Chapter 7
The Comet Assay

Elva I. Cortés-Gutiérrez, Martha I. Dávila-Rodríguez, and Carmen López-Fernández

7.1 Introduction

The main difference between a damaged DNA molecule in somatic and germ line cells is that while in somatic cells the DNA damage can be partially repaired, the DNA damage present in the germ line cells (this is true especially in spermatozoa, cells that possess a nonorthodox DNA molecule) cannot be repaired due to the absence of DNA repair mechanisms and a highly condensed chromatin structure. Repair of sperm DNA damage occurs within the oocyte after fertilization. There are four possible mechanisms that have been identified to play a role in the pathophysiology of sperm DNA damage: (i) abortive apoptosis: spermatozoa with defective DNA escape the physiological apoptotic pathway during meiosis I resulting in the ejaculate [1]; (ii) defective chromatin condensation during spermatogenesis: DNA breaks occur as a result of inappropriate protamination and insufficient chromatin packaging [2]; (iii) oxidative stress resulting from an imbalance between reactive oxygen species (ROS) production and antioxidant capacity [3]; and (iv) the existence of endogenous sperm nucleases that cleave the DNA into loop-sized fragments of about 50 kB [4]. This activity, in fact, resembles that of several nucleases in somatic cells that cleave the DNA into similar sizes during the activation of apoptosis [5–9]. The function of these nucleases in the mature spermatozoa is to carry the DNA to the oocyte without any damage. In humans, the nature of damaged DNA that occurs within certain patient populations is still poorly understood [10, 11].

E.I. Cortés-Gutiérrez (✉) • M.I. Dávila-Rodríguez
Department of Genetics, Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, IMSS, Monterrey, Mexico

Unit of Genetics, Department of Biology, Universidad Autónoma de Madrid, Madrid, Spain
e-mail: elvacortes@cibinmty.net; marthadavila@cibinmty.net

C. López-Fernández
Unit of Genetics, Department of Biology, Universidad Autónoma de Madrid, Madrid, Spain
e-mail: mariadelcarmen.lopez@uam.es

© Springer International Publishing AG 2018

A. Zini, A. Agarwal (eds.), *A Clinician's Guide to Sperm DNA and Chromatin Damage*, https://doi.org/10.1007/978-3-319-71815-6_7

One of the key aspects that needs to be investigated concerning the problem of cellular DNA damage is the discrimination between the presence of single-strand DNA breaks (SSBs) and double-strand DNA breaks (DSBs) or both affecting the same DNA thread. This is mainly related to the origin of the DNA damage. The causes of sperm DNA damage resulting in SSBs or DSBs are extremely variable and include exposure to adverse environmental factors such as pesticides, radiation, smoking, or pathological conditions such as cancer, varicocele, and infection (bacterial, viral) [12]. These and presumably other causes of sperm DNA breakage are mediated through one or a combination of the mechanisms mentioned above. With respect to the putative origin of DNA fragmentation in the sperm cells, we might also expect different types of DNA lesion that could possibly be predictive or diagnostic in nature. For example, nucleases, either endogenous or exogenous, usually produce SSBs and/or DSBs, whereas DNA breaks produced by chromatin remodeling during spermiogenesis appear to correspond to DSBs produced by topoisomerase II and SSBs produced by topoisomerase I [13]. On the other hand, we have ROS and other radical molecules such as those derived from nitric oxide which generate SSBs associated with the creation of abasic sites or the presence of 8-hydroxyguanine [14, 15]. It has been reported that more than 20 damaged DNA base lesions can be present in a cell exposed to oxidative stress [16].

The comet assay, also known as single-cell gel electrophoresis (SCGE), was developed in 1984 [17] and is known for its ability to detect DNA damage at a single-cell level. The rationale of the technique is very simple. The assay requires detergents to first lyse the cells embedded in an inert agarose matrix on a slide. A high salt concentration results in deproteinized nuclei recognized as nucleoids. Following this, DNA is electrophoresed. The idea of the technique is that nuclei containing DNA segments that are detached from the original chromosome migrate toward the anodes, resulting in an image resembling a comet that can be observed under the microscope. The comet is formed by a part of the original nucleoid retaining a large part of undamaged DNA and an emerging tail that putatively accumulates a large part of the DNA that presented DNA breaks (Fig. 7.1a, original image, and 7.1b, digitally enhanced images). Most of the DNA retained in the head consists of intact DNA that is not recognized by the technique, whereas the tail is made up of broken DNA or strands with heterogeneous molecular weights. The intensity of the comet represents the proportion of DNA that has been broken off, and the distance traveled by the comet relates to the relative size of the DNA fragments.

The comet assay commonly utilizes commercially available software programs to evaluate the extent of DNA damage at the single-cell level. These programs provide a large number of measurement outcomes, i.e., tail length (the length of the tail measured from the leading edge of the head), tail DNA percentage (the percentage of DNA in the tail compared to the percentage in the "head" or unfragmented DNA), and olive tail moment (OTM). OTM is the percentage of tail DNA × tail moment length (tail DNA percentage = 100 × tail DNA intensity/cell; the tail moment length is measured from the center of the head to the center of the tail). The OTM is expressed in arbitrary units. Each of these parameters describes endogenous DNA damage corresponding to DNA strand breakage and/or alkali-labile sites (ALSs). In

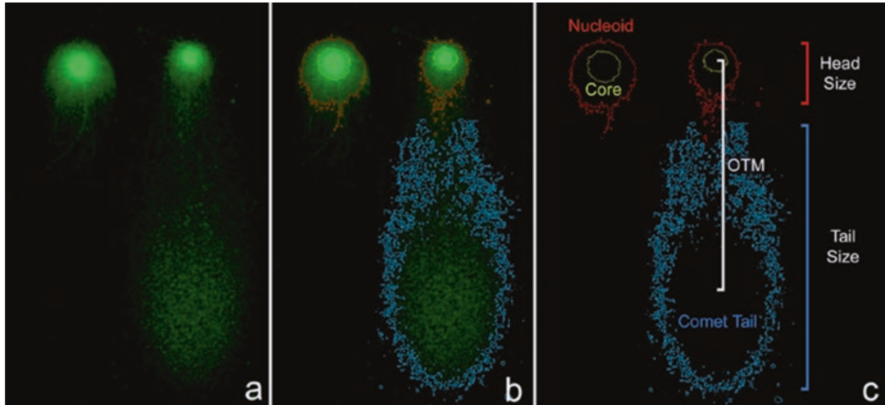

Fig. 7.1 Original (**a**) and image after application of a common electronic filter showing the comet head (consists of intact DNA) and emerging tail which possesses DNA breaks (**b**), features that are important for comet assay analysis (**c**)

the optimization of the alkaline comet for use with sperm, tail DNA was found to be the most reproducible parameter [18]; therefore, sperm DNA damage is expressed as tail DNA (Fig. 7.1c, digitally enhanced images).

The formation of a tail provides information on two important issues related to DNA damage concerning (i) the amount of DNA damage present in the original nucleoid and (ii) the type of DNA damage affecting the orthodox double-strand DNA conformation. For (i), it is generally assumed that the larger the tail and/or the higher the DNA density in the tail, the greater is the extent of DNA damage. For (ii), the presence of ssDNA breaks or dsDNA breaks or both affecting the same DNA thread is of crucial importance for understanding the images produced by the comet assay.

There are three types of comet assay techniques: (i) The neutral comet assay [18], in which DNA stretches originated from DSBs migrate under a neutral buffer according to the size of the DNA fragment. The larger the fragment, the lower the migration distance. (ii) The alkaline comet assay [19], in which DNA stretches containing both DSBs and SSBs migrate under alkaline conditions. Because alkaline conditions produce DNA denaturation, single-strand DNA threads resulting from DNA denaturation starting from the 5′ to 3′ free ends at the place of the DNA break migrate to the anode. (iii) The two-dimensional or two-tailed comet (2T-comet) assay combines the ability of the neutral and alkaline comet assay, allowing the differentiation within the same cell of the presence of DSB and SSB at the original nucleoid [20, 21].

These three techniques can be used on somatic cells, as well as in spermatozoa to assess DNA damage. However, as explained in detail later, given that the DNA is complexed with different proteins (histones, protamines, or both), depending on the cell type or cell activity, a general protocol does not exist to produce equivalent

results on different cells and the basic methodology requires technical adaptations to obtain the best results.

7.2 Neutral Comet Assay

DSBs are considered to be the most biologically lethal lesions affecting somatic and sperm DNA mainly because they are difficult to repair and often lead to genome instability even after being repaired by nonhomologous chromosome rejoining [22, 23].

The rationale of the technique is very simple, since is based on the principle that a naturally charged DNA molecule migrates when subjected to an electrophoretic field. Subsequently, identification of DNA damage is simple. Basically, when lysed cells with no DNA fragmentation are subjected to an electric field and by using a buffer under non-denaturing conditions, no substantial comet tails will be formed (Fig. 7.2a). In contrast, those spermatozoa containing sperm with a damaged DNA molecule tend to show an extensive migration of DNA fragments emerging from the original sperm nucleoid (Fig. 7.2b, c). These migrating DNA fragments are associated with the presence of DSBs at the origin, but it is not possible to know if the DNA threads that distribute along the comet tail contain SSBs.

Confirmation that comets visualized under neutral conditions are indeed large DNA molecules containing DSBs is based on the observation that similar comets can be produced after incubation with classic double-strand DNA cutters such as restriction endonucleases [24].

Double-stranded breaks may be a male infertility factor. Understanding its mechanism and its identification in different clinical groups (such as asthenoteratozoospermic (ATZ) with or without varicocele, oligoasthenoteratozoospermic (OAT), balanced chromosome rearrangements, and fertile donors) may be useful for determining the prognosis of male infertility associated with these conditions. Double-stranded DNA damage is also related to a higher risk of male factor-associated miscarriage, possibly due to the failure of repair of sperm DSB breaks by the oocyte [23].

7.3 Alkaline Comet Assay

The technical aspect of this version of the comet assay is similar to the previous methodology described with the principle based on the fact that DNA stretches containing 3′ 5′ free ends denaturize when the protein-depleted nuclei are subjected to an alkaline environment producing single-strand free threads of DNA. The alterations found in DNA such as strand breaks (single or double) result in the extension of DNA loops from lysed and salt-extracted nuclei; these in turn form a comet-like

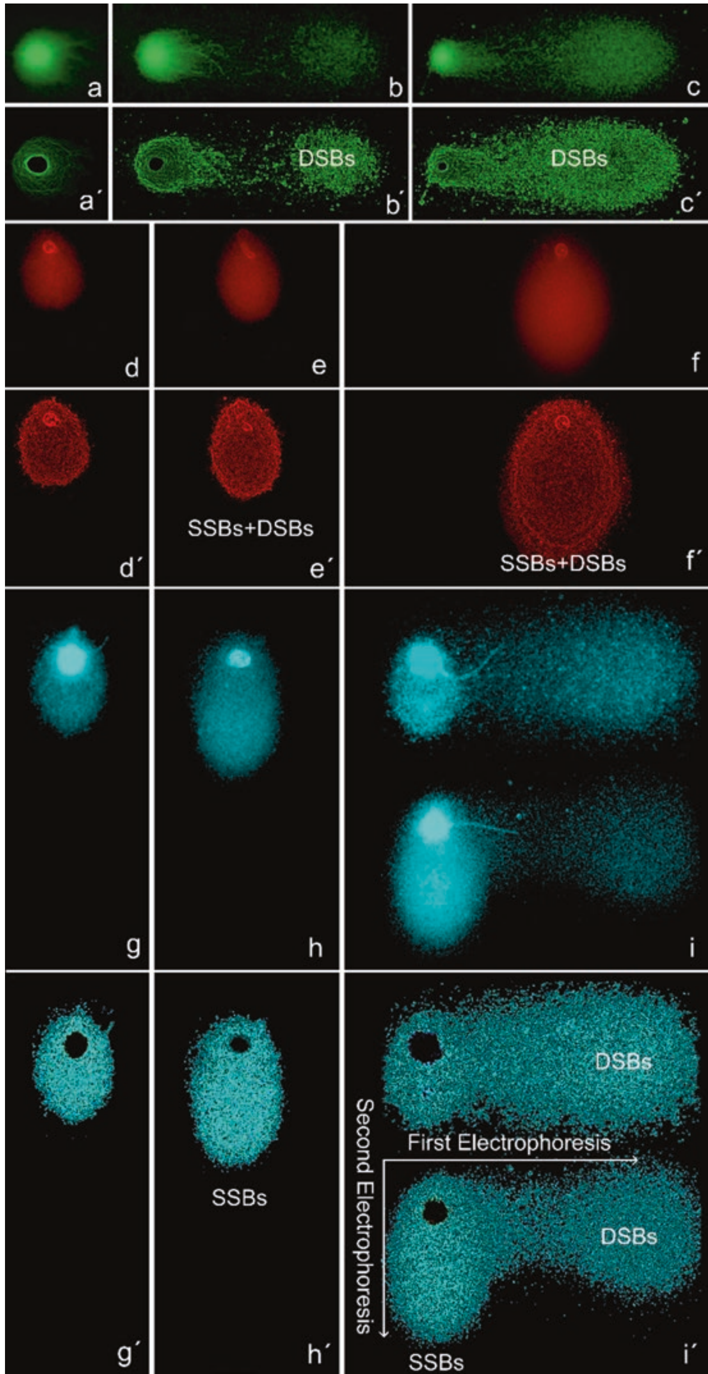

Fig. 7.2 Nonfragmented (**a**, **d**, and **g**) and fragmented spermatozoa (**b**, **c**, **e**, **f**, and **i**) in alkaline (**a**–**c**), neutral (**d**–**f**), and 2T-comet assays (**g**–**i**). Different levels of sperm DNA damage are shown for spermatozoa (SYBR® Green staining). The 2T-comet assay detected simultaneous DNA SSBs and DSBs in human spermatozoa. Undamaged (**g**) single-stranded DNA breaks (SSBs) (**h**), double-stranded DNA breaks (DSBs) (**i**), and SSBs/DSBs in the same cell (**i**). (**a′**–**i′**) Images after application of a common electronic filter scale

tail after alkaline electrophoresis, indicating global DNA damage identifying both single- (SSBs) and double-strand breaks (DSBs) [18, 25, 26].

DNA breaks are the starting points for alkaline DNA unwinding due to the disruption of hydrogen bonds between purines and pyrimidines. Moreover, mutagens may induce DNA base loss, and deoxyribose lesions may be transformed into SSBs by alkaline conditions, being designated as alkali-labile sites (ALS). Remarkably, when the spermatozoa of mammalian species are subjecting to denaturant alkaline conditions and electrophoresed, they exhibit a prominent comet tail (Fig. 7.2d–f) [25, 26]. Probably, the need of a highly compacted DNA molecule at the spermatozoa is favored by the presence of these ALS.

ALS can also be detected using DNA breakage detection-fluorescence in situ hybridization (DBD-FISH). This technique quantifies putative DNA breaks and ALS in situ within a single cell. It has the added advantage that it may also be utilized to scan the whole-genome or specific DNA sequences in sperm cells that have been embedded within an inert agarose matrix on a specifically prepared microscope slide [27]. The cells are then lysed to remove the membranes and proteins, and the resulting nucleoids are exposed to a controlled denaturation step using alkaline buffers. The alkali gives rise to ssDNA stretches starting from the 5′ to 3′ free DNA ends or from highly sensitive DNA motifs to alkaline conditions. These ssDNA threads may then be detected by hybridization with specific or whole-genome fluorescent DNA probes. Because DNA breaks increase in a target region, additional ssDNA are produced and further DNA probes are hybridized, resulting in a more intense FISH signal as additional ssDNA is produced (Fig. 7.3e, f). The resulting hybridized signal in the whole genome can be quantified using image analysis systems. The DBD-FISH signal obtained in the absence of exogenous DNA-damaging agents reflects the background level of ALS present in a genome (Fig. 7.3d) [28].

DNA damage detected by the comet assay suggests a possible high density of short unpaired DNA stretches that could act as origins of denaturation for alkaline treatment in the DBD-FISH procedure [28].

This same result has been seen in the large pericentromeric interstitial telomeric repeat sequence blocks from Chinese hamster cell lines. These short unpaired DNA segments could be a consequence of torsional stress of DNA loops during the process of chromatin packing, as they were initially found to be abundant in the chromatin of condensing mitotic chromosomes [29].

The comet assay is found to be a more sensitive technique in the evaluation of sperm DNA damage and fragmentation compared to the conventional TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end lebeling), the sperm chromatin dispersion test, or flow cytometry [30]. Particularly, the alkaline comet assay has been tested in vitro and in vivo in a wide variety of mammalian cells [19, 31, 32] employing a number of different genotoxic stimuli including UV radiation, carcinogens, radiotherapy, and chemotherapy [33]. It has been shown to be rapid [33, 34], reproducible [35], and with higher sensitivity than alkaline elution or nick translation assays, even with prior chromatin decondensation [36, 37]. The alkaline comet

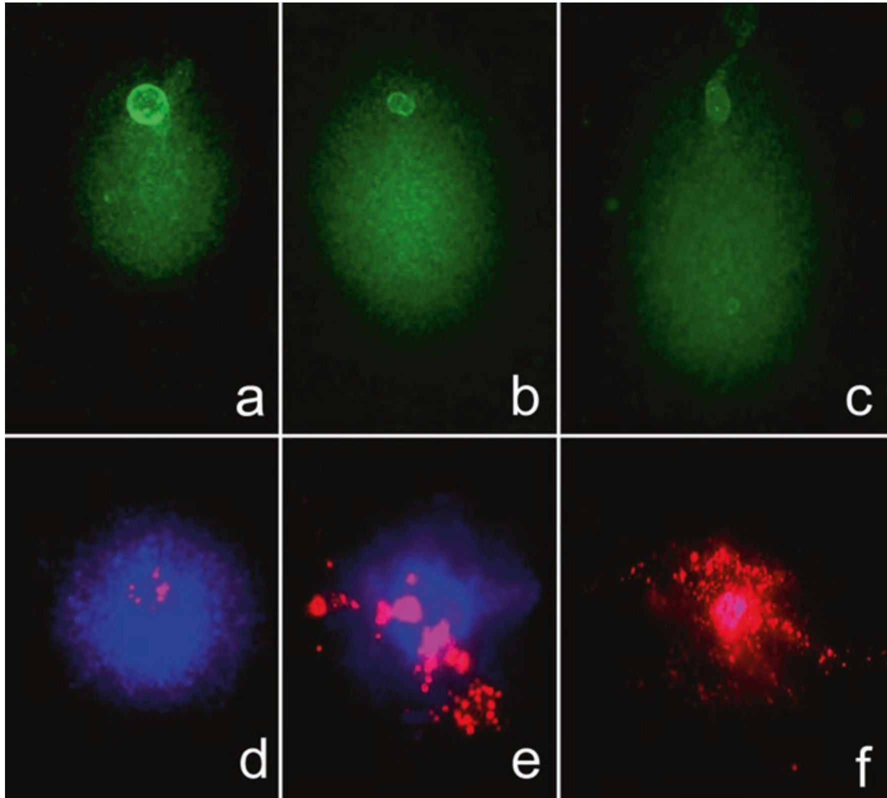

Fig. 7.3 Sperm cell classification according to DNA damage levels after alkaline comet assay (**a**–**c**) and DBD-FISH (**d**–**f**). Sperm with a "structural comet" or without DNA damage (**a**) exhibit a slightly fluorescent signal, (**d**) and nuclei with a prominent comet tail (**b** and **c**) result in a more intense FISH signal after DBD-FISH (**e** and **f**)

assay can detect damage equivalent to as few as 50 single-strand breaks (SSBs) per cell. One of its unique and powerful features is the ability to characterize the responses of a heterogeneous population of cells by measuring DNA damage within an individual (Fig. 7.2d), cells as opposed to just one overall measure of damaged cells versus undamaged (Fig. 7.2e, f).

Evaluation of sperm DNA damage by the alkaline comet assay is a more promising diagnostic test for male infertility as well as prognostic test for assisted reproductive technology (ART) outcomes. It has been shown to be closely associated with numerous fertility outcomes including negative relationships with fertilization, embryo quality, implantation, and positive relationships with miscarriage and childhood diseases [38].

7.4 Two-Tailed (2T)-Comet Assay

A modification of the original comet assay has been developed for the simultaneous evaluation of DNA SSBs and DSBs in human spermatozoa. The 2T-comet assay is a relatively fast, sensitive, and reliable technique for the quantification and characterization of whole DNA damage in spermatozoa [39–41].

In this protocol, cells are subject to an electrophoretic field under non-denaturing conditions to mobilize isolated free discrete DNA fragments produced from DSBs. This is similar to the neutral comet as explained above. Following mobilization of the DNA containing DSBs, a second electrophoresis which runs perpendicular to the first one but under alkaline conditions is performed to produce DNA denaturation. This process exposes both SSBs and DSBs existing in the comet head and tail formed during the first neutral electrophoresis. The result is a two-dimensional comet tail emerging from the core where SSBs and DBBs can be simultaneously discriminated (Fig. 7.2g–i). Three different comet figures may be produced (i) small comet representing a sperm free of DNA damage, resulting from the presence of large alkali-labile sites at each sperm (displacement at Y-axis) (Fig. 7.2g) (ii) comet at the Y-axis with equivalent size to the previous one but presenting additionally DNA displacement at the X-axis (Fig. 7.2i): this image is interpreted as a spermatozoa presenting DSBs at the origin and (iii) comet presenting displacement at the X- and Y-axis: in this case, the presence of SSBs is integrated at the Y-axis comet, while DSBs are mostly placed at the X-axis (Fig. 7.2i). The tail DNA produced after the first electrophoresis is formed because of the presence of DSBs at the origin but may contain SSBs distributed along the DNA threads. After denaturation, single-stranded DNA threads mobilize at the Y-axis, parallel to the migrating DNA remaining at the original comet head.

The 2T-comet assay is an innovative method for assessing whole sperm DNA integrity, which has not been extensively used for diagnostic purposes related to male infertility [39–42]. The technique can be used to assess highly damaged sperm DNA characterized by the presence of extensive presence of single- and double-strand breaks in some clinical situations such as Kartagener's syndrome [41] or in varicocele patients [40, 42].

7.5 The Clinical Relevance of the Comet Assay

Sperm DNA damage assays are important in assisting in the diagnosis of male infertility [36, 43–50] and may serve as prognostic tests for predicting ART outcomes. High levels of damaged DNA in the ejaculate of patients have been shown to have negative relationships with fertilization, embryo quality, and implantation failures and positive relationships with miscarriage and childhood diseases [38]. However, the optimal sperm DNA test and the value of these tests as predictors of reproductive outcomes still remain debatable.

The comet assay can be considered a reliable tool to assess sperm DNA damage, but most laboratories find it more difficult to perform compared to the Sperm Chromatin Structure Assay (SCSA), the TUNEL, or the Sperm Chromatin Dispersion (SCD) test. It is true that a standardized protocol with high quality controls for reproducibility is necessary in order to produce consistent and comparable results among different laboratories. With these limitations in mind, our experience using this technique is positive allowing a fine definition on the nature of the DNA break (SSBs or DSBs) present in the ejaculate. We must bear in mind that the presence of high level of DSBs in fertilizing spermatozoa shall be practically unrepairable at the oocyte with highly negative results on the embryo development even during the first stage [51, 52].

All types of comet assays have been used to assess different aspects of DNA quality in human sperm in order to find any possible correlation with pregnancy. However, as mentioned above, the alkaline comet assay has the capacity to visualize SSBs, DSBs, and additionally all constitutive alkali-labile sites present in the mammalian sperm. The proponents of the alkaline comet assay suggest that this assay is the most powerful to assess all DNA damage present in cells because it is based on the full capacity to denature the DNA when free 5–3′ breaks are present along the DNA molecule [53]. The neutral comet assay has not been used as widely as the alkaline comet assay but may be of relevance at the time of evaluating massive presence of DSBs. The 2T-comet assay is the least used methodology to assess sperm DNA damage, but important aspects on the nature of the DNA damage, such as the assessment of the relative presence of DSBs versus SSBs, are possible, and its relevance in fertility remains to be evaluated.

With respect to the neutral comet assay indicating the relative amount of DSBs affecting a certain proportion of the whole sperm population, some interesting information can be derived from published studies. Thus, SDF assessed by neutral comet has been found to be quite variable among different patient groups, with different sperm qualities [54]. While in normozoospermic individuals, Sperm DNA Fragmentation (SDF) values were around 10.5%, they were higher in other groups such as asthenozoospermic (15.2%), oligoteratozoospermic (18.3%), asthenoteratozoospermic (17.5%), or oligoasthenoteratozoospermic (21.3%). These results suggest that a single threshold value of SDF is probably not representative of the sperm's fertilizing capacity in achieving pregnancy, and the value needs to be tailored according to the type of patient under study.

Using the neutral comet assay, Ribas-Maynou et al. [55] studied the presence of DSBs and SSBs in sperm samples of patients with asthenoteratozoospermia (ATZ) with or without varicocele, oligoasthenoteratozoospermia (OAT), or balanced chromosome rearrangements. All of these patient groups were compared to fertile donors. The results revealed different sperm DNA damage profiles. Fertile donors presented low values for DSBs as well as for SSBs. OAT, ATZ, and ATZ presenting with an additional varicocele had higher SSB and DSB percentage compared with normozoospermic individuals. Interestingly in rearranged chromosome carriers, they presented with two different profiles: a high-equivalent comet assay profile, which could be compatible with a bad prognosis, and a nonequivalent comet assay

profile, which was found in three fertile donors. These results support the theory that a neutral comet assay profile applied to different clinical groups may be useful for characterizing different male infertility groups.

The predictive value of the neutral comet assay in pregnancy loss has also been reported [23]. The study included 25 fertile donors and 20 recurrent pregnancy loss (RPL) patients with at least two unexplained first-trimester miscarriages. SDF values were analyzed using both alkaline and neutral comet assays. The unexplained RPL patients showed a low SSB and high DSB profile. This profile was observed in 85% of unexplained RPL and 33% of fertile donors, suggesting that DSBs can be associated with a male factor-related RPL. Receiver operating characteristic (ROC) curve analysis done with respect to recurrent miscarriage set the cutoff value at 77.50% of DSBs.

The alkaline comet assay has been shown to have a significant clinical value in male reproductive health and in predicting the success of ART [56]. Although the assay is not included in routine infertility tests, some studies recognize this experimental approach as an advanced, accurate, and reliable test for analyzing all DNA damage affecting a genome. When the alkaline comet assay is used to assess human semen quality and sperm DNA damage in infertile and fertile males, a significantly lower sperm concentration, sperm viability, and sperm motility were observed in all of the infertile subjects presenting with a high level of SDF [57].

The alkaline comet assay is also suitable for obtaining information about the level of SDF present after density gradient centrifugation. This is crucial as the sperm selection for IVF influences the chances of achieving pregnancy. It was found that men with SDF higher than 25% had a high risk of infertility (OR, 117.33; 95% confidence interval [CI], 12.72–2731.84; RR, 8.75) [56]. Fertilization rates and embryo quality decreased as SDF increased in semen and in density gradient centrifugation sperm. These results suggest that the risk of failure to achieve a pregnancy increased when SDF exceeded a prognostic threshold value of 52% for semen (OR, 76.00; CI, 8.69–1714.44; RR, 4.75) and 42% for density gradient centrifugation sperm (OR, 24.18; CI, 2.89–522.34; RR, 2.16). In a different study, the alkaline comet assay was used to assess SDF in neat semen samples and in spermatozoa following density gradient centrifugation. In this case, 203 couples undergoing In Vitro Fertlization (IVF) and 136 couples undergoing ICSI were included to establish any relationship existing between SDF level and live-birth rate after IVF and intracytoplasmic sperm injection (ICSI). Following IVF, couples with <25% SDF had a live-birth rate of 33%. In contrast, couples with >50% SDF had a much lower live-birth rate of 13% following IVF. Following ICSI, there were no significant differences in levels of sperm DNA damage between any groups of patients [58].

Sperm DNA damage evaluated by an alkaline comet assay was also associated with implantation and embryo quality [59]. In a cross-sectional study of 215 men from infertile couples undergoing ART, the paternal effect of sperm DNA damage was observed at each stage of early embryonic development. In both the early and late paternal effect stages, the low DNA damage group had a higher percentage of good-quality embryos ($P < 0.05$) and a lower percentage of poor-quality embryos ($P < 0.05$) compared with the high DNA damage group. Implantation was lower in

the high DNA damage (33.33%) group compared with intermediate DNA damage (55.26%; $P < 0.001$) and low DNA damage (65.00%; $P < 0.001$) groups.

The implications of genomic damage in spermatozoa of type 1 diabetic patients were evaluated by alkaline comet assay [60] by comparing the SDF and the levels of oxidative DNA modifications with nondiabetic men. Spermatozoa from 11 patients with type 1 diabetes showed significantly higher levels of DNA fragmentation (44% versus 27%; $P < 0.05$) and concentrations of 8-OHdG (3.6 versus 2.0 molecules of 8-OHdG per 10(5) molecules of deoxyguanosine; $P < 0.05$) compared to 12 patients without diabetes. Furthermore, a positive correlation (rs = 0.7; $P < 0.05$) was observed between DNA fragmentation and concentration of 8-OHdG.

By using the alkaline comet assay, the actual damage load of small cohorts of sperm may be measured. As the alkaline comet only requires 100 cells for analysis, it has also been particularly useful for studies involving DNA of testicular sperm and for men with low sperm concentrations [61]. This is of importance as in addition to the low number of spermatozoa present in these samples, they are also contaminated with somatic cells. Other methodologies such as the SCSA are not operative in these cases. Using the alkaline comet assay, the apoptotic indices and SDF were compared in sperm collected after ejaculation from vasectomized men and fertile men undergoing vasectomy. Testicular biopsies from vasectomized ($n = 26$) and fertile men ($n = 46$) were used to calculate sperm/gram and also formalin-fixed to determine the numbers of developing sperm and incidence and intensities of testicular FasL, Fas, Bax, and Bcl-2. Increased intensities of FasL and Bax staining were observed in the seminiferous tubules of vasectomized men. FasL positivity also increased in Sertoli cells, and both FasL and Fas positivity increased in primary spermatocytes and round spermatids of vasectomized men [62]. These results demonstrate that SDF can be considered an end point marker of apoptosis with significantly higher sperm SDF in vasectomized men compared to fertile men. Another study [63] concluded that an inverse relationship between pregnancy and SDF is observed for both testicular and ejaculated sperm. However, no relationships were observed between SDF and fertilization rates.

According to the information we have summarized previously, it seems that a high level of SDF in the ejaculate or in the selected sample for fertilization purposes is negatively correlated with reproductive outcomes. However, it is not only the amount of detected damaged DNA but also the nature of the DNA breaks that is of importance in explaining certain reproductive outcome failures [39, 40]. In this scenario, the 2T-comet assay may provide additional information not provided by the neutral or alkaline comets regarding the nature of the DNA damage. This methodology may provide important and singular information understanding a part of andrological pathology as is the case for Kartagener's syndrome [41].

Some studies have obtained interesting results using the 2T-comet assay. Enciso et al. [21] studied the frequency of sperm cells containing SSBs and DSBs in the ejaculates of a group of ten infertile patients with abnormal semen parameters such as volume, concentration, and sperm motility and compared them with those obtained in a group of ten normozoospermic fertile men. The infertile patient group had a significantly higher percentage of spermatozoa containing DSBs, compared to

the group of fertile subjects. Nevertheless, no significant differences were found in the percentage of spermatozoa with SSBs between infertile patients and fertile men.

In another study, Gosálvez et al. [41] established SSB and DSB profiles in infertile patients with varicocele and compared them to fertile normozoospermic subjects by 2T-comet assay. In this study, the authors analyzed a particular sperm class observed after applying the sperm chromatin dispersion test that was referred to as "degraded" sperm because they showed relatively low amounts of chromatin remaining in the nucleoid after protein removal when compared to normal or even sperm containing fragmented DNA. The 2T-comet assay demonstrated that degraded sperm containing both massive double- and single-strand DNA breaks coexist in the same spermatozoa. Recently, it has been reported that these types of spermatozoa, also present in the ejaculate of normal individuals, are fully covered with ALS which can be used as another indication of the presence of sperm DNA damage [27]. These "degraded" spermatozoa, a distinctive subpopulation in varicocele patients (six times more than fertile men), probably occur due to the fact that both DNA and protein fractions are affected by intratesticular oxidative stress. The 2T-comet assay has been also used to assess SSBs and DSBs in one patient with Kartagener's syndrome with four failures of fertilization after ISCI using testicular sperm obtained with testicular sperm aspiration [41]. The authors concluded that in addition to a failure of sperm motility, this patient was infertile because of a high level of unrepairable DBSs (85.2%) present in the ejaculate.

The clinical implication of the information provided by the comet assay in fertilization and embryo development depends on the balance between the DNA damage in sperm and the oocyte's repair capacity. Moreover, the type and/or complexity of DNA lesions in the different sperm can vary, and this would influence the embryonic development. After penetration into the oocyte, sperm with extensive DSBs, associated with apoptotic-like processes, will lead to a delayed paternal DNA replication, paternal DNA degradation, and arrest of embryo development if this exceeds the repair capacity of the oocyte [63]. Conversely, when sperm DNA damage is composed mainly of a low level of DSBs, SSBs, abasic sites, and/or DNA base modifications, the oocyte's various specific DNA repair pathways are likely to be more effective, resulting in functional male pronucleus DNA and normal early embryonic development. Nevertheless, some misrepaired or unrepaired DNA lesions could still potentially lead to mutations or chromosome aberrations. Unrepaired SSBs or other lesions types may also result in DSBs when DNA is replicating, leading to structural chromosomal abnormalities [64]. If these chromosome aberrations are unstable, they are more likely to affect the normal mitotic segregation of chromosomes, resulting in genomic instability and cell death, and thereby adversely affect embryo development [65]. When DNA repair is complete, the morula and blastocyst stages can be achieved. In contrast, if the repair processes are defective, blastocyst arrest or spontaneous abortion may then result [66]. SDF can lead to congenital malformations and genetic illnesses, as well as potentially increase the risk of certain cancers in related offspring [67]. The long-term consequences on development and behavior of mice generated by ICSI with fragmentation sperm were investigated [66]. Anatomopathological analysis of animals at

Table 7.1 Types of comet assay and their clinical importance

Comet assay		
Type	DNA break detected	Clinical relevance
Neutral	DSBs	DSBs have been associated with male infertility [54, 55] and pregnancy loss [23]. The identification of DSBs has biological importance at the sperm because of deficiency of DNA damage reparation mechanisms. Standardization, reproducibility, and validation of this technique are necessary
Alkaline	SSBs + DSBs + ALS	Clinical relevance in infertility [38], embryo quality [58, 59], implantation [59], and miscarriage [66] pregnancy [56].Used to assess SDF in obstructive azoospermia [61], varicocele [40, 55] vasectomy [62], chromosomal abnormalities [64], childhood diseases cancer [66], and diabetes mellitus [60]. The methodology is not included in routine infertility tests
Two-tailed comet (neutral + alkaline)	Discrimination of DSBs and SSBs	An innovative method to assess the simultaneous presence of DSB + SSB profiles within the same cell. It has been used to assess infertility [39], varicocele [40], and in a case of Kartagener's syndrome [41]. This methodology may provide important and singular information to understand a part of human fertility and andrological associated problems

SSBs single-strand breaks, *DSBs* double-strand breaks, *ALS* alkali-labile sites, *SDF* sperm DNA fragmentation

16 months of age showed that 33% of females produced with fragmented sperm presented some solid tumors in the lungs and the dermis of the back or neck.

The comet assay, in its different versions, offers the possibility of discriminating between single- and double-strand breaks, and this aspect is of relevance in predicting the fate of the embryo prior to implantation. However, further studies are necessary to understand the mechanisms of paternal DNA damage as a cause of early loss of developmental stages and congenital malformations. The technical problems inherent to the comet assay represent the only bottleneck that limits its wide use in reproduction. In Table 7.1 we have summarized the different types of comet assay and the main clinical relevance associated with each one.

7.6 Conclusions

(i) Assessment of DNA damage (DSBs) can be obtained using a neutral comet assay; whole (SSBs and DSBs) sperm DNA damage can be performed using an alkaline comet assay; the study of whole DNA damage and discrimination between SSBs and DSBs is possible using a 2T-comet assay. (ii) DNA damage evaluation by comet assay – or alternative strategies – as a predictor of male fertility is highly appreciated to assist in the diagnosis of recurrent spontaneous abortions or failures in ART. However, until we demonstrate with certainty which is the best protocol to assess sperm DNA damage, the acceptability and widespread application of any version of the comet assay will limit the application of this test as a research tool.

References

1. Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N, Bizzaro D. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. Biol Reprod. 2002;66:1061–7.
2. Marcon L, Boissonneault G. Transient DNA strand breaks during mouse and human spermiogenesis: new insights in stage specificity and link to chromatin remodelling. Biol Reprod. 2004;70:910–8.
3. Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril. 2003;79:829–43.
4. Sotolongo B, Lino E, Ward WS. Ability of hamster spermatozoa to digest their own DNA. Biol Reprod. 2003;69:2029–35.
5. Li TK, Chen AY, Yu C, Mao Y, Wang H, Liu LF. Activation of topoisomerase II-mediated excision of chromosomal DNA loops during oxidative stress. Genes Dev. 1999;13:1553–60.
6. Yakovlev AG, Wang G, Stoica BA, Boulares HA, Spoonde AY, Yoshihara K, Smulson ME. A role of the Ca21/Mg21-dependent endonuclease in apoptosis and its inhibition by Poly(ADP-ribose) polymerase. J Biol Chem. 2000;275:21302–8.
7. Boulares AH, Zoltoski AJ, Sherif ZA, Yakovlev AG, Smulson ME. The Poly (ADP-ribose) polymerase-1-regulated endonuclease DNAS1L3 is required for etoposide-induced internucleosomal DNA fragmentation and increases etoposide cytotoxicity in transfected osteosarcoma cells. Cancer Res. 2002;62:4439–44.
8. Boulares AH, Zoltoski AJ, Contreras FJ, Yakovlev AG, Yoshihara K, Smulson ME. Regulation of DNAS1L3 endonuclease activity by poly (ADP-ribosyl)ation during etoposide-induced apoptosis. Role of poly (ADP-ribose) polymerase-1 cleavage in endonuclease activation. J Biol Chem. 2002;277:372–8.
9. Solovyan VT, Bezvenyuk ZA, Salminen A, Austin CA, Courtney MJ. The role of topoisomerase II in the excision of DNA loop domains during apoptosis. J Biol Chem. 2002;277:21458–67.
10. Ribas-Maynou J, García-Peiró A, Martínez-Heredia J, Fernández-Encinas A, Abad C, Amengual MJ, Navarro J, Benet J. Nuclear degraded sperm subpopulation is affected by poor chromatin compaction and nuclease activity. Andrologia. 2015;47:286–94.
11. Sotolongo B, Huang TT, Isenberger E, Ward WS. An endogenous nuclease in hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments. J Androl. 2005;26:272–80.
12. Cortés-Gutiérrez EI, López-Fernández C, Fernández JL, Dávila-Rodríguez MI, Johnston SD, Gosálvez J. Interpreting sperm DNA damage in a diverse range of mammalian sperm by means of the two-tailed comet assay. Front Genet. 27. 2014;5:404.
13. Laberge RM, Boissonneault G. On the nature of DNA strand breaks in elongating spermatids. Biol Reprod. 2005;73:289–96.
14. Reiter TA. NO∗ chemistry: a diversity of targets in the cell. Redox Rep. 2006;11:194–206.
15. Dizdaroglu M. Oxidative damage to DNA in mammalian chromatin. Mutat Res. 1992;275:331–42.
16. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J. 2003;17:1195–214.
17. Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun. 1984;123:291–8.
18. Olive PL, Wlodek D, Banath JP. DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. Cancer Res. 1991;51:4671–6.
19. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175:184–91.
20. Fernández JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl. 2003;24:59–66.
21. Enciso M, Sarasa J, Agarwal A, Fernández JL, Gosálvez J. A two-tailed comet assay for assessing DNA damage in spermatozoa. Reprod BioMed Online. 2009;5:609–16.

22. McMillan TJ, Tobi S, Mateos S, Lemon C. The use of DNA double-strand break quantification in radiotherapy. Int J Radiat Oncol Biol Phys. 2001;49:373–7.
23. Ribas-Maynou J, García-Peiró A, Abad C, Amengual MJ, Navarro J, Benet J. Alkaline and neutral comet assay profiles of sperm DNA damage in clinical groups. Hum Reprod. 2012;27:652–8.
24. Brooks JE. Properties and uses of restriction endonucleases. Methods Enzymol. 1987;152:113–29.
25. Singh NP, Stephens RE. X-ray induced DNA double-strand breaks in human sperm. Mutagenesis. 1998;13:75–9.
26. Cortés-Gutiérrez EI, Dávila-Rodríguez MI, López-Fernández C, Fernández JL, Crespo F, Gosálvez J. Localization of alkali-labile sites in donkey (Equus asinus) and stallion (Equus caballus) spermatozoa. Theriogenology. 2014;81:321–5.
27. Fernández JL, Vázquez-Gundin F, Delgado A, Goyanes VJ, Ramiro-Díaz J, de la Torre J, Gosálvez J (2000) DNA breakage detection-FISH (DBD-FISH) in human spermatozoa: technical variants evidence different structural features. Mutat Res 453:77–82.
28. López-Fernández C, Arroyo F, Fernández JL, Gosálvez J. Interstitial telomeric sequence blocks in constitutive pericentromeric heterochromatin from *Pyrgomorpha conica* (Orthoptera) are enriched in constitutive alkali-labile sites. Mutat Res. 2006;599:36–44.
29. Rivero MT, Mosquera A, Goyanes V, Slijepcevic P, Fernández JL. Differences in repair profiles of interstitial telomeric sites between normal and DNA double-strand break repair deficient Chinese hamster cells. Exp Cell Res. 2004;295:161–72.
30. Evenson DP, Kasperson K, Wixon RL. Analysis of sperm DNA fragmentation using flow cytometry and other techniques. Soc Reprod Fertil. 2007;65:93–113.
31. Tice RR, Andrews PW, Singh NP. The single cell gel assay: a sensitive technique for evaluating intercellular differences in DNA damage and repair. Basic Life Sci. 1990;53:291–301.
32. Olive PL, Johnston PJ, Banath JP, Durand RE. The alkaline comet assay: a new method to examine heterogeneity associated with solid tumours. Nat Med. 1998;4:103–5.
33. Fairbairn DW, Olive PL, O'Neill KL. The alkaline comet assay: a comprehensive review. Mutat Res. 1995;339:37–59.
34. McKelvey-Martin VJ, Green MH, Schmezer P, Pool-Zobel BL, De Meo MP, Collins A. The single cell gel electrophoresis assay (alkaline comet assay): a European review. Mutat Res. 1993;288:47–63.
35. Hughes CM, Lewis SE, McKelvey-Martin VJ, Thompson W. Reproducibility of human sperm DNA measurements using the alkaline single cell gel electrophoresis assay. Mutat Res. 1997;374:261–8.
36. Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA, Aitken RJ. DNA integrity in human spermatozoa: relationships with semen quality. J Androl. 2000;21:33–44.
37. Leroy T, Van Hummelen P, Anard D, Castelain P, Kirsch Volders M, Lauwerys R, Lison D. Evaluation of three methods for the detection of DNA single-strand breaks in human lymphocytes: alkaline elution, nick translation and single-cell gel electrophoresis. J Toxicol Environ Health. 1996;47:409–22.
38. Lewis SE, Simon L. Clinical implications of sperm DNA damage. Hum Fertil. 2010;13:201–7.
39. Enciso M, Sarasa J, Agarwal A, Fernàndez JL, Gosálvez J. A two-tailed comet assay for assessing DNA damage in spermatozoa. Reprod BioMed Online. 2009;18:609–16.
40. Gosálvez J, Rodríguez-Predreira M, Mosquera A, López Fernández C, Esteves SC, Agarwal A, Ferández JL. Characterisation of a subpopulation of sperm with massive nuclear damage, as recognised with the sperm chromatin dispersion test. Andrologia. 2014;46:602–9.
41. Nuñez R, López-Fernández C, Arroyo F, Caballero P, Gosálvez J. Characterization of sperm DNA damage in Kartagener's syndrome with recurrent fertilization failure: case revisited. Sex Reprod Health. 2010;1:73–5.
42. Esteves SC, Gosálvez J, López-Fernández C, Núñez-Calonge R, Caballero P, Agarwal A, Fernández JL. Diagnostic accuracy of sperm DNA degradation index (DDSi) as a potential noninvasive biomarker to identify men with varicocele-associated infertility. Int Urol Nephrol. 2015;47:1471–7.

43. Gatewood JM, Cook GR, Balhorn R, Schmid CW, Bradbury EM. Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. J Biol Chem. 1990;265:20662–6.
44. Hughes CM, Lewis SE, McKelvey-Martin VJ, Thompson W. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. Mol Hum Reprod. 1996;2:613–9.
45. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod. 1999;14:1039–49.
46. Spano M, Bonde JP, Hjollund HI, Kolstad HA, Cordelli E, Leter G. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. Fertil Steril. 2000;73:43–50.
47. Carrell DT, Liu L. Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. J Androl. 2001;22:604–10.
48. Zini A, Bielecki R, Phang D, Zenzes MT. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. Fertil Steril. 2001;75:674–7.
49. Zini A, Fischer MA, Sharir S, Shayegan B, Phang D, Jarvi K. Prevalence of abnormal sperm DNA denaturation in fertile and infertile men. Urology. 2002;60:1069–72.
50. Zhang X, San Gabriel M, Zini A. Sperm nuclear histone to protamine ratio in fertile and infertile men: evidence of heterogeneous subpopulations of spermatozoa in the ejaculate. J Androl. 2006;27:414–20.
51. Marchetti F, Bishop JB, Cosentino L, Moore D 2nd, Wyrobek AJ. Paternally transmitted chromosomal aberrations in mouse zygotes determine their embryonic fate. Biol Reprod. 2004;70:616–24.
52. Nanassy L, Carrell DT. Paternal effects on early embryogenesis. J Exp Clin Assist Reprod. 2008;5:2.
53. Lewis SEM, Agbaje IM. Using the alkaline comet assay in prognostic tests for male infertility and assisted reproductive technology outcomes. Mutagenesis. 2008;23:163–70.
54. Chi HJ, Chung DY, Choi SY, Kim JH, Kim GY, Lee JS, Lee HS, Kim MH, Roh SI. Integrity of human sperm DNA assessed by the neutral comet assay and its relationship to semen parameters and clinical outcomes for the IVF-ET program. Clin Exp Reprod Med. 2011;38:10–7.
55. Ribas-Maynou J, Gardía-Peiró A, Fernández-Encinas A, Amengual MJ, Prada E, Cortés P, Navarro J, Benet J. Double stranded sperm DNA breaks, measured by comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor. PLoS One. 2012;7:e44679.
56. Simon L, Lutton D, McManus J, Lewis SE. Sperm DNA damage measured by the alkaline comet assay as an independent predictor of male infertility and in vitro fertilization success. Fertil Steril. 2011;95:652–7.
57. Ramzan MH, Ramzan M, Khan MM, Ramzan F, Wahabb F, Khan MA, Jillani M, Shah M. Human semen quality and sperm DNA damage assessed by comet assay in clinical groups. Turk J Med Sci. 2015;45:729–37.
58. Simon L, Proutski I, Stevenson M, Jennings D, McManus J, Lutton D, Lewis SE. Sperm DNA damage has negative association with live-birth rates after IVF. Reprod BioMed Online. 2012;26:68–78.
59. Simon L, Murphy K, Shamsi MB, Liu L, Emery B, Aston KI, Hotaling J, Carrell DT. Paternal influence of sperm DNA integrity on early embryonic development. Hum Reprod. 2014;29:2402–12.
60. Agbaje IM, Rogers DA, McVicar CM, McClure N, Atkinson AB, Mallidis C, Lewis SE. Insulin dependant diabetes mellitus: implications for male reproductive function. Hum Reprod. 2007;22:1871–7.
61. Dalzell LH, McVicar CM, McClure N, Lutton D, Lewis SE. Effects of short and long incubations on DNA fragmentation of testicular sperm. Fertil Steril. 2004;82:1443–5.

62. O'Neill DA, McVicar CM, McClure N, Maxwell P, Cookie I, Pogue KM, Lewis SE. Reduced sperm yield from testicular biopsies of vasectomized men is due to increase apoptosis. Fertil Esteril. 2007;87:834–41.
63. Fatehi AN, Bevers MM, Schoevers E, Roelen BA, Colenbrander B, Gadella BM. DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages. J Androl. 2006;27:176–88.
64. Marchetti F, Essers J, Kanaar R, Wyrobek KJ. Disruption of DNA repair increases sperm derived chromosomal aberrations. Proc Natl Acad Sci U S A. 2007;104:17725–9.
65. Gawecka JE, Marh J, Ortega M, Yamauchi Y, Ward MA, Ward WS. Mouse zygotes respond to severe sperm DNA damage by delaying paternal DNA replication and embryonic development. PLoS One. 2013;8:e56385.
66. Fernandez-Gonzalez R, Moreira PN, Perez-Crespo M, Sanchez-Martin M, Ramirez MA, Pericuesta E, Bilbao A, Bermejo-Alvarez P, de Dios Hourcade J, de Fonseca FR, Gutiérrez-Adán A. Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. Biol Reprod. 2008;78:761–72.
67. Hales BF. DNA repair disorders causing malformations. Curr Opin Genet Dev. 2005;15:234–40.