., Chen, Z., Lewis, C., Hunag, T. and Hauser, R. (2002) Reliability of the comet assay in cryopreserved human sperm. *Hum. Reprod.*, **17**, 1274–1280.

Elder, K.T., Wick, K.L. and Edwards, R.G. (1990) Seminal plasma antisperm antibodies and IVF: the effect of semen sample collection into 50% serum. *Hum. Reprod.*, **5**, 179–184.

Emiliani, S., Van den Bergh, M., Vannin, A.S., Biramane, J., Verdoodt, M. and Englert, Y. (2000) Increased spermatozoa motility after *in vitro* culture of testicular biopsies from obstructive azoospermic patients results in better post thaw recovery rate. *Hum. Reprod.*, **15**, 2371–2374.

Emiliani, S., Van den Bergh, M., Vannin, A.S., Biramane, J., Verdoodt, M. and Englert Y. (2001) Evidence of reduced single-stranded testicular sperm DNA from obstructive azoospermic men after 3 days of *in vitro* culture. *Hum. Reprod.* **16**, 1200–1203.

Esterhuizen, A.D., Franken, D.R., Becker, P.J., Lourens, J.G.H., Muller, I.I. and Van Rooyen, L.H. (2002) Defective sperm decondensation: a cause for fertilization failure. *Andrologia*, **34**, 1–7.

Evenson, D.P., Drazynkiewicz, Z. and Melamed, M.R. (1980) Relation of mammalian sperm chromatin heterogeneity to fertility. *Science*, **210**, 1131–1133.

Evenson, D.P., Baer, R.K., Turner, T. and Schrader, S. (1991) Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. *Reprod. Toxicol.*, **5**, 115–125.

Evenson, D.P., Jost, L.K., Marshall, D., Zinaman, M.J., Clegg, E., Purvis, K., de Angelis, P. and Claussen, O.P. (1999) Utility of sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum. Reprod.*, **14**, 1039–1049

Evenson, D.P., Jost, L.K., Corzett, M. and Balhorn, R. (2000) Characteristics of human sperm chromatin structure following an episode of influenza and high fever: a case study. *J. Androl.*, **21**, 739–746.

Evenson, D.P., Larson, K.L. and Jost, L.K. (2002) Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with the other techniques. *J. Androl.*, **23**, 25–43.

Fernandez, J.L. and Gozalvez, J. (2002) Application of FISH to detect DNA damage: DNA breakage detection-FISH (DBD-FISH). *Methods Mol. Biol.*, **203**, 203–216.

Fernandez, J.L., Mouriel, L., Rivero, M.T., Goyanes, V., Vasquez, R. and Alvarez, J.L. (2003) The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. *J. Androl.*, **24**, 59–66.

Finch, J.T. and Klug, A. (1976) Solenoid model superstructure in chromatin. *Proc. Natl Acad. Sci. USA*, **73**, 1897–1901.

Floyd, R.A., Watson, J.J., Harris, J., West, M. and Wong, P.K. (1986) Formation of 8-hydroxy-2-deoxyguanosine, hydroxyl free radical adduct of DNA in granulocytes exposed to the tumor promoter. *Biochem. Biophys. Res. Commun.*, **137**, 841–846.

Foresta, C., Zorzi, M., Rossato, M. and Varotto, A. (1992) Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in infertile men. *Int. J. Androl.*, **15**, 330–337.

Fraga, C.G., Motchnik, P.A., Wyrobek, A.J., Rempel, D.M. and Ames, B.N. (1996) Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat. Res.*, **351**, 199–203.

Francavilla, S., D'abrizio, P., Rucci, N., Silvano, G., Properzi, G., Straface, E., Cordeschi, G., Necozione, S., Gnessi, L., Arizzi, M. and Ulisse, S. (2000) Fas and Fas ligand expression in fetal and adult human testis with normal or deranged spermatogenesis. *J. Clin. Endocrinol. Metab.*, **85**, 2692–2700.

Fuentes-Mascorro, G., Serrano, H. and Rosado, A. (2000) Sperm chromatin. *Arch. Androl.*, **45**, 215–225.

Gandini, L., Lombardo, F., Paoli, D., Caponechia, L., Familiari, G., Verlengia, C., Dondero, F. and Lenzi, A. (2000) Study of apoptotic DNA fragmentation in human spermatozoa. *Hum. Reprod.*, **15**, 830–839.

Gardiner-Garden, M., Ballesteros, M., Gordon, M. and Tam, P.P. (1998) Histone and protamine-DNA association: conservation of different patterns within the beta-globin domain in human sperm. *Mol. Cell. Biol.*, **18**, 3350–3356.

Genesca, A., Caballin, M.R., Miro, R., Benet, J., Germ, J.R. and Egoscue, J. (1992) Repair of human sperm chromosome aberrations in the hamster egg. *Hum. Genet.*, **89**, 181–186.

Golan, R., Shochat, L., Weissenberg, R., Soffer, Y., Marcus, Z., Oschry, Y. and Lewin, L.M. (1997) Evaluation of chromatin condensation in human spermatozoa: a flow cytometric assay using acridine orange staining. *Mol. Hum. Reprod.*, **3**, 47–54.

Golden, A.L., Moline, J.M. and Bar-Chama, N. (1999) Male reproduction and environmental and occupational exposures: a review of epidemiologic methods. *Salud. Publica. Mex.*, **41** (Suppl 2), S93–S105.

Gorczya, W., Traganos, F. and Jesionowska, H. (1993a) Presence of DNA strand breaks and increased sensitivity of DNA *in situ* to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. *Exp. Cell Res.*, **207**, 202–205.

Gorcyza, W., Gong, J. and Drazynkiewicz, Z. (1993b) Detection of DNA strand breaks in individual apoptotic cells by the *in situ* terminal deoxynucleotidyl transferase and nick translation assay. *Cancer Res.*, **53**, 1945–1951.

Haaf, T. and Ward, D.C. (1995) Higher order nuclear structure in mammalian sperm revealed by *in situ* hybridization and extended chromatin fibers. *Exp. Cell Res.*, **219**, 604–611.

Hallak, J., Kolettis, P.N., Sekhon, V.S., Thomas, A.J., Jr and Agarwal, A. (1999) Sperm cryopreservation in testicular cancer. *Urology*, **54**, 894–899.

Hammadeh, M.E., Al-Hassani, S., Stieber, M., Gauss, C., Rosenbaum, P., Georg, T., Diedrich, K. and Schmidt, W. (1996) The effect of chromatin condensation (Aniline Blue staining) and morphology (strict criteria) of human spermatozoa on fertilization, cleavage and pregnancy rates in an intracytoplasmic programme. *Hum. Reprod.*, **13**, 2468–2471.

Hofman, N. and Hilscher, B. (1991) Use of aniline blue to assess chromatin condensation in morphologically normal spermatozoa in normal and infertile men. *Hum. Reprod.*, **6**, 979–982.

Holmes, R.P., Goodman, H.O., Shihabi, Z.K. and Jarow, J.P. (1992) The taurine and hypotaurine content of human semen. *J. Androl.*, **13**, 289–292.

Hoshi, K., Katayose, H., Yanagida, K., Kimura, Y. and Sato, A. (1996) The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. *Fertil. Steril.*, **66**, 634–639.

Host, E., Lindenberg, S., Ernst, E. and Christensen, F. (1999a) DNA strand breaks in human spermatozoa: a possible factor to be considered in couples suffering form unexplained infertility. *Acta Obstet. Gynecol. Scand.*, **78**, 622–625.

Host, E., Lindenberg, S., Kahn, J.A. and Christensen, F. (1999b) DNA strand breaks in human sperm cells: a comparison between mean with normal and oligozoospermic sperm samples. *Acta Obstet. Gynecol. Scand.*, **78**, 336–339.

Host, E., Lindenberg, S. and Smidt-Jensen, S. (2000) The role of DNA strand breaks in human spermatozoa used for IVF and ICSI. *Acta Obstet. Gynecol. Scand.*, **79**, 559–563.

Hughes, C.M., Lewis, S.E.M., McKelvey-Martin, V.J. and Thompson, W. (1996) A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Mol. Hum. Reprod.*, **2**, 613–619.

Hughes, C.M., Lewis, S.E.M., McKelvey-Martin, V.J. and Thompson, W. (1998) The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. *Hum. Reprod.*, **13**, 1240–1247.

Huszar, G., Sbracia, M., Vigue, L., Miller, D.J. and Shur, B.D. (1997) Sperm plasma membrane remodeling during spermiogenic maturation in men: relationship among plasma membrane beat 1,4 galactosyltransferase, cytoplasmic creatine phosphokinase and creatine phosphokinase isoform ratios. *Biol. Reprod.*, **56**, 1020–1024.

Irvine, D.S., Twigg, J.P., Gordon, E.L., Fulton, N., Milne, P.A. and Aitken, R.J. (2000) DNA integrity in human spermatozoa: relationships with semen quality. *J. Androl.*, **21**, 33–44.

Jurisicova, A., Lopes, S., Meriano, J., Oppedisano, L., Casper, R.F. and Varmuza, S. (1999) DNA damage in round spermatids of mice with a targeted disruption of the Pp1c gamma gene and in testicular biopsies of patients with non-obstructive azoospermia. *Mol. Hum. Reprod.*, **5**, 323–330.

Keel, B.A. and Webster, B.W. (1990) The standard semen analysis. In *CRC Handbook of the Laboratory Diagnosis and Treatment of Infertility*. CRC Press, Boca Raton, FL, pp. 27–69.

Khalifa, E., Oehninger, S., Acosta, A.A., Morshedi, M., Veeck, L., Bryvyski, R.G. and Muasher, S.J. (1992) Successful fertilization and pregnancy outcome in in-vitro fertilization using cryopreserved/thawed spermatozoa from patients with malignant disease. *Hum. Reprod.*, **7**, 105–108.

Kim, J.M., Ghosh, S.R., Weil, A. and Zirkin, B.R. (2001) Caspase-3 and caspase-activated deoxyribonuclease are associated with testicular germ cell apoptosis resulting from reduced intratesticular testosterone. *Endocrinology*, **142**, 3809–3816.

Klaude, M., Eriksson, S., Nygren, J. and Ahnstrom, G. (1996) The comet assay: mechanism and technical considerations. *Mutat. Res.*, **363**, 89–96.

Kobayashi, H., Larson, K., Sharma, R.K., Nelson, D.R., Evenson, D.P., Toma, H., Thomas, A.J., Jr and Agarwal, A. (2001) DNA damage in cancer patients before treatment as measured by the sperm chromatin structure assay. *Fertil. Steril.*, **75**, 469–475.

Kodama, H., Yamaguchi, R., Fukuda, J., Kasai, H. and Tanak, T. (1997) Increased deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil. Steril.*, **65**, 519–524.

Kunzle, R., Mueller, M.D., Hanggi, W., Birkahuser, M.H., Drescher, H. and Bersinger, N.A. (2003) Semen quality of male smokers and nonsmokers in infertile couples. *Fertil. Steril.*, **79**, 287–291.

Kuroda-Kawaguchi, T., Skaltesky, H., Brown, L.G., Minx, P.J., Cordum, H.S., Waterson, R.H., Wilson, R.K., Silber, S., Oates, R., Rozen, S. and Page, D.C. (2001) The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. *Nature Genet.*, **29**, 279–286.

Labbe, C., Martoriati, A., Devaux, A. and Maisse, G. (2001) Effects of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout. *Mol. Reprod. Dev.*, **60**, 397–404.

Larson, K.L., Brannian, J.D., Timm, B.K., Jost, L.K. and Evenson, D.P. (1999) Density gradient centrifugation and glass wool filtration of semen remove spermatozoa with damaged chromatin structure. *Hum. Reprod.*, **14**, 2015–2019.

Larson, K.L., Dejonge, C.J., Barnes, C.M., Jost, L.K. and Evenson, D.P. (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Hum. Reprod.*, **15**, 1717–1719.

Lee, J., Richburg, J.H., Younkin, S.C. and Boekelheide, K. (1997) The Fas system is a key regulator of germ cell apoptosis in the testis. *Endocrinology*, **138**, 2081–2088.

Li, H., Jian, Y., Rajpurkar, R., Dunbar, J. and Dhabuwala, C.B. (1999) Cocaine induced apoptosis in rat testes. *J. Urol.*, **162**, 213–216.

Linfor, J.J. and Meyers, S.A. (2002) Detection of DNA damage in response to cooling injury in equine spermatozoa using single-cell gel electrophoresis. *J. Androl.*, **23**, 107–113.

Loir, M. and Lanneau, M. (1984) Structural function of the basic nuclear proteins in rat spermatids. *J. Ultrastruct. Res.*, **86**, 262–272.

Lopes, S., Jurisicova, A., Sun, J. and Casper, R.F. (1998a) Reactive oxygen species: a potential cause for DNA fragmentation in human spermatozoa. *Hum. Reprod.*, **13**, 896–900.

Lopes, S., Sun, J., Jurisicova, A., Meriano, J. and Casper, R.F. (1998b) Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil. Steril.*, **69**, 528–532.

Manicardi, G.C., Bianchi, P.G., Pantano, S., Azzoni, P., Bizzaro, D., Bianchi, U. and Sakkas, D. (1995) Presence of endogenous nicks in DNA of ejaculated human spermatozoa and it relationship to chromomycin A3 accessibility. *Biol. Reprod.*, **52**, 864–867.

Manicardi, G.C., Tombacco, A., Bizzaro, D., Bianchi, U., Bianchi, P. and Sakkas, D. (1998) DNA strand breaks in ejaculated human spermatozoa: comparison of susceptibility to the nick translation and terminal transferase assays. *Histochem. J.*, **30**, 33–39

Matsuda, Y. and Tobari, I. (1989) Repair capacity of fertilized mouse eggs for X-ray damage induced in sperm and mature oocytes. *Mutat. Res.*, **210**, 35–47.

McKelvey-Martin, V.J., Melia, N., Walsh, I.K., Johnston, S.R., Hughes, C.M., Lewis, S.E.M. and Thompson, W. (1997) Two potential clinical applications of the alkaline single-cell gel electrophoresis assay: (1) human bladder washings and transitional cell carcinoma of the bladder: and (2) human sperm and male infertility. *Mutat. Res.*, **375**, 93–104.

McPherson, S.M.G. and Longo, F.J. (1992) Localization of Dnase I-hypersensitive regions during rat spermatogenesis: stage dependent patterns and unique sensitivity of elongating spermatids. *Mol. Reprod. Dev.*, **31**, 268–279.

McPherson, S.M.G. and Longo, F.J. (1993) Chromatin structure-function alterations during mammalian spermatogenesis: DNA nicking and repair in elongating spermatids. *Eur. J. Histopathol.*, **37**, 109–128.

Molina, J., Castilla, A., Gil, T., Hortas, M.L., Vergara, F. and Herruzo, A. (1995) Influence of incubation on the chromatin condensation and nuclear stability in human spermatozoa by flow cytometry. *Hum. Reprod.*, **10**, 1280–1286.

Morris, I.D., Ilott, S., Dixon, L. and Brison, D.R. (2002) The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. *Hum. Reprod.*, **17**, 990–998.

Nagata, S. (1997) Apoptosis by death factor. *Cell*, **88**, 355–365.

Ollero, M., Gil-Guzman, E., Lopez, M., Sharma, R., Agarwal, A., Larson, K., Evenson, D., Thomas, A.J., Jr and Alvarez, J.G. (2001) Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis of male infertility. *Hum. Reprod.*, **16**, 1912–1921.

Ondrizek, R., Chan, P.J., Patton, W.C. and King, A. (1999) An alternative medicine study of herbal effects on the penetration of zona-free hamster oocytes and the integrity of sperm deoxyribonucleic acid. *Fertil. Steril.*, **71**, 517–522.

Ostling, O. and Johanson, K.J. (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem. Biophys. Res. Commun.*, **123**, 291–298.

Padron, O.F., Brackett, N.L., Sharma, R.K., Kohn, S., Lynn, C.M., Thomas, A.J., Jr and Agarwal, A. (1997) Seminal reactive oxygen species, sperm motility and morphology in men with spinal cord injury. *Fertil. Steril.*, **67**, 115–1120.

Palermo, G., Cohen, J., Alikani, M., Adler, A. and Rosenwaks, Z. (1995) Development and implementation of intracytoplasmic sperm injection (ICSI). *Reprod. Fertil. Dev.*, **7**, 211–218.

Pienta, K.J. and Coffey, D.S. (1984) A structural analysis of the role of the nuclear matrix and DNA loops in the organization of the nucleus and chromosome. *J. Cell Sci. Suppl.*, **1**, 123–135.

Poccia, D. (1986) Remodeling of nucleoproteins during gametogenesis, fertilization, and early development. *Int. Rev. Cytol.*, **105**, 1–65.

Potts, R.J., Newbury, C.J., Smith, G., Notarianni, L.J. and Jefferies, T.M. (1999a) Sperm chromatin damage associated with male smoking. *Mutat. Res.*, **423**, 103–111.

Potts, R.J., Jefferies, T.M. and Notarianni L.J. (1999b) Antioxidant capacity of the epididymis. *Hum. Reprod.*, **14**, 2513–2516.

Raman, R.S., Chan, P.J., Corselli, J.U., Patton, W.C., Jacobson, J.D., Chan, S.R. and King, A. (2001) Comet assay of cumulus cell DNA status and the relationship to oocyte fertilization via intracytoplasmic sperm injection. *Hum. Reprod.*, **16**, 831–835.

Ramos, L., Kleingeld, P., Meulman, E., Van Kooy, R., Kremer, J., Braat, D. and Wetzels, A. (2002) Assessment of DNA fragmentation of spermatozoa that were surgically retrieved from men with obstructive azoospermia. *Fertil. Steril.*, **77**, 233–237.

Reichart, M., Kahane, I. and Bartoov, B. (2000) In vivo and *in vitro* impairment of human and ram sperm nuclear chromatin integrity by sexually transmitted *Ureaplasma urealyticum* infection. *Biol. Reprod.*, **63**, 1041–1048.

Richardson, L.L., Pedigo, C. and Handel, A. (2000) Expression of deoxyribonucleic acid repair enzymes during spermatogenesis in mice. *Biol. Reprod.*, **62**, 789–796.

Richter, M.A., Haning, R.V., Jr and Shapito, S.S. (1984) Artificial donor insemination: fresh versus frozen semen; the patient as her own control. *Fertil. Steril.*, **41**, 277–280.

Roberts, K.P. (1998) Y chromosome deletions and male infertility: state of the art and clinical applications. *J. Androl.*, **19**, 255–259.

Rodriguez, I., Ody, C., Araki, K., Garcia, I. and Vassali, P. (1997) An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO J.*, **16**, 2262–2270.

Sailer, B.L., Jost, L.K. and Evenson, D.P. (1995) Mammalian sperm DNA susceptibility to in-situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *J. Androl.*, **16**, 80–87.

Sakkas, D., Umer, F., Bizzaro, D., Manicardi, G., Bianchi, P.G., Shoukir, Y. and Campana, A. (1998) Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. *Hum. Reprod.*, **12** (Suppl. 4), 11–19.

Sakkas, D., Mariethoz, E., Manicardi, G., Bizzaro, D., Bianchi, P.G. and Bianchi, U. (1999a) Origin of DNA damage in ejaculated human spermatozoa. *Rev. Reprod.*, **4**, 431–437.

Sakkas, D., Mariethoz, E. and St John, J.C. (1999b) Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. *Exp. Cell Res.*, **251**, 350–355.

Sakkas, D., Manicardi, G.C., Tomlinson, M., Mandrioli, M., Bizzaro, D., Bianchi, P.G. and Bianchi, U. (2000) The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. *Hum. Reprod.*, **15**, 1112–1116.

Sakkas, D., Moffatt, O., Manicardi, G.C., Mariethoz, E., Tarozzi, N. and Bizzaro, D. (2002) *Nature* of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol. Reprod.*, **66**, 1061–1067.

Saleh, R. and Agarwal, A. (2002) Oxidative stress and male infertility: from research bench to clinical practice. *J. Androl.*, **23**, 737–752.

Saleh, R., Agarwal, A., Sharma, R., Nelson, D. and Thomas, A.J., Jr (2002a) Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. *Fertil. Steril.*, **78**, 491–499.

Saleh, R., Agarwal, A., Nelson, D., Nada, E., El-Tonsy, M., Alvarz, J.G., Thomas, A.J., Jr and Sharma, R. (2002b) Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study. *Fertil. Steril.*, **78**, 313–318.

Saleh, R., Agarwal, A., Nada, E., El-Tonsy, M.H., Sahram, R.K., Meyer, A., Nelson, D.R. and Thomas, A.T., Jr (2003) Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil. Steril.* (in press).

Sharma, R.K. and Agarwal, A. (1996) Role of reactive oxygen species in male infertility. (Review). *Urology*, **48**, 835–850.

Shen, H.M., Chia, S.E. and Ong, C.N. (1999) Evaluation of oxidative DNA damage in human sperm and its association with male infertility. *J. Androl.*, **20**, 718–723.

Shoukir, Y., Chardonnes, D., Campana, A. and Sakkas, D. (1998) Blastocyst development from supernumerary embryos after intracytoplasmic injection: a paternal influence? *Hum. Reprod.*, **13**, 1632–1637.

Siciliano, L., Tarantino, P., Longobardi, F., Rago, V., De Stefano, C. and Caprino, A. (2001) Impaired seminal antioxidant capacity in human semen with hyperviscosity or oligoasthenozoospermia. *J. Androl.*, **22**, 798–803.

Sierens, J., Hartley, J.A., Campbell, M.J., Leathem, A.J. and Woodside, J.V. (2002) *In vitro* isoflavone supplementation reduces hydrogen peroxide-induced DNA damage in sperm. *Teratogen. Carcinogen. Mutagen.*, **22**, 227–234.

Sigman, M. and Lopes, L. (1993) The correlation between round cells and white blood cells in the semen. *J. Urol.*, **388**, 573–574.

Sikka, S.C. (2001) Relative impact of oxidative stress on male reproductive function. *Curr. Med. Chem.*, **8**, 851–862.

Silber, S.J., Van Steirteghem, A.C., Liu, J., Naguy, Z., Tournaye, H. and Devroey, P. (1995) High fertilization and pregnancy rates after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. *Hum. Reprod.*, **10**, 148–152.

Singh, N. and Stephens, R. (1998) X-ray-induced double strand breaks in human sperm. *Mutagenesis*, **13**, 75–79.

Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L. (1988) A simple technique for quantification of low levels of DNA damage in individual cells. *Exp. Cell Res.*, **175**, 184–191.

Sinha Hikim, A.P. and Swerdloff, R.S. (1999) Hormonal and genetic control of germ cell apoptosis in the testis. *Rev. Reprod.*, **4**, 38–47.

Snager, W.G., Olson, J.H. and Sherman, J.K. (1992) Semen cryobanking for men with cancer-criteria change. *Fertil. Steril.*, **58**, 1024–1027.

Sofikitis, N., Miyagawa, I., Dimitriadis, D., Zavos, P., Sikka, S. and Hellstrom, W. (1995) Effects of smoking on testicular function, semen quality and sperm fertilizing capacity. *J. Urol.*, **154**, 1030–1034.

Spano, M., Bonde, J.P., Hjollund, H.I., Kolstad, H.A., Cordelli, E. and Leter, G. (2000) Sperm chromatin damage impairs human fertility. *Fertil. Steril.*, **73**, 43–50.

Steele, E.K., McClure, N., Maxwell, R.J. and Lewis, S.E. (1999) A

comparison of DNA damage in testicular and proximal epididymal spermatozoa in obstructive azoospermia. *Mol. Hum. Reprod.*, **5**, 831–835.

Steele, E.K., McClure, N. and Lewis, S.E. (2000) Comparison of the effects of two methods of cryopreservation on testicular sperm DNA. *Fertil. Steril.*, **74**, 450–453.

Suda, T., Takahashi, T., Goldstein, P. and Nagata, S. (1993) Molecular cloning and expression of Fas ligand, a novel member of tumor necrosis factor family. *Cell*, **75**, 1169–1178.

Sun, J.G., Jurisicova, A. and Casper, R.F. (1997) Deletion of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization *in vitro*. *Biol. Reprod.*, **56**, 602–607.

Tejada, R., Mitchell, J.C., Norman, A., Marik, J.J. and Friedman, S. (1984) A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil. Steril.*, **42**, 87–91.

Tesarik, J., Greco, E., Cohen-Bacrie, P. and Mendoza, C. (1998) Germ cell apoptosis in men with complete and incomplete spermiogenesis failure. *Mol. Hum. Reprod.*, **4**, 757–762.

Tesarik, J., Mendoza, C. and Greco, E. (1999) *In vitro* culture facilitates the selection of healthy spermatids for assisted reproduction. *Fertil. Steril.*, **72**, 809–813.

Thiele, J.J., Friesleben, H.J., Fuchs, J. and Ochsendorf, F.R. (1995) Ascorbic acid and urate in human seminal plasma: determination and interrelationships with chemiluminescence in washed semen. *Hum. Reprod.*, **10**, 110–115.

Thornberry, N.A. and Lazebnik, Y. (1998) Caspases: enemies within. *Science*, **281**, 1312–1316.

Tomlinson, M.J., White, A., Barratt, C.L., Bolton, A.E. and Cooke, I.D. (1992) The removal of morphologically abnormal sperm forms by phagocytes: a positive role for seminal leukocytes. *Hum. Reprod.*, **7**, 517–522.

Tomlinson, M.J., Moffatt, O., Manicardi, G.C., Bizzaro, D., Afnan, M. and Sakkas, D. (2001) Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. *Hum. Reprod.*, **16**, 2160–2165.

Tomsu, M., Sharma, V. and Miller, D. (2002) Embryo quality and IVF treatment outcomes may correlate with different sperm comet assay parameters. *Hum. Reprod.*, **17**, 1856–1862.

Twigg, J., Irvine, D.S., Houston, P., Fulton, N., Michael, L. and Aitken, R.J. (1998a) Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. *Mol. Hum. Reprod.*, **4**, 439–445.

Twigg, J., Fulton, N., Gomez, E., Irvine, D.S. and Aitken, R.J. (1998b) Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of the human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum. Reprod.*, **13**, 1429–1436.

Twigg, J.P, Irvine, D.S and Aitken, R.J. (1998c) Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Hum. Reprod.*, **13**, 1864–1871.

van Holde, K. and Zlatanova, J. (1996) What determines the folding of the chromatin fiber? *Proc. Natl Acad. Sci. USA*, **93**, 10548–10555.

Van Loon, A.A., Den Boer, P.J., Van Der Schans, G.P., Mackenbach, P., Grootegoed. J.A., Baan, R.A. and Lohman, P.H. (1991) Immunochemical detection of DNA damage induction and repair at different cellular stages of spermatogenesis of the hamster after *in vitro* or in vivo. *Exp. Cell Res.*, **193**, 303–309.

Vaux, D.L. and Korsmeyer, S.J. (1999) Cell death in development. *Cell*, **96**, 245–254.

Vogt, P., Chandley, A.C., Hargreave, T.B., Keil, R., Ma, K. and Sharkey, A. (1992) Microdeletions in interval 6 of Y chromosome of males with idiopathic sterility point to disruption of AZF, a human spermatogenesis gene. *Hum. Genet.*, **89**, 491–496.

Ward, W.S. and Coffey, D.S. (1990) Specific organization of genes in relation to the sperm nuclear matrix. *Biochem. Biophys. Res. Commun.*, **173**, 20–25.

Ward, W.S. and Coffey, D.S. (1991) DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol. Reprod.*, **44**, 569–574.

Warren, J.S., Johnson, K.J. and Ward, P.A. (1987) Oxygen radicals in cell injury and cell death. *Pathol. Immunopathol. Res.*, **6**, 301–315.

World Health Organization (1999) *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*, 4th edition, Cambridge University Press, Cambridge.

Younglai, E.V., Holt, D., Brown, P., Jurisicova, A. and Casper, R.F. (2001) Sperm swim-up technique and DNA fragmentation. *Hum. Reprod.*, **16**, 1950–1953.

Zalata, A., Hafez, T. and Comhaire, F. (1995) Evaluation of the role of reactive oxygen species in male infertility. *Hum. Reprod.*, **10**, 1444–1451.

Zamboni, L. (1992) Sperm structure and its relevance to infertility. *Arch. Pathol. Lab. Med.*, **116**, 325–344.

Zini, A., Finelli, A., Phang, D. and Jarvi, K. (2000) Influence of semen processing technique on human sperm DNA integrity. *Urology*, **56**, 1081–1084.

Zini, A., Kamal, K., Phang, D., Willis, J. and Jarvi, K. (2001a) Biologic variability of sperm DNA denaturation in infertile men. *Urology*, **58**, 258–261.

Zini, A., Bielcki, R., Phang, D. and Zenzes, M.T. (2001b) Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil. Steril.*, **75**, 674–677.

Zini, A., Fischer, M.A., Sharir, S., Shayegan, B., Phang, D. and Jarvi, K. (2002) Prevalence of abnormal sperm DNA denaturation in fertile and infertile men. *Urology*, **60**, 1069–1072.

Zollner, U., Zollner, K.P., Dietl, J. and Steck, T. (2001) Semen sample collection enhances the implantation rate following ICSI in patients with severe oligoasthenoteratozoospermia. *Hum. Reprod.*, **16**, 1110–1114.