Basic and Clinical Aspects of Sperm Comet Assay

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Luke Simon and Sheena E.M. Lewis

Abstract

Sperm DNA damage is associated with poorer assisted reproductive treatment (ART) outcomes including reduced fertilization rates, embryo quality, and pregnancy rates and higher rates of spontaneous miscarriage and childhood diseases. It shows promise as a more robust biomarker of infertility than conventional semen parameters. Among the sperm DNA testing methods, the alkaline comet assay is a sensitive, reliable, and powerful tool to detect even low levels of DNA damage within individual sperm. The present chapter provides an overview of the use of the alkaline comet assay in sperm. This includes the need for standardization of the alkaline comet assay protocol and its present strengths and weaknesses. Since sperm DNA damage is often the result of increased oxidative stress in the male reproductive tract, primarily formed due to an imbalance between reactive oxygen species generation and antioxidant depletion, a novel addition to the comet assay to measure oxidized bases is explored. The potential use of antioxidant therapy to protect against such damage is also described. Finally, the diagnostic and prognostic values of sperm DNA damage measures in determining the assisted reproductive technology (ART) success are discussed.

Keywords

Sperm comet assay • Sperm DNA • Assisted reproductive treatment • Comet assay • Oxidative stress in male reproduction

The Need for Novel Diagnostic and Prognostic Tests

Male infertility is implicated in more than 40% of couples presenting for treatment with assisted reproductive technology (ART). Conventional

S.E.M. Lewis (✉)
Centre for Public Health, Queen's University of Belfast, Room 208, Institute of Clinical Science, Belfast, Northern Ireland, UK
e-mail: s.e.lewis@qub.ac.uk

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semen analysis continues to be the only routine test to diagnose male infertility. However, semen analysis cannot discriminate between the sperm of fertile and infertile men [1]. Recent evidence has suggested that instability in the genomic material of the sperm nuclei is a more robust parameter in measuring the fertility potential of sperm, either in vivo or in vitro. For a test to be useful diagnostically or prognostically, it must have a threshold value that provides a discriminatory power above or below the threshold value with little overlap between groups of fertile and infertile men and couples with ART success and failure. However, neither the routine semen analysis nor the available sperm DNA tests yet meet these standards (reviewed in references [2, 3]).

The primary function of the sperm is to deliver the paternal genome to the oocyte. Recent studies have shown a number of sperm nuclear abnormalities such as DNA strand breaks, Y chromosome microdeletions, alterations in chromosome number, distorted epigenetic regulation and sperm's environmental milieu during epididymal transport and ejaculation. Factors such as increased oxidative stress or low levels of antioxidants may have implications on male reproductive health [4]. As the structural organization of the sperm chromatin is also essential for the normal function of the sperm [5], characterization of sperm DNA quality has gained importance. In recent years, comet assay, TUNEL, SCSA, and SCDA or Halo assay, in situ nick end labeling have been studied extensively to analyze sperm chromatin integrity. Each of these tests determines different aspects of DNA integrity, but to date, combining all the studies available in meta-analysis shows that these tests lack the statistical power and diagnostic potential necessary to incorporate them into routine clinical use.

Causes of Sperm DNA Damage

In recent years, the generation of reactive oxygen species (ROS) has been widely studied in the male reproductive tract and reported to be a concern because of their toxic effects on sperm quality and function (reviewed by Saleh and Agarwal [6]). They have been shown to cause DNA fragmentation in the reproductive tract as well as damage in ejaculated sperm [7]. High levels of ROS have also been reported in the seminal plasma of infertile men [8]. Sperm are vulnerable to the oxidative-stress-mediated damage, due to their structure with a high proportion of polyunsaturated fatty acids in their plasma membranes [9]. As sperm cannot repair such damage, sperm DNA has evolved to protect itself by compact packaging of the sperm DNA by protamines [10, 11].

The exact mechanisms by which ROS induces DNA damage are poorly understood, However, ROS-induced sperm DNA damage is exemplified by DNA cross-links, frameshifts, production of base free sites, chromosomal rearrangements and DNA base-pair oxidation [12–14]. It is also well known to cause strand breaks, with the levels of ROS correlated with increased percentage of single and double-strand damage in sperm [15–17]. ROS-mediated DNA damage is also seen in the formation of modified bases, which are often converted into strand breaks and considered to be important biomarkers for oxidative DNA damage [18]. Finally, ROS cause gene mutations such as point mutations and polymorphism [19, 20].

Seminal plasma is contaminated with ROS [21, 22] primarily produced by leukocytes and defective sperm [23]. The presence of elevated levels ($>1 \times 10^6$/mL) of leukocytes in the semen is defined as leukocytospermia [24] and is associated with increased levels of ROS, leading to sperm DNA damage [25]. Cytoplasmic droplets are also associated increased ROS generation and poor sperm quality [26, 27].

Environmental and Lifestyle Hazards

It has recently been reported that male fertility declines with age, even though spermatogenesis continues [28]. An increase in male age has been associated with increased genetic and chromosomal defects [29, 30]. Men over 37 years have been shown to three times more sperm DNA

damage then men aged <37 years [31, 32]. Male germ cells are particularly vulnerable to environmental chemicals and xenobiotics that cause DNA damage [33]. Studies also show the adverse impact of some occupations to increase the sperm DNA damage, for example among coke oven workers [34]. Oh et al. [35] concluded that there are elevated levels of DNA damage among waste incineration workers, when compared with men from similar origin. Further, men working in the factories with organic molecules such as styrene show a significant amount of increase in sperm DNA damage [36]. Similarly, men working in the insecticide and pesticide industries have higher levels of sperm DNA damage [37, 38].

A further hazard for sperm DNA is by pharmacological exposure to drugs. This has become very common as molecular medicine advances, especially in the field of cancer. Chemotherapeutic drugs are genotoxic to the male germ cells. A well-known example for such an intervention is the cyclophosphamide [39, 40] in animal model. Hellman et al.'s [41] cyclophosphamide treatment resulted in a five fold increase in DNA damage. Environmental exposure of xenobiotics cannot be avoided in our contemporary lifestyle because these pollutants are present in our food, water, and air. Studies have shown the association between environmental estrogens and derived compounds and male infertility through elevated sperm DNA damage [42]. Environmental pollutants such as organochlorides [43] and smog [44] also have the ability to induce DNA damage. Bennetts et al. [45] showed that estrogenic compounds such as 2-hydroxyestradiol induce redox cycling activities and concomitant sperm DNA damage. These examples support the belief that exposure to xenobiotics has powerful impacts on sperm DNA and sperm functions, leading to male infertility.

Lifestyle choices also play an important role in male infertility. For example, smoking and consumption of alcohol and caffeine have been associated to the increase in nuclear DNA damage of the white blood cells [46, 47]; on the contrary, very little is known about their effect on sperm DNA [48]. There is a very strong and significant correlation between smoking and genetic defects in the sperm [49, 50]. Smoking increases oxidative stress, which results in depletion of antioxidants in the seminal plasma, thereby inducing oxidative DNA damage to the sperm [15] and mutagenic adducts [51]. Recent studies have also suggested a possible link between cell-phone use associated with electromagnetic radiations and sperm DNA damage [52–56]. Finally, physical factors such as mild scrotal heating [57] and radio frequencies [55] have also been proven to diminish sperm DNA integrity.

The Comet Assay: What Does It Measure?

For a sperm DNA test to be clinically useful, (a) it should measure both single- and double-strand breaks, as both may be important and the oocyte has limited ability to repair fragmented paternal DNA, (b) it should measure the level of DNA fragmentation in each sperm, as an ejaculate is known to show a high degree of variation, (c) the methodology should be appropriate for cell lysis and DNA decondensation for full extent of damage to be determined, (d) the test must have strong predictive capacity for pregnancy outcome and little overlap between fertile and infertile samples. Among the tests currently available, the alkaline comet assay addresses the first three above-mentioned issues but useful thresholds have not been established yet to validate the assay.

Initially, the comet assay [58] was designed to characterize the structure of the nucleus. However, when electrophoresis of DNA strands after alkaline denaturation came into existence in 1988 by Singh et al., the detection of DNA damage within the nucleus became a possibility. Collins et al. [59] suggested that the migrated comet tail after electrophoresis consists of fragments originated from relaxation of supercoiled loops and single-stranded DNA formed under alkaline conditions. Some studies suggest that double-strand DNA breaks alone may be detected under neutral conditions

(pH 8–9) [60, 61], and in these studies the level of measurable DNA damage is low compared to the alkaline comet assay. This is due to either the measurement of additional DNA damage by the alkaline condition or the relatively higher migration of DNA strands under alkaline conditions [62].

The extent of DNA damage in individual cells could be monitored by the use of image analyzing system. Presently, different commercial software packages are available to measure the comet parameters. A fully automated comet analyzing system has also been developed [63]. In the past, different methodologies were used to measure the extent of DNA damage such as the proportion of cells with altered tail DNA migration, approaches classifying comets into several categories based on the tail migration [64, 65]. However, these approaches are generally limited to electrophoretic conditions. Hughes et al. [66] reported that the evidence for intact DNA is considered more important in relation to fertility status than measurement of other comet parameters that could be altered by the experiment conditions.

The commonly used comet parameters are percentage head DNA, percentage tail DNA, tail length, and olive tail moment. The software system analyzes the light intensities (fluorescence) in the head relative to the tail to determine the percentage of DNA present in the head and tail. The background light intensity is subtracted from head and tail intensities to get the actual value. Also, the sperm populations are known to be more heterogeneous, and the baseline values of DNA damage of sperm population in an ejaculate are substantially higher than those in somatic cells [67]. Although, few number of sperm could be analyzed in the comet assay, Hughes et al. [66] demonstrated that the analysis of 50 sperm is sufficient to provide a measurement of DNA damage of the total sperm population with a coefficients of variation lower than 4%.

The comet assay is highly sensitive to detect extensive fragmented cell in the form of nonexistent heads or a large diffused tail termed as "ghost" or "clouds" or "hedgehogs" [68]. In such cases, the comet image system cannot interpret the full extent of DNA damage [69]; therefore, it is advisable to consider the ghosts as completely damaged cells. In sperm, such highly damaged cells should not be excluded during analysis [70]. The DNA-specific fluorescent dyes are used for comet visualization. The most frequently used fluorescent dyes are ethidium bromide, propidium iodide, DAPI, SYBR Green I, and benzoxazolium-4-quinolinum oxazole yellow homodimer [71]. Addition of an antifade reagent along with fluorescent dyes could significantly reduce fluorescence quenching [72]. No fluorescent dyes such as silver nitrate are also reported for comet assay; however, the efficiency of the assay is reduced [73]. Excess of fluorescence dye could increase the background intensity of the slides thereby very low-molecular-weight DNA fragments could not be measured. Hence, standardization of the comet assay is required for accurate performance.

Strengths of Comet Assay

The comet assay is one of the most sensitive techniques available to measure DNA damage, and according to Aravindan et al. [74], the results of comet assay are also related to the results obtained from the TUNEL assay. The alkaline comet assay could be used in all the cell types and also in the sperm [75]. The assay requires only a few numbers of cells; hence, the assay is possible in cases of oligospermia and testicular biopsy. The DNA damage data can be collected at the level of individual cells, making the analysis efficient. The removal of protamines and histones during the assay reveals the total DNA damage in the cell. The range of DNA damage measured in sperm using the alkaline comet assay varies from 0–100% showing its capacity to identify sperm with much or little damage. A further advantage is that, unlike the TUNEL and SCSA, which detect primarily breaks in histone-associated chromatin, the comet assay has a broader use in detecting breaks in both protamine- and histone-bound chromatin equally.

Weaknesses of Comet Assay

One disadvantage of the comet assay is that it lacks standardized protocols, which makes it difficult to combine the results from different

laboratories [76]. This should be resolved by agreement on an optimal protocol (see next section). The assay is criticized for the use of high pH conditions, which is known to denature the alkaline-labile sites measurable after electrophoresis [77], making it difficult to discriminate between endogenous and induced DNA breaks. However, labile sites may be considered as another form of potential damage, and some consider this as a strength, in that an indication of existing and potential damage may be more important clinically. The assay is also criticized for an underestimation of DNA damage that may occur through entangling of DNA strands or the presence of proteins and cross-linked DNA strands, which could restrict the movement of DNA fragments during electrophoresis. In some protocols, incomplete chromatin decondensation will not allow all strand breaks to be revealed. Overlapping comet tails decrease the accuracy of the assay, and few small tail fragments are lost or too small fragments are difficult to be visualized. As in other DNA tests, strong reducing agents are sometimes used to remove protamines, and they may increase what is perceived to be baseline damage. Also, the assay requires a laborious process of analysis and shows high interlaboratory variation and, hence, is not used clinically [78]. Owing to a labor-intensive and sensitive protocol, the assay requires skilled technicians for accuracy. Finally, the available software to measure DNA damage cannot recognize "Ghost cells" without head DNA and overlapping comet tails, making the scoring difficult. However, most of these weaknesses can be corrected with appropriate protocols and training.

Need for Standardized Methodology for the Comet

The comet assay is currently used primarily for genotoxic studies, although it is a test with great potential for ART [79]. For use with sperm, a number of academic and methodological issues need to be addressed, as there is no generally accepted protocol for the assay, even though international groups of scientists [53, 80–82] have used it extensively.

The first variation relates to lysis conditions. Absence of cytoplasm in sperm makes it difficult to optimize lysis conditions compared to the somatic cells. For example, in some labs, lysis of plasma membranes is performed by incubating cells with a buffer (usually containing Proteinase K, Triton X-100, and high concentrations of NaCl) for a short time (3 h), in others a long, even overnight period (18 h) [83–85].

As discussed previously, the sperm genomic DNA is more highly condensed than somatic cells preventing the migration of the comet tail, so for use with sperm it requires the use of additional steps to decondense the tightly packed DNA. A wide range of strong reagents (Proteinase K, Triton X-100, Dimethyl Sulfoxide, DTT, and LIS) have been used to remove protamines and histones [67, 83, 84, 86–88], but these agents may also induce damage. The presence of these different approaches prevents interlaboratory comparisons.

To reduce the level of laboratory-induced damage and make the assay more reproducible, our group has replaced Proteinase K with DTT and LIS and for a shorter duration of 3 h [85].

Another difference between labs is the pH at which the assay is performed. Currently, electrophoresis is carried out with wide range of buffers with pH ranging from pH 8.0 to 13.5 [66, 67, 84–86, 89]. Such a wide range of pH conditions again makes results difficult to compare, as the extent of DNA migration is highly influenced by the degree of alkali denaturation and the pH value.

A further confusion from "comet" studies comes from the lack of standardization of comet parameters described in different studies. There are several parameters used in comet studies. McKelvey et al. [90] described it as "DNA migration can be determined visually by the categorization of comets into different "classes" of migration. The percentage of DNA in the tail (percent migrated DNA), tail length and tail moment (fraction of migrated DNA multiplied by some measure of tail length). Of these, tail moment and/or tail length measurements are the most commonly reported, but there is much to recommend the use of per cent DNA in tail, as this gives a clear indication of the appearance

of the comets and, in addition, is linearly related to the DNA break frequency over a wide range of levels of damage. The approach or parameter used must be clearly defined and, if not typical, be justified."

Hughes et al. [67] recommended the use of percent tail DNA, as its coefficients of variation was less than 4%. Measurement of fifty comets from a single slide is reported to have a coefficient of variation of less than 6% within a sperm population [67]. They also reported the reproducibility of the image analysis software with repeated analysis of individual sample showed a coefficient of variation of less than 5.4%.

Tice et al. [71] recommended the measurement of tail length, percent tail DNA and tail moment, finding different results between tail DNA and tail moment. However, Kumaravel and Jha [91] did not find any statistical difference with olive tail moment and percentage tail DNA to analyze the extent of DNA damage. The percentage of tail DNA is reported to be directly proportional to the dosage of radiation and concentration of hydrogen peroxide. By contrast, the olive tail moment is highly influenced by the study conditions, so it is not consistent between labs and, thus, not advisable for use.

In summary, agreement on a standardized protocol for the comet to necessary to compare results between groups. To reduce the additional DNA damage caused during the assay procedure, the duration of lysis, the composition of the lysis buffer, the method of decondensation, the pH for unwinding, and electrophoresis condition and parameters to be reported should be standardized.

Clinical Significance of DNA Fragmentation Measured by the Comet Assay

The alkaline comet assay is proving to be a useful diagnostic tool for male infertility. The clinical importance of the comet assay in assessing male infertility has been demonstrated by a number of authors [79, 92–94]. However, until recently, its predictive value in assisted reproduction outcome has been assessed by few [86, 95].

In a recent study from our group [82] of 360 couples having IVF or ICSI we reported that sperm DNA damage is associated with poorer ART outcomes and promises to be a more robust biomarker of infertility than conventional semen parameters. We found significant inverse correlations between DNA fragmentation, fertilization rate, and embryo quality assessed by the alkaline comet assay (to detect both double and single strand breaks) following IVF treatment. A decrease in fertilization rates were observed as DNA damage of native semen and DGC sperm increased. Low DNA damage (0–20%) showed a significantly higher fertilization rate compared with DNA damage >60%. Our work supports that of Morris et al. [88] who also reported a significant correlation between fertilization and DNA damage when measured by the neutral comet assay (measuring double-strand breaks only). However, by contrast, no correlations were observed between fertilization rates and DNA fragmentation measured in alkaline comet assay by Tomsu et al. [95].

Our study [82] also showed a decrease in embryo quality following IVF treatment, as DNA fragmentation increased both in native semen and DGC sperm. The embryo quality showed a significant decrease, when DNA damage was greater than 60% in the native semen. The embryo cumulative score calculated according to Steer et al. [96] was 15.5 in the group where sperm DNA fragmentation was <20% and was only 7.3 where sperm DNA fragmentation was >60% in DGC sperm. Similarly, Tomsu et al. [95] showed a negative correlation between embryo quality and DNA fragmentation in both the native semen and the DGC sperm. However, Morris et al. [88] did not find any association in embryo quality and DNA damage. In contrast to associations following IVF, we did not find any correlation between sperm DNA damage and fertilization rate or embryo quality when ICSI was used as a treatment of choice [82].

Using pregnancy as the outcome measure, Morris et al. [88] did not find an association between clinical pregnancy and sperm DNA

fragmentation measured by the neutral comet assay. Similarly, Tomsu et al. [95] in a small study ($n=40$) no associations were found. However, we found a significant difference in DNA fragmentation of clinically pregnant and nonpregnant couples following IVF [82]. By contrast, although couples undergoing ICSI who failed to achieve a clinical pregnancy tended to have more DNA fragmentation but it was not statistically significant.

Further Uses of the Comet to Measure DNA Adducts

A major cause of sperm DNA damage is oxidative stress due to the generation of the ROS from contaminating leukocytes, defective sperm, and antioxidant depletion [23, 97]. FPG is the commonly used bacterial repair enzyme that could recognize and excise 8-OHdG and other modified bases generated by ROS. This FPG enzyme has been shown to possess affinities toward the various modified DNA bases [98, 99]. The catalytic activity of FPG involves a three-step process: (a) hydrolysis of the glycosidic bond between the damaged base and the deoxyribose, (b) incision of DNA at abasic sites, leaving a gap at the 3′ and 5′ ends by phosphoryl groups, and (c) removal of terminal deoxyribose 5′-phosphate from 5′ terminal site to excise the damaged base showed by Kuznetsov [100].

When a eukaryotic or prokaryotic base repair enzyme or glycosylase is introduced as an intermediate step during the alkaline comet assay, the modified bases can be converted into single-strand breaks [101, 102]. Addition of base repair enzymes can increase the sensitivity of the assay by including the modified bases, resulting in total DNA damage measured after the alkaline comet assay [103]. Among the modified bases, 8-OHdG is the most commonly studied biomarker and is often selected as a representative of oxidative DNA damage due to its high specificity, potent mutagenicity, and relative abundance in DNA [33, 104]

Clinical Significance of Existing Strand Breaks Plus Adducts Measured by the Comet Assay

To analyze modified bases in the sperm DNA, we have used the prokaryotic repair enzyme (FPG) as an intermediate step during the alkaline comet assay, to introduce breaks at sites of modified bases [82]. We found inverse relationships between total DNA damage (existing strand breaks plus modified bases) and IVF and ICSI outcomes after conversion of modified bases to DNA strand breaks by FPG. There was a significant increase in DNA damage after treatment with the DNA glycosylase FPG in both native and DGC samples. In the IVF patients, addition of the FPG enzyme showed a significant increase in mean percentage of sperm DNA fragmentation in nonpregnant compared with that from pregnant couples (55 vs. 72) in the native semen and (42 vs. 56) in DGC sperm. Similarly, in ICSI couples, when modified bases were included, the percent DNA damage between pregnant and nonpregnant couples was markedly different (63 vs. 80 in native semen, and 50 vs. 65 in DGC sperm), in contrast to comet assay without FPG where it was not significant.

The Risks of Using Sperm with Damaged DNA

Sperm DNA damage measured by SCSA, TUNEL, and alkaline and neutral comet assays has been closely associated with all the stages of ART outcome such as fertilization, embryo quality, implantation pregnancy, and spontaneous abortion [105, 106]. A limited amount of sperm DNA damage can be repaired by the oocyte post fertilization, but above a threshold limit this process is either incomplete or inappropriate, resulting in genetic mutations and may impact the viability of the embryo and the health of the offspring [107]. Men suffering from male infertility have high levels of sperm with DNA damage, which result in an negative impact on their ART outcome [25, 108–112].

In recent years, sperm DNA damage has gained interest to understand the fertilization process to improve fertility diagnostics. The influence of DNA damage on fertilization rates in assisted reproduction is still controversial. A number of papers have analyzed the possible association between sperm DNA damage and fertilization rates in vitro [16, 106, 111, 113–127]. But, many of these papers suggest that sperm DNA damage does not affect fertilization rates [106, 111, 115, 117–120, 126, 127]. Sperm with damaged DNA are still capable of fertilization [93] but its effect is prominent in the later stages [128]. Sperm with abnormal chromatin packing and DNA damage is showed to result in decondensation failure, which results in fertilization failure [25]. It is also showed that that a significantly proportion of nondecondensed sperm in human oocytes has a higher DNA damage, compared to decondensed sperm and higher degree of chromatin damage, this may prevent the initiation or completion of decondensation, and may be an important factor leading to a failure in fertilization [129]. A negative correlation between the proportion of sperm having DNA strand breaks and the proportion of oocytes fertilized after IVF is established [114].

Measurement of sperm DNA damage has been shown to have a significant negative effect on the developing embryo [130]. Poor sperm DNA quality is associated with poor blastocyst development and the failure to achieve a clinical pregnancy. Sperm DNA damage has a significant impact on embryo development [16, 95, 105, 114, 126, 129, 131–133]. However, a number of studies have contradicted the influence of DNA damage on embryo development [106, 108, 109, 112, 115–119, 122, 123, 126, 127]. Abnormalities in the embryo seen in vitro can be more directly related to male factors because the results can be assessed without the interference of female factors such as uterine and endocrine abnormalities that may lead to miscarriage after embryo transfer [134]. The embryonic genome is activated on day three, and its transcriptional products take over from the regulatory control provided by maternal messages stored in the oocyte [132]. The effect of sperm DNA damage has been attributed to embryo development, particularly between four and eight cell stage of preimplantation development until which the embryonic genome is transcriptionally inactivated and the paternal genome plays a significant contributory role in embryo function during the transcriptional activity [133]. Therefore, the effect of sperm DNA damage impacts more on pregnancy rates than embryo quality [115].

Couples who failed to achieve a pregnancy are known to have a higher mean level of DNA fragmentation than pregnant couple after IVF treatment [105, 112, 115, 118–120, 122, 133, 135]. This implies that sperm with DNA fragmentation can still fertilize an oocyte but that when paternal genes are "switched on," further embryonic development stops, resulting in failed pregnancy [121]. In contrast to these reports, no significant association between sperm DNA damage and clinical pregnancies has been reported [88, 95, 114, 116, 123, 124, 126, 127, 136, 137]. Studies using animal models show that oocytes and developing embryos can repair sperm DNA damage; however, there is a threshold beyond which sperm DNA cannot be repaired [138]. They also reported that sperm with defective DNA can fertilize an oocyte and produce high-quality early-stage embryos, but then, as the extent of the DNA damage increases, the likelihood of a successful pregnancy decreases. Virro et al. [132] have shown that high levels of sperm DNA damage significantly decrease the pregnancy rates and results in higher rate of spontaneous abortions. An increase in sperm DNA damage is associated with decreased implantation, thereby a decrease in pregnancy rates [118]. By contrast, Bungum et al. [136] and Boe-Hansen et al. [137] showed a decrease in implantation rates with increase in DNA damage but no effect is seen on clinical pregnancies. Frydman et al. [106] showed increase in DNA damage not only decrease implantation and pregnancy rates but also increase spontaneous miscarriage rates. Lin et al. [127] also observed an increase in miscarriage rates with an increase in DNA damage.

It is also shown that damage in the paternal genome could result in abnormalities occur during postimplantation development [139]. Genetic abnormalities in the paternal genome in the form of strand breaks are a significant cause of miscarriages [134]. Sperm DNA damage could likely be the cause of infertility in a large percentage of patients [140]. However, these studies may not causal, but simply associations between DNA damage and reduced ART outcomes. Are the tests clinically useful?

The Clinical Usefulness of the Comet Test

Two recent systematic reviews have shown that the impact of sperm DNA damage on ART outcomes decreases from IUI to IVF and is least useful in ICSI [3, 141]. In IVF, using TUNEL and SCSA assays, the odds ratios is 1.57 (95% CI 1.18–2.07; $p < 0.05$). However, in our study using the alkaline comet [82] we obtained an odds ratio of 4.52 (1.79–11.92) in native semen and 6.20 (1.74–26.30) in DGC sperm for clinical pregnancy following IVF, indicating its promise as a prognostic test. Owing to the high sensitivity of the test and level of damage observed when both strand breaks and modified bases were measured it was not possible to establish thresholds for our novel combined test. Following ICSI, the odds ratio for clinical pregnancy was 1.97 (0.81–4.77) using native semen and 2.08 (0.93-4.68) in DGC sperm showing less strength and supporting the combined odds ratio of 1.14 from the meta-analyses by Collins et al. [141] and Zini and Sigman [3]. This supports the belief that ICSI bypasses genetic, as well as functional defects, but the results are counterintuitive. Given the many animal studies showing adverse effects of DNA damage on the long-term health of offspring (reviewed by Aitken et al. [142]; Fernadez-Gonzalez et al. [143]), we need to follow-up the children born by ISCI to make sure that this genetic heritage does not have long-term adverse effects of these children's health even if short-term success in terms of pregnancies is achieved.

Two People but Just One Prognostic Test

The quest for one perfect test to predict a outcome with multifactorial input is particularly unachievable when this outcome involves not just one individual but, in the case of ART, two partners. Since female factors such as age, occyte and embryo quality, and uterine competence all impact significantly on pregnancy, it is not surprising that if one test on the male partner is not acceptably strong. The current literature exemplifies how the controversies as to the usefulness of sperm DNA testing are exacerbated by flawed experimental design. Couples undergoing IVF treatment can be divided into those with female, male, and unexplained infertility. A large proportion of couples undergoing IVF treatment are due to female causes.

In many studies, couples with male, female, and idiopathic infertility have been grouped together. In order to assess the clinical usefulness of a test for one partner of the infertile couple, the appropriate patient population should be identified. Future studies should be designed to minimize the variation in these female factors. Only then can we accurately determine the effects of sperm DNA and thereby maximize the usefulness of the test.

Protection of DNA from Damage

In the male reproductive tract, oxidative stress is due to the increase in the production of ROS, rather than the decrease in the seminal antioxidants. Owing to the lack of cytoplasm excluded during spermatogenesis, there is no self DNA repair mechanism in the sperm; therefore, antioxidants in the seminal plasma are essential to reduce the oxidative stress, and it is the only available mechanism for the sperm to protest against oxidative-stress-mediated DNA damage. Naturally, the concentration of antioxidants in seminal plasma is 10 times greater than in blood plasma [144], and the presence of antioxidants in the seminal plasma protects the functional

integrity of the sperm against the oxidative stress [145]. Several other studies showed the role of antioxidants against ROS [21, 146–148]. However, some studies show limited protection of antioxidants against induced ROS [149].

Low levels of antioxidants in semen are associated with suboptimal semen parameters (Kao et al. [189]) and increased sperm DNA damage [150]. Oral administration of the antioxidants has been shown to significantly increase antioxidant levels in the seminal plasma and an improvement in the semen quality [151–155]. Specifically, antioxidant treatment to infertile patients by oral administration of vitamins significantly improved their sperm motility [152, 154, 156–158], sperm concentration [12, 159, 160], and normal morphology of the sperm [152, 159]. Improvement in semen parameters by administration of oral antioxidants were seen in volunteers as well as patients [154, 161]. Studies by Lenzi et al. [162–164] reported a protective function of antioxidants on semen quality due to a reduction of ROS and a reduction in the lipid peroxidation of the membrane. By contrast, other studies have shown no significant effects of oral antioxidant treatment on semen parameters [165–167]. The absence of effects in these studies may be due to shorter duration of treatment [167, 168] and/or very low dosage of antioxidants used [169].

Administration of oral antioxidants had been shown to significantly decrease sperm DNA damage [12, 170–173] and to reduce sperm DNA adducts [174] and the incidence of aneuploidy in sperm [175], thereby increasing the assisted reproductive success [176, 177].

Protection of sperm from DNA damage should also be monitored during sperm processing and cryopreservation when they are especially vulnerable. The absence of antioxidant protection in these procedures has been shown to increase sperm DNA damage [15]. Zalata et al. [178] showed that high-speed centrifugation and removal of sperm from the protective seminal plasma resulted in ROS-mediated DNA damage. Addition of antioxidants in the sperm medium could decrease oxidative stress [179] and damage to sperm [180]. Donnelly et al. [181] showed that addition of vitamins in the sperm suspension media could protect the sperm from DNA damage. This in turn would have a positive effect on male infertility [174]. Cryopreservation of sperm is known to increase the level of sperm DNA damage [93, 182–184].

Oxidative stress occurs when the level of ROS exceeds the antioxidant protection resulting in sperm DNA damage. Approximately, half of infertile men exhibit oxidative stress [185]. In light of these considerations, future research to determine the best regime of antioxidant therapy so be pursued to find an effective treatment [186–188].

Conclusions and Future Recommendations

Clinical evidence shows the negative impact of sperm DNA fragmentation on reproductive outcomes, and sperm from infertile men show higher levels of DNA fragmentation than the sperm of fertile or donor men. Recent studies have shown that the use of alkaline comet assay to test sperm DNA fragmentation is a useful tool for male infertility diagnosis and early predictor of ART outcomes. Below novel "comet" threshold values of sperm DNA fragmentation in both native semen and DGC sperm obtained from the alkaline comet assay, there is evidence of infertility in vivo and in vitro. Therefore, it is beneficial to assess sperm DNA fragmentation in couples presenting with infertility problems and also in patients undergoing ART. We encourage studies to analyze the impact of sperm DNA fragmentation and to validate the current protocol of the alkaline comet assay through large multicenter trials, using good quality control, with standardized protocols.

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