Iranian Journal of Reproductive Medicine Vol.5. No.4. pp:137-150, Autumn 2007

Review article

Human sperm DNA damage in the context of assisted reproductive techniques

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Received: 11 May 2007; accepted: 27 December 2007

Abstract

Fertilization involves direct interaction of the sperm and oocyte, fusion of the cell membranes and union of male and female gamete genomes. The completion of this process and subsequent embryo development depend in part on the inherent integrity of the sperm DNA. Sperm genome quality has been emphasized for several years as playing a major role in early embryogenesis. There is clinical evidence showing that human sperm DNA damage may adversely affect reproductive outcomes and that spermatozoa of infertile men possess substantially more DNA damage than do spermatozoa of fertile men. Testing DNA integrity may help selecting spermatozoa with intact DNA or with the least amount of DNA damage for use in assisted reproductive techniques (ARTs). This review will focus on how sperm DNA is organized, what causes sperm DNA damage and what impact this damage may have on reproductive outcome.

***Key words**: Fertilization, Sperm DNA damage, Assisted reproductive technique (ART).*

Introduction

Sperm DNA is organized in a specific manner that keeps the nuclear chromatin compact and stable (1). This DNA organization not only permits the very tightly packaged genetic information to be transferred to the egg but also insures that the DNA is delivered in a physical and chemical form that allows the developing embryo to easily access the genetic information. Fertile sperm have stable DNA, which is capable of decondensation at the appropriate time in the fertilization process and transmitting the DNA without defects. The positive relationship between poor sperm parameters and DNA damage in human spermatozoa points to inherent problems in spermatogenesis in specific patients. Various hypotheses have been proposed as the molecular mechanism of sperm DNA damage. The most important ones are abnormal chromatin packaging, oxidative stress and apoptosis (2). Semen samples that contain high levels of DNA damage are often associated with decreased fertilization rates and/or embryo cleavage after in vitro fertilization (IVF) and intra cytoplasmic sperm injection (ICSI) and may be linked to early embryo death. Although the most normal appearing and motile spermatozoa are selected during ART, there is always a chance that sperm containing varying degrees of DNA damage may be used.

The cause of infertility in infertile men with normal semen parameters could be related to abnormal sperm DNA(3).Therefore, the evaluation of sperm DNA integrity, in addition to routine sperm parameters, could add further information

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on the quality of spermatozoa. The damaged Sperm DNA is critical in the context of ART which are increasingly used to treat infertile couples. This review aims to summarize the impact of human sperm DNA damage in the context of the different damage origins on male infertility and prognosis of ART.

Etiology of sperm DNA damage

A variety of several etiological factors such as cigarette smoking, leukocytospermia, drugs, irradiation and varicoceles have been correlated with increased levels of human sperm DNA damage, and in turn, affect the status of male fertility.

Cigarette smoking

Cigarette smoking has mutagenic properties, having been associated with an overall reduction in semen quality, and specifically a reduction in sperm count and motility and increase in number of abnormal cells (4). Also it was reported that the DNA fragmentation index (% DFI) was significantly higher in fertile men who smoked (p=0.02) (5). This observation was first described in 35 smokers included in 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 ± 0.2 %; P=0.01) (6). A possible explanation for these finding could be the increased leucocytes-induced oxidative stress (OS) on developing or mature sperm.

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 leucocytes (5). In turn, activated leucocytes can generate high levels of reactive oxygen species (ROS) in semen which may overwhelm the antioxidant strategies and result in OS (7). Another causative factor would be the fact that the seminal plasma in smokers contains lower levels of antioxidants than that of non-smokers (8).

Leukocytospermia

Leucocytes in general are present in most ejaculates and are thought to play an important role in immunosurveillance and phagocytic clearance of abnormal sperm (9). However increased concentrations of leucocytes in semen indicate the presence of a genital tact infection or inflammation and have been reported to be associated with an increase in immature germ cell concentration (10). Higher amounts of DNA-damaged cells were reported in the raw semen samples of leukocytospermic patients compared with normal donors (39 ± 10.9 versus 24.9 ±10.2 %; P<0.01). Following the fractionation of semen samples into different portions according to their stage of maturation, it was also reported that chromatin alterations were highest in the immature fraction (11).

Iatrogenic sperm DNA damage

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 (12). Also, seminal plasma contains chain breaking antioxidants such as ascorbate, urate, albumin, glutathione and taurine (13).Thus, the seminal plasma plays a crucial protective role against ROS and its removal during sperm preparation may be hazardous to sperm DNA integrity (14).

Another form of iatrogenic interference that might lead to DNA damage is that of cryopreservation, which is used extensively in ART programmes. Although it was once proved that the cryopreservation of testicular sperm does not increase baseline levels of DNA damage, most other studies indicate that the freeze-thaw process significantly damages spermatozoal DNA from infertile men (15).

Testicular hyperthermia and varicocele

A febrile illness has been shown to cause an increase in the histone protamine ratio and DNA damage in ejaculated spermatozoa. Direct testicular hyperthermia has also been shown to cause these effects (16). Varicoceles have been associated with sperm DNA damage. The level of sperm DNA damage is related to the high levels of OS found in the semen of infertile men with this condition (17). Recent studies have demonstrated that varicoceles are associated with the abnormal retention of sperm cytoplasmic droplets (a morphologic feature associated with high levels of ROS) and that these retained droplets are correlated with sperm DNA damage in infertile men (18). Furthermore, sperm DNA integrity has been shown to improve after varicocele repair (19).

Drugs and irradiation

Chemotherapeutic drugs such as fludarabine, cyclophosphamide and busolphane can cause testicular damage as manifested by reduced testicular volume, oligospermia, elevated FSH and

LH and lower testosterone concentrations (20). 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 (21). Male germ cells are sensitive to the mutagenic effects of irradiation. Although sperm DNA damage exits following radiotherapy, no increase in genetic defect or congenital malformations was detected among children conceived by parents who had previously undergone treatment (22).

Origin of sperm DNA damage

Damage of sperm DNA or its chromatin structure can be occurred at any step of the whole spermatogenesis (23). The positive relationship between poor sperm parameters and DNA damage in mature spermatozoa points to inherent problems in spermatogenesis in specific patients (23).

Three theories have been proposed to explain DNA anomalies in the ejaculated human spermatozoa.

The first theory is correlated with poor chromatin packaging or abnormal making due to under protamination which results in the presence of endogenous nicks in DNA (2). The second one is the OS mechanism that has been studied extensively, which is caused by the overproduction of ROS (24,25). The last one proposes that the presence of endogenous nicks is characteristic of programmed cell death aiming to the functional elimination of possibly defective germ cells from the genetic pool. Recent models of apoptosis include receptor mediated pathways and intrinsic triggered apoptosis, as well as cytotoxic or stress induced forms (2).

All these mechanisms, either individually or together, have some bearing on the presence of abnormal spermatozoa in the ejaculate, and they may or may not be interrelated.

Human sperm chromatin structure and abnormal chromatin packing

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 6 times more condensed than in mitotic chromosomes (26). It occupies almost the entire nucleus volume, whereas somatic cell DNA only partially fills the nucleus.

The DNA in somatic cell nuclei is first packed into nucleosomes (27). These structures consist of a protein core formed by an octamer of stones with 2 laps of wrapped DNA around base pairs. The nucleosomes are then further coiled into regular helixes also called solenoids (28). These 2 types of DNA packaging increase the volume of the (29). 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 (29). In order for the sperm nucleus to evolve and become highly condensed with a species-specific shape, it undergoes a complicated series of reaction through which somatic histones and non-histones chromatin proteins are replaced during a variable period of time by one or more protamine types (30). In the first step, the transition nuclear proteins (TP1 and TP2) replace the somatic cell histones. In the second step, during the elongated spermatid stage, the sperm protamine proteins replace the transition proteins. The result is a highly compact sperm chromatin, which fosters DNA stability and transcriptional quiescence. In humans there are 2 forms of sperms protamine: protamine-1 (P1) and protamie-2 (P2), which occur in a strictly regulated 1-to-1ratio (31).

Sperm epididymal maturation involves a final stage of chromatin organization in which protamine cross-linking by disulfide bond formation occurs-a step that is supported by the fact that protamins contain a significant number of cysteine residues that participate in sperm chromatin compaction by forming multiple inter- and intra-protamine disulfide cross-links. All these interactions make mammalian DNA the most condensed eukaryotic DNA (32).

Therefore, more than two third of the chromatin structure of human sperm is thus packaged by protamines, only up to 15% of the human DNA are less tightly compacted and packaged by histones. It has shown that infertile men have an increased sperm histone: protamine ratio than fertile counterparts. This alteration of histone:protamine ratio, that is also called as abnormal packing, increases susceptibility of sperm DNA to external stresses due to poorer chromatin compaction. Furthermore, complete deficiency of protamine has been demonstrated in about 5%-15% of infertile men. The studies conducted by Carrell and Liu, and Yebra et al describe a population with fertile

males with undetectable sperm P2 (33). Recently, P1deficiency has also been identified in a population of subfertile males (34). It has been postulated that protamine deficiency is related to DNA damage in human sperm.

The mitochondrial DNA of human sperm is a small, circular DNA which is not bound to special proteins it has been demonstrated that sperm motility is directly related to the mitochondrial volume within the sperm mid piece. The mitochondrial DNA exhibits a high rate of mutation or deletions those have been associated with reduced sperm motility. The inheritances of mitochondrial DNA is primarily maternal and only in 1% of cases paternal transmission of mitochondrial DNA mutations have been reported (35).

Although the first study on mitochondrial DNA inheritance after ICSI suggested that human embryos eliminate the mitochondrial DNA of the injected sperm (36), another study has shown that abnormal paternal mitochondrial DNA transmission may not be uncommon when poor-quality gametes are used. It is also of interest that populations of human spermatozoa exhibiting evidence of mitochondrial dysfunction also show high rates of nuclear DNA fragmentation (37). Abnormal sperm samples revealed high incidence of mitochondrial DNA damage, which confirms their role in male infertility (38). Although the biological significance of sperm DNA damage remains unclear, it appears to be detrimental to fertility in humans and has been linked to lower embryo quality, blastulation rates, and IVF pregnancy rates (39-41).

The role of apoptosis of human spermatozoa in DNA damage

Apoptosis is a mode of cellular death based on a genetic mechanism that induces a serious of cellular, morphological and biochemical alteration, leading the cell to suicide (42). 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 (43). In mammalian testes, germ cells expand clonally through many rounds of mitosis before undergoing the differentiation steps that result in mature spermatozoa. This clonal expansion is excessive and thus requires a mechanism such as apoptosis (44).

Apoptosis can be postulated to have two putative roles during normal spermatogenesis: limitation of the germ cell population to numbers that can be supported by the Sertoli cells and, possibly, selective depletion of abnormal spermatozoa. During apoptosis the cells shrink and exhibit several typical features, including cell membrane disruption, cytoskeletal rearrangement, nuclear condensation, and intra nucleosomal DNA fragmentation (45). Apoptosis in the human spermatozoa is a result of DNA strand breaks induced by a cascade of regulatory mechanisms with infertility (46).The degradation of DNA into fragments approximately 185 bp and its multiples in size is one of the best characterized biochemical features of apoptotic cell death and is used as the basis for the commonly used labeling techniques for detecting apoptotic cells (47).

Pathways involving the cell surface protein Fas (a member of the tumor necrosis factor receptor family) may mediate apoptosis in sperm. Binding of Fas legend (FasL) or agonistic anti-Fas antibody to Fas kills cells by apoptosis (48). 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%. Therefore, the presence of spermatozoa that posses' apoptotic markers, such as Fas positivity and DNA damage, indicate that in men with abnormal semen parameters, an "abortive apoptosis" has taken place (49).

Failure to clear Fas-positive spermatozoa may be due to dysfunction at one more levels. Because Sertoli cells can limit this proliferation by producing FasL, it has been postulated that oligospermic men with reduced spermatogenesis may not produce enough spermatozoa to trigger this action (50). In these men, Fas-positive spermatozoa may escape the signal to undergo apoptosis. 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 posses a higher percentage of spermatozoa containing DNA damage and abnormal spermatozoa that display markers of apoptosis (51).

Another major component of apoptosis machinery that contributes to sperm DNA damage involves specific proteinase, called Caspases (cysteinyl aspartate-specific proteinases), which have been claimed to play a major role in the regulation of apoptosis. More than a dozen of specific proteinase has been reported to be related to apoptosis in the human seminiferous epithelium that expressed as inactive proenzymes and participate in a cascade triggered in response to

pro-apoptotic signals. Among these, caspase-3 is considered to be a major executioner protease (52). Caspases share the ability to cleave their substrates on the carboxyl side of aspartate residues (48). Cell-surface death receptors such as Fas or tumor necrosis factor-a receptor 1 (TNFR 1) are activated by ligand binding resulting in the proteolytic activation of caspases, in the destruction of vital proteins and finally in the death of the cell.

The FasL/Fas ligation in the inner mitochondrial membrane leads to activation of Capases 8 & 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 (53). In addition, caspases activate other proteins needed for the achievement of apoptosis such as caspase-activated Dnase which is responsible for DNA fragmentation (47).

The Bcl-2 family proteins (Bcl-x, Bcl-w, Bax, Bak, Bid, Bad), the tumor suppressor p53, the nuclear factor kB (NF-kB) and the heat shock proteins (HSPs) have been shown to be regulators of apoptosis (54). Having in mind the ultimate purpose of apoptosis, spermatozoa exhibiting apoptotic features should be eliminated in the ejaculate. Several other studies have also found that other apoptotic markers such as Bcl-x, p53 and annexin V are also present on ejaculated human spermatozoa and show distinct relationships with abnormal semen parameters (51, 55). It has been postulated that in these sub fertile men spermatozoa that have been assigned to undergo apoptosis escape this process, so that the correct clearance of spermatozoa via apoptosis is not occurring. The final outcome is the production of spermatozoa that possess a range of anomalies including abnormal levels of apoptotic proteins and/or cytoplasmic retention, abnormal chromatin packaging (indicated by low levels of protamine) and the presence of DNA strand breaks.

Oxidative stress

OS and its role in the origins of male infertility were first appreciated in 1943, when the Scottish andrologist John MacLeod demonstrated that catalase could support the motility of human spermatozoa incubated under aerobic conditions (56). His explanation for these findings that human spermatozoa are vulnerable to OS created by ROS has been confirmed in a number of independent studies (57). OS at high levels are potentially toxic to sperm quality and function (58). 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 posses one or more unpaired electrons”. The presence of high ROS levels has been reported in the semen of 25-40% of infertile men (59).

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 shift, DNA cross-links and chromosomal rearrangement (60). Two factors protect the sperm DNA from oxidative insult: the characteristic tight packaging of the DNA, and the antioxidants present in seminal plasma. However, OS may develop as a result of an imbalance between ROS generation and antioxidant scavenging (61).

It has been shown that the amount of ROS generation well controlled by seminal antioxidants in the semen of fertile men. Thus initially, the pathogenic effects of ROS presumed to occur in cases of excessive production that can not be tolerated by antioxidant capabilities of the male reproductive tract or seminal plasma (62). Subsequently, it has been claimed that there is not a significant reduction in the total antioxidant capacity associated with increased levels of ROS. Furthermore, the pathological levels of ROS detected in the semen of infertile men was reported to be more likely caused by increased ROS production than by reduced antioxidant capacity of seminal plasma (63).

Morphologically abnormal spermatozoa and leukocytes are the main source of excess ROS generation in semen (62). Activated leukocytes are capable of producing 100-fold higher amounts of ROS than non-activated leukocytes (64).Sperm DNA thus is more prone to leukocyte-induced ROS damage in infertile men with abnormal semen parameters likely possessing “masked” DNA damage and/or more fragile chromatin structure which are under the sensitivity threshold of the assays used for the sperm DNA damage assessment (65).

Assessment of DNA damage

Sperm DNA fragmentation can be evaluated in a variety of ways. These assays include single cell gel electrophoresis (COMET) assay, terminal deoxynucleotidyl transferase (TdT)-mediated-deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay, In-situ nick translation (NT assay), and acridine orange staining technique

(AOT). The COMET assay measures DNA damage by quantifying the single- and double-stranded breaks associated with DNA damage (65). 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 (66). The characteristics that have been used for analysis include the diameter of the nucleus and the COMET length (67). It is a useful technique because it allows for the distinction between the different kinds of DNA fragmentation, apoptotic or necrotic. Apoptotic cells produce teardrop shaped comets due to the migration and accumulation of the short DNA fragments, and the intensity of the tail represents the amount of DNA fragments present (68).

Apoptotic DNA fragmentation is characterized by double stranded DNA breaks. Tomsu et al (39) noted that the COMET head and tail DNA parameters could be considered potentially useful predictors of embryo quality and IVF outcomes, especially in couples with unexplained infertility. It has also been shown that high loads of DNA damage were predictive of embryo development failure after ICSI (69). On the other hand, Abu-Hassan et al (70) do not report any correlation between apoptosis levels assessed by Comet assay and the outcome of ICSI as far as fertilization and embryo quality are concerned.

The TUNEL assay detects both single- and double-stranded DNA breaks by labeling the free 3'-OH terminus with modified nucleotides in an enzymatic reaction with TdT and can be analyzed microscopically or using flow cytometry. It was introduced by Gorczyca et al (71) to identify a population of spermatozoa in the ejaculate that were believed to be apoptotic. Muratori et al (72) demonstrated that DNA fragmentation assessed by the TUNEL method was not associated with an apoptosis-like phenomenon in ejaculated sperm and that DNA fragmentation should be considered a sign of defective sperm maturation probably dating back to the time of DNA packaging. Sakkas et al (51) support that TUNEL positivity and apoptotic markers do no always exist simultaneously in spermatozoa, however, semen samples that had a low sperm concentration and poor morphology were more likely to show high levels of TUNEL positivity and Fas and p53 expression.

Sun et al (6) reported that, using the TUNEL assay, a negative association was found between the percentage of sperm with DNA fragmentation and embryo cleavage rates after IVF. Lopes et al (73) reported a negative association between sperm with DNA fragmentation and ICSI fertilization rate. Benchaib et al (74) found that a high proportion of sperm with fragmented DNA (>10%) was a negative factor for achievement of pregnancy when ICSI was performed, but there was no relationship when conventional IVF was carried out. COMET and TUNEL assays are commonly used in research applications for detecting apoptotic DNA and both correlate well with fertility outcome in ART (75).

The NT assay quantifies the incorporation of biotinylated- dUTP at single stranded DNA breaks in a reaction that is catalyzed by the template dependent enzyme, DNA polymerase I. The NT assay identifies spermatozoa that contain appreciable and variable levels of endogenous DNA damage. The clinical value of the NT assay is severely limited because no correlation has been proven with fertilization during in vivo studies, and because of its lack of sensitivity compared with other assays.

Sperm chromatin structure assay (SCSA) is a flow cytometric assay that relies on the fact that abnormal sperm chromatin is highly susceptible to physical induction of partial DNA denaturation in situ (76). It measures the intensity of acridine orange (AO) fluorescence using flow cytometry. AO fluoresces green when binding to native DNA and red when it binds to the fragmented DNA. The ratio of red/red+green yields the percentage of DFI. While the SCSA is a statistically robust test (77). Not all laboratories have access to a flow cytometery or the technical expertise to perform this assay. Most methods currently used to assess apoptosis and sperm DNA damage lack a threshold between normal levels in the average fertile population and the minimal levels of sperm DNA integrity required for achieving pregnancy, except for the SCSA. The most important parameter of the SCSA is the DFI, which represents the population of cells with DNA damage (40).

Evenson et al (77) and Spano et al (78) studied the relationship between SCSA results and sperm fertilization capacity. Both demonstrated that when >30% of sperm have abnormal chromatin as evaluated by SCSA; human male infertility is hampered independent of sperm number, morphology and motility. The categories proposed by Evenson et al (40) for individual fertility potential according to DFI fraction are: excellent <15%, good 15-24%, fair 25-30% and poor >30% DFI, and if HDS (high DNA staining) is >15% the fertility potential is downgraded at least one category.

The Acridine orange test (AOT) is a simple microscopic procedure based on the same principle as the SCSA but indistinct colors, rapid fading of fluorescence, and heterogeneous staining of slides makes AOT a test of questionable value in clinical practice (79).Recently, a new method, the sperm chromatin dispersion test (SCD), was introduced for evaluating sperm DNA fragmentation (80).The SCD test is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo of dispersed DNA loops that is observed in sperm with non fragmented DNA following acid denaturation and removal of nuclear proteins.

Other methods include high performance liquid chromatography which is used to measure the level of 8-OhdG, enzyme-linked immunosorbent assay (ELISA), Electron microscopy, and FISH (81).

Impact of DNA damage on fertilization, cleavage, implantation, and live birth

Several studies have correlated the degree of DNA damage with various indices of fertility such as the fertilization rate, embryo cleavage rate, implantation rate, pregnancy rate and live birth rate of the offspring. If sperm DNA is unable to decondense after entering the ooplasma, fertilization may not take place or a post fertilization failure may occur when sperm DNA is defective. Pregnancy loss may occur with increase in degree of sperm DNA damage and may be the cause of unexplained pregnancy loss in some patients (82). In addition, the degree of DNA damage can also affect the ability of a couple to conceive naturally (80,81).The relationship between conventional semen parameters and sperm DNA fragmentation is not strong enough to eliminate DNA fragmentation as a potential source of infertility in normozoospermic men and requires a distinct assessment of sperm DNA fragmentation in male infertility (83).

Saleh et al (17) in a prospective study, they examined the relationship between sperm DNA damage and ART outcomes in 33 couples with male factor infertility and whether this damage was related to seminal oxidative stress. They found that, clinical pregnancy was achieved in 27 % (9/33) of couples who underwent ART [26 % (5/19) with IUI, 30 % (3/10) with IVF and 25 % (1/4) with ICSI]. The sperm DFI was negatively correlated with sperm concentration ($r = -0.31$; $P = 0.001$), percentage motility ($r = -0.47$; $P < 0.001$) and normal sperm morphological forms ($r = -0.40$; $P < 0.0001$). In addition to higher DNA fragmentation index and oxidative stress were found in men who failed to initiate a pregnancy after assisted reproductive techniques (n = 24), compared with the cases of those who succeeded and of the fertile donors. DNA fragmentation index was correlated positively with oxidative stress ($r = 0.27$), and negatively with fertilization ($r = -0.70$) and embryo quality ($r = -0.70$). In a blinded study, Høst and colleagues (84) studied DNA damage in four clinically different groups of infertile couples. DNA damage was correlated with semen parameters, the fertilization rate and IVF outcome. In group I (n=75), the female partner had tubal obstruction. Group II consisted of men with unexplained infertility (n =50). Group III consisted of men with oligozoospermia undergoing IVF with their partner (n=50). The proportion of spermatozoa having DNA strand breaks was negatively correlated with the proportion of oocytes that were fertilized after IVF in all 3 groups ($r = -0.39$, $P < 0.01$; $r = -0.61$, $P < 0.01$; $r = -0.39$, $P < 0.01$, respectively). Group IV consisted of men with oligozoospermia (n=50) undergoing ICSI with their partner.

They presented negative correlations between the proportion of spermatozoa with DNA strand breaks and the fertilization rates in all groups except for the ICSI group. This might be attributed to the fact that, on performing ICSI, there is selection of morphologically normal spermatozoa, diminishing the chance of injecting spermatozoa having DNA strand breaks (73).

Interestingly, the number of spermatozoa with DNA strand breaks was significantly higher in the group of men where the females had tubal obstruction compared to proven fertile men which suggest that a male factor may also be included. They suggest that if sperm samples from couples with unexplained infertility exhibit more than 4% DNA strand breaks in the spermatozoa, these couples should have ICSI as the impact of DNA strand breaks will be reduced. (85). Huang et al (86) correlated sperm DNA fragmentation rates >10 % with lower fertilization rates but not with pregnancy outcome.

In another study conducted by Muriel et al (87) they Analyzed DNA fragmentation by the SCD test in 170 aliquots obtained from the ejaculate and from the processed semen used for ART. Fertilization rate was inversely correlated with DNA fragmentation ($r= -0.245$ $P= 0.045$). Higher DNA fragmentation rate gave an increased proportion of zygotes showing asynchrony between the nucleolar precursor bodies of zygote pronuclei (73.8% vs. 28.8% $P < 0.001$). In addition, the slower embryo development and worst morphology on day 6 was correlated with

higher sperm DNA fragmentation (47.7% vs. 29.4% P = 0.044). They also observed a negative correlation between DNA fragmentation and the implantation rate (r = -0.250 P = .042). However, SCD test values were not statistically different in cycles that resulted in a pregnancy compared with those that did not (33.2 vs. 28.2 and 32.4 vs. 34.7).

DFI levels >30% - 40% were incompatible with fertility in vivo, whatever sperm concentration, morphology and motility (77, 78). Bungum et al (41) examined the relationship between the outcome of intrauterine insemination (IUI), and IVF / ICSI and sperm chromatin defects evaluated by SCSA. Two groups were studied for each ART procedure; one with DFI ≤ 27% and another with DFI > 27% since a DFI of 27% previously was reported to be the cut off level for achieving a pregnancy by in vitro ART (83). In IUI patients, there were 20.2% clinical pregnancies per ET and 17.6% deliveries per started cycle for DFI≤ 27%, while the respective rates for DFI > 27% were 4.5% and 4.5%. While in the IVF / ICSI patients there were 38.2% clinical pregnancies, 32.7% implantation rate and 31.4% deliveries for DFI ≤ 27%, while for DFI >27% the corresponding values were 38.2%, 28.6% and 34.3% . A result also confirmed in the study of Payne et al (88) who reported that nine of nineteen couples with DFI >27% achieved clinical pregnancy with IVF / ICSI. On the contrary, other studies reported that, no pregnancy after in vitro ART procedures, both standard IVF and ICSI, when the DFI in raw semen was more than 27% (83, 89).

Larson-Cook et al (83) studied the correlation between the fertilization, embryo development, implantation and pregnancy rates after conventional IVF and ICSI with sperm nuclear DNA fragmentation assessed by the (SCSA) test .The fertilization rate (72.5±0.2%) was not related to DFI. This means that normal fertilization does not ensure high quality DNA in the paternal genome and supports previous studies that showed no relationship between DNA fragmentation and fertilization rate (69). On the contrary, other investigators have shown a significant negative correlation between sperm DNA fragmentation and IVF and ICSI fertilization rates (6, 73).

In the study of Larson – Cook et al (86) cleavage rates were not related to SCSA parameters. Blastocyst formation rate (36.5±5.2%) was also not significantly related to SCSA parameters. All patients who achieved pregnancy had DFI <27% which is contradictory with the results of Payne et al (88) reporting that only 2 out of 22 couples achieved clinical pregnancy when DFI was ≤9%. One patient achieved a chemical pregnancy with DFI >27%, but subsequently lost the pregnancy before ultrasound (90).

Supporting the results of Larson – Cook et al (83), Gandini et al (91) studied the relationships between SCSA parameters evaluated on both neat and processed semen used in ART procedures and fertilization rate, embryo quality and pregnancy rate following IVF and ICSI .No differences were seen in SCSA parameter values between patients initiating pregnancies and not doing so in either conventional IVF or ICSI. Pregnancies and normal delivery were obtained even with high levels of DFI. The mean DFI value for men who had a child was 32.1%, which was not different from the other group of men not having a child (25.1%).

Therefore results of this study were similar with those reported by Larson- Cook et al (83) stating that fertilization rate, cleavage rate and blastocyst formation rate were not significantly related to SCSA parameters and contradict the results of Saleh et al (17) who found that DFI levels were negatively correlated with fertilization and embryo quality after IVF and ICSI.

In a study of Benchaib et al (92) statistically significant negative relationship was found for sperm DNA fragmentation and fertilization when ICSI and IVF were compared. With ICSI, a statistically significant negative relationship was found between fertilization rate and percentage of sperm DNA fragmentation (DFI). The risk of non transfer due to blocked embryo development increased when the DFI exceeded 15% (18.2% for ICSI vs 4.2% for IVF) with an odds ratio of 5.05. The miscarriage risk increased four fold when the DFI exceeded 15% (37.5% for ICSI vs 8.8% for IVF). Sperm DNA fragmentation measured 2 to 5 months before the assisted reproduction procedure was a prognostic indicator of the fertilization, pregnancy, and miscarriage rates and the pregnancy outcome.

An interesting study published by Greco et al (93), they reported that the incidence of DNA fragmentation was markedly lower in testicular spermatozoa compared with ejaculated spermatozoa, and there were no differences in fertilization rate and cleavage rates and in embryo morphological grade between the ICSI attempts performed with ejaculated and with testicular spermatozoa. However, eight ongoing pregnancies were achieved by ICSI with testicular spermatozoa (44.4% pregnancy rate; 20.7%implatation rate), whereas ICSI with ejaculated spermatozoa led to only one pregnancy which was spontaneously aborted.

Li et al (94) in a meta analysis study mentioned that for articles using the TUNEL assay, the pooled results of IVF outcomes indicated that the clinical pregnancy rate (RR 0.68, 95% CI 0.54 to 0.85, P = 0.006), but not the fertilization rate (RR 0.79, 95% CI 0.54 to 1.16, P = 0.23) decreased significantly for patients with high degree of sperm DNA damage compared with those with low degree of sperm DNA damage. In addition to, there was no significant difference in either fertilization rate (RR 1.03, 95% CI 0.89 to1.18, P = 0.70) or clinical pregnancy rate (RR 0.76, 95% CI 0.55 to 1.04, P = 0.09) between these two groups. As for the SCSA papers, the pooled results s howed no significant effects of sperm DNA damage on the clinical pregnancy rate after IVF (RR 0.58, 95% CI 0.25 to 1.31, P = 0.19) or ICSI (RR 1.18, 95% CI 0.81 to 1.74, P=0.38). Thus, there are still some controversies on the effect of sperm DNA damage and ART outcomes. However, majority of the studies indicated sperm DNA damage have negative impact on pregnancy rate, embryo quality, live birth and early pregnancy loss (Table I).

Table I. The results of studies on sperm DNA damage and outcomes in the context of assisted reproductive techniques.

Method of diagnose	Pregnancy rate	Embryo quality	Early pregnancy loss	Live birth
Studies assessed by TUNEL				
Tomlinson et al., 2001(95)	Decrease	No relation	-	-
Benchaib et al.,2003(96)	Decrease	Decrease	-	-
Huang et al., 2005 (86)	No relation	No relation	-	-
Borini et al., 2006 (90)	Decrease	-	Increase	Decrease
Benchaib et al., 2006 (92)	Decrease	Decrease	Increase	Decrease
Studies assessed by Comet assay				
Morris et al., 2002(69)	-	Decrease	Increase	Increase
Tomsu et al., 2002 (39)	Decrease	Decrease	-	-
Nasr-Esfahani et al., 2005 (97)	-	Decrease	-	-
Studies assessed by SCSA or SCD				
Larson-Cook et al., 2003 (83)	Decrease	No relation	-	-
Gandini et al., 2004 (91)	No relation	Decrease	-	-
Bungum et al., 2004 (41)	No relation	-	-	-
Payne et al., 2005 (88)	No relation	-	-	-
Muriel et al., 2006 (87)	Decrease	Decrease in implantation rate	-	-
Bungum et al., 2007 (98)	Decrease	-	- Decrease	Decrease

- : not determined
TUNEL: Terminal transferase dUTP nick end labeling
SCD: Sperm chromatin dispersion test
SCSA: Sperm chromatin structure assay
COMET assay performed by single cell electrophoresis.

Is it safe to use DNA damaged human sperm in ART?

The safety of the ICSI procedure in severely compromised semen characteristics and DNA damage has been questioned. In the study of Bungum et al (41) the men in the ICSI group had significantly higher DFI levels compared with the men in the IVF group (median 18% versus 15%). Consequently, concern arises as to the fact that the most efficient ART techniques are used to treat males with the highest level of sperm DNA damage. Larson- COOK et al (83) support that ICSI overrides safeguards that typically prevent sperm with damaged DNA to fertilize via spontaneous pregnancy or conception after conventional IVF.

Aitken and Krausz (25) proposed that sperm DNA damage is promutagenic and can give rise to mutations after fertilization as the oocyte attempts to repair DNA damage prior to the initiation of the first cleavage. Mutations occurring at this point will be fixed in the germline and may be responsible for infertility, childhood cancer in the offspring, and imprinting diseases (99). The significant decrease in implantation and pregnancy rates using sperm with high DFI indicates that the damaged paternal genome is selected against during embryonic development which provides a possible explanation for the lack of evidence for an increased incidence of major congenital malformations among children born after ICSI (100).

Gandini et al (91) stated that the biological impact of an abnormal sperm chromatin structure depends on the combined effects of extend of DNA damage in the spermatozoa and the capacity of the oocyte to repair that damage. Therefore, if spermatozoa selected from samples with extensively damaged DNA are used for IVF, the oocyte repair capacity may be inadequate leading

to a low rate of embryonic development and high early pregnancy loss. However, although we are now reasonably able to assess the damage level of a sperm population we cannot assess the repair capability of the oocyte, neither can the possibility of selection of sperm with limited DNA damage compensated by the oocyte repair capabilities, in a sample characterized by a high DFI , be excluded.

In general, studies have not shown an increased risk of major birth defects in children conceived with either ICSI or standard IVF (101). Much of this research, however, has had methodological problems, including inadequate sample sizes and a lack of appropriate data for comparison. On the contrary, some studies showed that, the increased risk for major and minor birth defects after ICSI described in some studies might be attributes to parental background factors that required the use of ICSI and not to the technique itself (102).

Michèle Hansen et al (103) documented that twenty-six of the 301 infants conceived with ICSI (8.6%) and 75 of the 837 infants conceived with IVF (9.0%) had a major birth defect diagnosed by one year of age, as compared with 168 of the 4000 naturally conceived infants (4.2%) As compared with natural conception, the odds ratio for a major birth defect by one year of age, after adjustment for maternal age and parity, the sex of the infant, and correlation between siblings, was 2.0 (95% confidence interval, 1.3 to 3.2) with ICSI, and 2.0 (95%confidence interval, 1.5 to 2.9) with IVF. Therefore, infants conceived with use of ICSI or IVF have twice as high a risk of a major birth defect as naturally conceived infants. ART raises specific concerns about the health of sperm used for fertilization. It seems that the development of new methods for identification, selection and use of spermatozoa with intact DNA during ART would eliminate the risk of inheriting genetic diseases.

Cryopreservation and DNA human spermatozoa

Despite various advances in cryopreservation methodology, the recovery rate of functional post-thaw spermatozoa remains mediocre, with sperm motility being significantly decreased after freezing. The most commonly reported detrimental effect of cryopreservation on human spermatozoa is a marked reduction in motility (104). The primary cause of cellular damage during cryopreservation is the formation of intracellular ice (105). Whenever cells, or culture media, are cooled below their freezing point, water is removed from the solution in the form of ice. The concentration of solutes remaining in the unfrozen fraction increases, thereby both depressing the freezing point and increasing the osmotic pressure of the remaining solution. Hence, biological systems freeze progressively over a wide temperature range, during which the solute becomes gradually more concentrated as the temperature falls (106). This leads to irreversible rupturing of plasma and nuclear membranes and disturbance of cellular organelles. The nucleus has generally been considered to be a stable constituent of the cell, but that inappropriate chromatin condensation can occur with freezing Further cellular damage may be caused during the thawing process as the ice melts or re-crystallizes. Slow thawing is most likely to induce injury, as it allows time for consolidation of microscopic ice crystals into larger forms which are known to be damaging (107).

Duru et al (108) reported that cryopreservation-thawing of human sperm from patients was associated with membrane change, as revealed by membrane translocation of phosphatidylserine, while having no major impact on DNA fragmentation. Probably the DNA of spermatozoa obtained from infertile men is more susceptible to be damaged by freeze-thawing rather than the fertile men sperm DNA (20). On contrary, de Paula et al (109) suggested that cryopreservation induces apoptotic sperm DNA fragmentation regardless of sperm concentration and the increase in DNA fragmentation was found to be similar in both normozoospermic and oligozoospermic men. However, men with oligozoospermia presented with higher pre- and post-cryopreservation apoptotic sperm DNA fragmentation.

DNA damage of human sperm has been reported to be less with flash-freezing in liquid nitrogen that performed without the use of cryopreservative. This technique gives the closest results to those reproduced by cryopreservation of fresh human semen samples (68). Isachenko et al (110) compared the results of slow-rate freezing and vitrification also showed that the vitrification of human spermatozoa in the absence of conventional cryoprotectants is indeed feasible. Thus, DNA integrity of vitrified sperm is comparable with the results obtained in spermatozoa that cryopreserved by standard slow-freezing/thawing. As well, the same group also suggests that optimal regimes for the cryoprotectant-free cryopreservation of spermatozoa should not restricted to only very fast cooling, but a wide range of cooling rates can be acceptable before storage in liquid nitrogen (111).

Conclusion

Traditional semen parameters have become less important in the evaluation of sperm quality. Clinical evidences now point to sperm DNA damage as a detrimental factor to reproductive outcomes and spermatozoa of infertile men have more DNA damage than do spermatozoa of fertile men. Testing sperm DNA integrity may help in selection of spermatozoa with the minimal damage for use in assisted conception. ART procedures bypass the natural selection process, subsequently increases the chance of sperm with abnormal genomic material fertilizing an oocyte. The impact of sperm DNA damage on fertilization rates remains controversial, but there is a kind of agreement about its negative effects on embryo development and pregnancy rates. Additional studies are needed to fully clarify the clinical value of testing of sperm DNA damage and its impact on reproduction.

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