Sperm Chromatin Dispersion Test: Technical Aspects and Clinical Applications

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Abstract

Sperm DNA damage has been connected, among other things, with an increased incidence of miscarriage and enhanced risk of disease in the offspring. However, its occurrence is multifaceted and many of the variable consequences it has for fertility are as yet not fully understood. Tests that assess sperm quality should identify not only the ability of spermatozoa to reach the oocyte with an intact DNA molecule but also their ability to fertilize the oocyte and activate embryo growth. Sperm DNA fragmentation should be considered a parameter of sperm quality. Compared to other methods of assessing DNA fragmentation, the sperm chromatin dispersion (SCD) test can be conducted promptly and without the need for complex and expensive laboratory equipment. The SCD test is a powerful and versatile approach for investigating DNA fragmentation, allowing for the assessment of damaged DNA over a diverse range of clinical situations. The technique can be easily adapted to incorporate new research directions, and the analysis of sperm DNA can be performed on a wide range of species.

Keywords

Sperm DNA damage • Sperm DNA fragmention • DNA fragmentation in sperm • Sperm chromatin dispersion test

Sperm DNA Fragmentation: Now and Then

After more than 30 years using different approaches to assess sperm DNA fragmentation (SDF), the scientific community still has serious doubts about which technique produces the most

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reliable results, and most importantly, what value these results have in a clinical context [1–4]. Several techniques have been used effectively to detect SDF in humans and several animal species: (1) The sperm chromatin structure assay (SCSA; [5–7]) was one of the first experimental approaches performed to assess SDF. The underlying principle for this method involves subjecting the DNA to mild acid in order to denature double-stranded or single-stranded breaks. Subsequent staining with acridine orange, which fluoresces green with double-stranded non-denatured DNA or red with single-stranded denatured DNA, allows for the quantification of sperm cells with fragmented DNA using a flow cytometer. (2) Another approach that has been successfully implemented to assess sperm DNA breakage is based upon the enzymatic addition of labelled nucleotides to the end of a DNA break. This includes techniques such as terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labelling (TUNEL) or in situ nick translation (ISNT) using *E. coli* DNA polymerase [8, 9]. (3) The comet assay consists in performing single-cell gel electrophoresis (SCGE). Because of the differential resistance encountered by DNA molecules of different sizes when moving through the gel, a characteristic "comet" distribution is formed after fluorescent staining, with a dense head containing long molecules of DNA and a tail of varying length with shorter fragments of DNA. Thus, DNA breakage can be evaluated by measuring the number of cells with migration tails, as well as the length of the tail and/or percentage of DNA contained in the tail [10, 11]. A modification of this technique based on a two-dimensional displacement of the DNA fragments offers the possibility of differentiating single- and double-strand breaks on the DNA molecule [12, 13]. (4) Lastly, the sperm chromatin dispersion (SCD) test [14–16] and the improved commercially available version of this test, Halosperm® (Halotech, Madrid, Spain), constitute a fast method based on a controlled DNA denaturation and protein depletion to determine SDF. As detailed in the following section, this procedure gives rise to halos of chromatin dispersion due to the spreading of nuclear DNA loops and/or fragments of DNA when the spermatozoa contain fragmented DNA. The size of the halo is related to the amount of sperm DNA damage. Other approaches to measure sperm DNA damage and chromatin alterations have also been described but warrant no further mention due to their restricted use.

As researchers, we are aware that there exists a tendency in the laboratory to use those methods or techniques with which we feel most confident, even though these may present certain constraints. This is the reason why, in our opinion, a sterile debate has evolved over the capacity of the different technologies to measure "real" vs. "potential" sperm DNA damage [17]. It has been claimed that tests that measure "real" DNA damage, such as TUNEL, ISNT or the comet assay (neutral conditions), have a higher predictive value than tests that measure "potential" DNA damage, such as the SCSA, SCD, DBD-FISH, Chromomycin A3 staining or the comet assay (alkaline conditions). It is important to clarify whether DNA breakage is simply present or not; it can exist as a single-strand or double-strand DNA break. In either case, this damage is "real". A similar debate has arisen over how the different techniques measure this damage – whether by a "direct" or "indirect" method. We believe that all existing techniques to assess SDF are "indirect", and that each one has its own particular set of limitations. The TUNEL assay, for instance, is not "direct", as it requires an enzymatic mediator to incorporate labelled nucleotides into DNA breaks. The substrate for the terminal transferase must be a clean hydroxyl 3′ end that has not been chemically modified, and so, the TUNEL assay may underestimate the amount of DNA damage. In addition, the TUNEL protocol used in most laboratories has been designed for use with DNA from somatic cells where the chromatin is arranged with histones, but this protocol may not be as effective when used on highly protected protaminated sperm DNA, given that the enzymes used in this assay are large molecules that may not reach all DNA targets equally [18]. For example, in Fig. 10.1g, a TUNEL labelled sperm cell is shown after partial protein removal. The efficiency of DNA labelling is notably improved

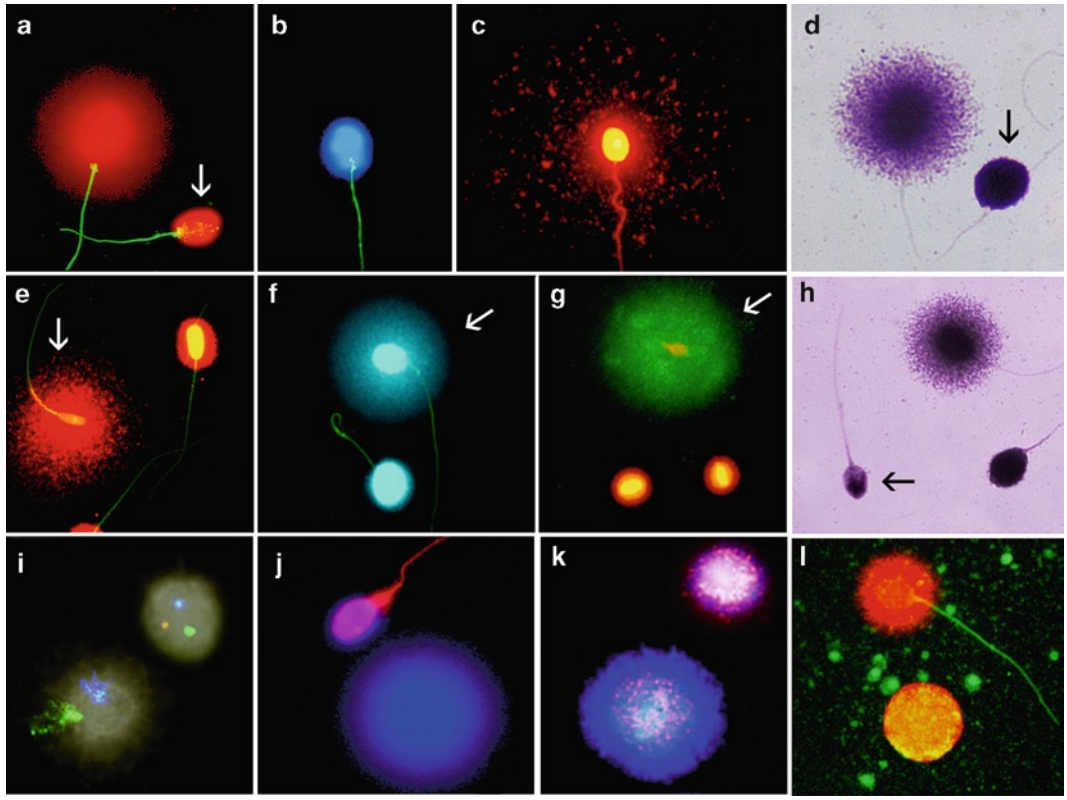

Fig. 10.1 Versatility of the SCD test. (**a**) Classical SCD in human sperm showing normal sperm (halo of dispersed chromatin) or a fragmented sperm (absence of halo; *arrow*) under fluorescence microscopy. (**b**, **c**) A comparison of two fragmented human sperm stained with DAPI (**b**) and GelRed (**c**) to highlight the presence of a massive halo of dispersed and atomized chromatin remnants in a fragmented sperm cell. (**d**) Classical SCD under bright-field microscopy (*arrow* denotes fragmented sperm cell). (**e**) Modified SCD for animal species (boar). The presence of a halo is correlated with sperm DNA fragmentation (SDF) (*arrow*). (**e–g**) Direct correlation between the presence of haloes of chromatin dispersion and in situ DNA labelling. In animal species, the expanded halo of dispersed chromatin (**f**; koala) could be highly labelled by in situ extension of the DNA breaks using polymerase (**g**; deer). (**h**) Classical SCD in human sperm under bright-field microscopy showing the presence of a degraded spermatozoon (*arrow*). (**i–k**) Direct correlation of SDF and specific DNA targets in human sperm. (**i**) SCD combined with FISH for aneuploidy detection. (**j**) SCD combined with McAbs for detection of 8-oxoguanosine. (**k**) SCD combined with McAbs for detection of 5-methyl citosine. (**l**) Dual staining (DNA *red*, proteins *red*) to differentiate histonized (*yellow*) and protaminized (*red*) cells. In this case, yellow fluorescence corresponds to a leukocyte

with respect to that obtained using paraformaldehyde-fixed samples. In fact, a recent report has demonstrated this very point by showing that there is increased TUNEL labelling when sperm samples are treated with the disulphide bond reducing agent DTT [19]. The only explanation for this is that the terminal transferase is not reaching all the available DNA breaks in the damaged sperm. Lastly, differences in SDF levels have been reported when the results of the TUNEL assay are assessed by flow cytometry or optical microscopy [20]. On the other hand, the so-called "indirect" methodologies, based on the susceptibility of DNA to denaturation, have been extensively used in mutagenesis [21]. The established dogma is that acid denaturation does not create any "potential" DNA break, but rather DNA breakage makes DNA more susceptible to DNA denaturation, DNA mobilization or enzyme-mediated incorporation of nucleotides.

Despite their different approaches and their limitations, the techniques have been shown to

produce results that are highly correlated [8, 22, 23]. Thus, the main advantages or disadvantages of each procedure will largely depend on the time to obtain results, cost and the requirement for technical equipment or qualified personnel. The SCSA is not easily implemented in every laboratory, since it is a complex procedure that requires an expensive flow cytometer and highly specialized personnel. Alternatively, the samples may be shipped and analyzed in reference laboratories, but this prolongs considerably the time to obtain results. The comet assay requires trained personnel to perform the methodology with a certain level of reproducibility. The requirement of an electrophoresis unit and specific software for image analysis also limit the quick production of results. The methods based on in situ hybridization or enzyme-mediated extension of the DNA molecule also have the limitation of being complex, time-consuming and requiring specialized personnel. As a result, these procedures are best suited for research purposes and are therefore considered unsuitable for routine use in the andrology laboratory.

Technical Basis of the SCD Test

The technical basis of the SCD test rests on two observations: the first is that DNA strands that contain breaks or nicks are more easily denatured, since the ends of the breaks behave as origins of denaturation. This is the rationale for the classical unwinding assays that have been employed for many years for the quantification of DNA breaks in radiobiology and mutagenesis [24]; the second is that partial protein depletion from chromatin results in a characteristic pattern of DNA loops, spreading around a nucleoid of DNA that remains attached to protein residues, as described by Cook and Brazell [25].

The SCD test has been adapted for the nuclei of human spermatozoa and the methodology comprises three main steps: (1) inclusion of sperm cells in an inert semi-solid medium spread over a glass slide, (2) sperm sample incubation in HCl for acid denaturation, (3) treatment in a lysing solution for controlled nuclear protein removal and a final staining step [14]. The acid solution produces a controlled DNA denaturation only when this DNA contains extensive breakage. The subsequent incubation in the lysis solution removes protamines. If the sperm DNA is intact, a characteristic halo of DNA loops is formed around a dense central core (Fig. 10.1a). On the other hand, if the sperm nucleus contains fragmented DNA, the halos are absent or they are very small (arrow in Fig. 10.1a). This differential chromatin behaviour is the base of the SCD test. In actual fact, halos are also produced when the DNA is fragmented and susceptible to denaturation by acid (Fig. 10.1c). In this case, however, the DNA fragments diffuse further from the central core and because they are smaller, they are faintly stained to the point that they remain invisible using standard fluorochromes such as propidium iodide, diamidino phenyl indole (DAPI) or Diff-Quick under bright-field microscopy (Fig. 10.1b). Nevertheless, this pattern can be revealed using more efficient fluorescent DNA binding molecules such as GelRed (Biotium, Hayward, CA, USA) or Synergy Brand derived molecules (Invitrogen, Carlsbad, CA, USA) and captured with high-performance CCD (cooled charge-coupled-device; Fig. 10.1c).

This methodology has also been used with sperm from other mammalian species including Eutheria [26–29], Metatheria [30, 31] and Prototheria [32] to produce similar halos of chromatin dispersion. The methodology needs to be adapted for each species, although commercial procedures have been developed for each mammalian species (Halomax®, Halotech, Madrid, Spain). For mammalian species, the SCD test was simplified so that only a species-specific modified lysing solution is used for protein depletion. This is because each species contains different protamine residues that require a different strength of lysis solution to produce efficient protein removal, and this is enough to produce a differential chromatin dispersion pattern without the need to subject the DNA to acid denaturation. The result is that, unlike the SCD test adapted for use with human sperm, large halos of spotty

dispersed chromatin are associated to fragmented DNA (arrow in Fig. 10.1e, f) and small, compact halos of chromatin loops correspond to sperm cells with intact DNA (Fig. 10.1e, f; [33]). Therefore, the expanded halos of dispersed chromatin are positive for TUNEL labelling (arrow in Fig. 10.1g). This serves a direct control to demonstrate that the presence of halos is associated to DNA damage. Similarly, the presence of halos of chromatin dispersion in this test is correlated with the characteristic migration tails denoting DNA fragmentation in the comet assay [31, 34, 35].

Validation of the SCD Test

The SCD test has the unique advantage that it can be directly validated by other techniques applied on the same sperm cell. Such experiments have been conducted using DNA breakage detection-fluorescence in situ hybridization (DBD-FISH). In this procedure, breaks in the DNA molecule are transformed into restricted single-stranded DNA areas by a denaturing acid or alkaline solution. These areas are targets for hybridization with a fluorescent-labelled whole genome probe or even using DNA probes for specific genome domains [15, 36]. The intensity of fluorescence after hybridization is related to the amount of DNA damage [15]. Incubation with a whole-genome probe following the SCD test – the acid used in the SCD test is sufficient to reveal the single-stranded targets for the probe – results in strong hybridization only in those nucleoids with a small or absent halo, demonstrating in situ that these sperm cells contain fragmented DNA. Validation was also obtained using enzymatic labelling of DNA breaks on SCD-processed nucleoids. The sequential incubation with the TdT, DNA polymerase I or the Klenow fragment, following the TUNEL, ISNT or Klenow-end labelling procedures, respectively, also resulted in intense labelling of those nucleoids that presented a small or no halo [36–38].

The SCD test was also validated using agents that are known to induce DNA breakage. When sperm samples were exposed to hydrogen peroxide, sodium nitroprusside (SNP) or DNaseI, a concomitant dose-dependent increase was observed in the frequency of sperm cells with no halo or small halos [15, 39]. Lastly, the SCD test was validated indirectly by comparing the results with those obtained using other techniques with the aliquots from the same semen sample. The percentage of sperm cells with fragmented DNA as measured with TUNEL and SCSA correlated highly with the number obtained using the SCD test adapted for human sperm samples [23, 40] and for other animal species [13, 27, 32]. Results obtained with ISNT and the comet assay also correlate with those obtained with the SCD test adapted for stallion [27], ram [29], marsupials [30, 32] rhinoceros [41] or fish [35].

Methodological Versatility

Assessing DNA Damage Intensity

The amount of DNA damage differs from one sperm cell to another in any given semen sample. Such variation accounts for the dispersion in colour ratio values obtained with SCSA and the different amount of DNA labelling obtained with the TUNEL assay. Similarly, the different halo sizes produced by the SCD test are indicative of the level of DNA damage [15]. In addition to the differences in halo size, the SCD test also reveals a distinct class of sperm cells referred to in the literature as “degraded sperm”, which are characterized by a residual nuclear core after protein depletion (arrow in Fig. 10.1h; [42]). This extreme level of nuclear damage may involve damage of the nuclear matrix. Such degraded sperm cells have been observed in both fertile and infertile patients but are especially prevalent in cases of varicocele [15, 42].

Assessing Chromosomal Abnormalities

Conventional FISH may be performed on sperm cells that have been previously processed by the

SCD test because the protein-depleted sperm chromatin exposes the DNA in such a way as to allow efficient hybridization of fluorescent DNA probes. Thus, it is possible to simultaneously determine the level of fragmentation and the presence of aneuploidies (Fig. 10.1i) or structural chromosome rearrangements [43] in the same sperm cell. In patients presenting genomic unbalances in their sperm, SCD-processed slides were subjected to FISH against chromosomes X, Y and 18. The authors describe a 4.4 ± 1.9-fold increase in diploidy rate, and a 5.9 ± 3.5-fold increase in disomy rate in sperm containing fragmented DNA, with the overall aneuploidy rate being 4.6 ± 2.0-fold higher in sperm with fragmented DNA (Wilcoxon rank test: $p < 0.001$ in the three comparisons; Muriel et al. [43]). A similar correlation between SDF and the incidence of aneuploidies has been shown using FISH and SCSA, although this study did not measure both parameters simultaneously in the same cell and so the correlation is only indirect [44]. These results suggest that the occurrence of numerical chromosome abnormalities during meiosis may lead to SDF as part of a genomic screening mechanism conducted to genetically inactivate sperm with a defective genomic background.

Assessing Oxidative DNA Base Damage

Intense oxidative stress may give rise to DNA modifications such that the guanine residues at C-8 are hydroxylated to form 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoG) [45]. Thus, the presence of 8-oxoG is considered an indirect marker of oxidative stress [46], and monoclonal antibodies have been developed against these modified residues [47]. The anti-8-oxoG antibodies have been effectively used to show the presence of 8-oxoG in somatic tissue samples using liver sections [48]. The SCD test may be used together with specific antibodies against 8-oxoG to investigate the link between oxidative stress and DNA damage (Fig. 10.1j). A recent study has shown that increased levels of 8-oxoG were mostly present in those spermatozoa that had fragmented DNA, suggesting a close relationship between both DNA lesion types [39]. The presence of 8-oxoG was also associated with decreased sperm motility and lower embryo quality after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) [49]. As a positive control, sperm cells were subjected to H_2O_2, to produce DNA fragmentation and a concomitant 8-oxoG base modification. As a negative control, SNP produced similar DNA damage, but an 8-nitroguanine base modification rather than 8-oxoG, and DNAase I produced only DNA breakage.

Assessing DNA Methylation

DNA methylation is an important base modification closely related to gene regulation during mammalian development, and its presence is related with diverse processes such as gene expression and genomic imprinting [50, 51]. Abnormal DNA methylation levels in sperm have been associated with decreased pregnancy rates in IVF [52]. The SCD method can be combined with the use of antibodies directed against 5-methylcytosine for the sequential assessment of DNA methylation and DNA fragmentation. The intensity of the signal can be quantified to provide a semi-quantitative estimate of DNA methylation levels in each sperm cell (Fig. 10.1h; Kumar, personal communication).

Assessing Sperm Protein Matrix

The classical SCD protocol can be modified to omit the acid denaturation step resulting in an extensive spreading of DNA loops [53]. With this protocol, the use of a fluorochrome specific for proteins enriched in disulphide bonds (2,7-dibrom-4-hydroxy-mercury-fluorescein) reveals that remnants of other nuclear proteins tend to remain within the core of the nucleoid only in those spermatozoa with fragmented DNA [53]. This suggests that the nuclear matrix of sperm containing fragmented DNA is more resistant to protein removal by the lysis solution. Spermatozoa with fragmented DNA may thus have a modified nuclear protein matrix, suggesting that the processes that initiate DNA fragmentation are also

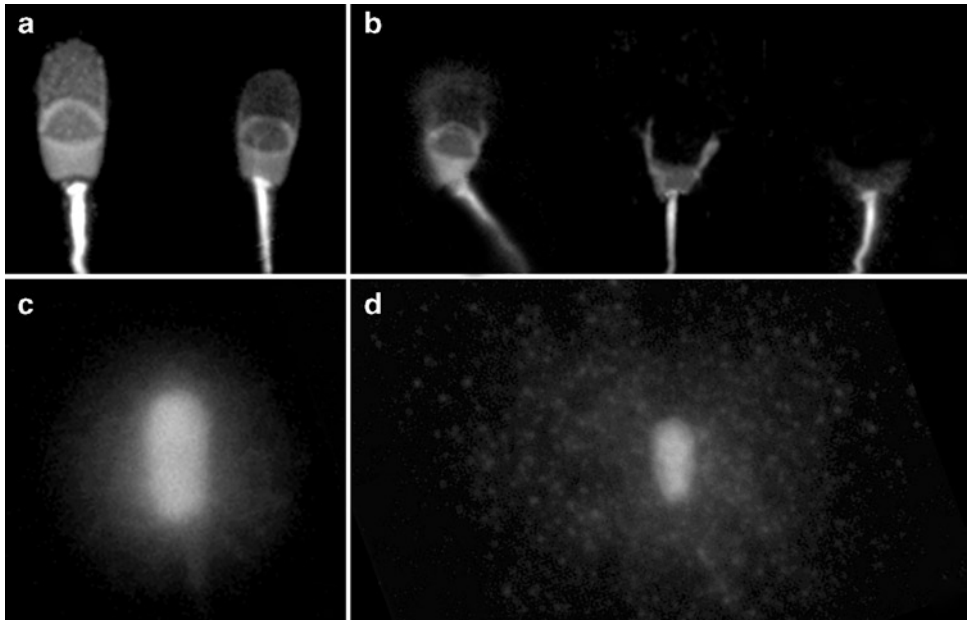

Fig. 10.2 Residual protein matrix (**a**, **b**) and SDF (**c**, **d**) in boar spermatozoa after the SCD test. Fragmented sperm (**d**) show an altered residual protein scaffold (**b**) when compared with unfragmented spermatozoa (**c**)

expressed at the nuclear matrix level. In other mammalian species such as the boar, this effect is also present. Thus, the residual protein matrix is more intensely damaged when the sperm DNA is more fragmented ([54]; Fig. 10.2).

In leukocytes or other somatic cells, the DNA is coiled around histones rather than the protamines of sperm cells. The DNA denaturing and protein lysis treatments of the SCD do not remove the nuclear histone proteins in these cell types. The leukocyte nucleoids, therefore, show no halos of chromatin dispersion. Double fluorescent staining can thus be used on SCD-processed slides to discriminate, for example, leukocytes from sperm cells in patients with leukocytospermia. If SCD processed slides are stained with a mixture of fluorochromes directed against proteins (green emission) and DNA (red emission), cells that contain histones will have overlapping protein and DNA labelling and exhibit yellow fluorescence, while sperm cell heads will exhibit red fluorescence. This methodological approach was used to analyze a Kartagener syndrome patient. In this case, a baseline SDF of 76.4% and a proportion of 1:4 germ cells to somatic cells were observed [55]. This methodological variant may be used to study those patients with high leukocyte counts, since these cells may release reactive oxygen species (ROS) or stimulate their production by spermatozoa, thus producing DNA fragmentation [56]. The scenario could be of particular interest, since Henkel et al. [57] have suggested that the threshold value of leukocytospermia of 1×10^6/mL should be re-evaluated because lower leukocyte counts can compromise DNA integrity.

The SCD and Low Sperm Counts

The SCD can easily be applied to assess SDF in sperm samples obtained from critical clinical situations where the number of spermatozoa is very low. Thus, this should be the procedure of choice in severe oligozoospermia, immotile sperm samples, TESA/TESE samples [55], sorted spermatozoa for sexed semen production [58], samples to be selected using intracytoplasmic morphologically selected sperm injection (IMSI) or even post-mortem epididymal samples.

In the case of IMSI or high magnification sperm selection, a direct correlation can be established between the selected sperm and SCD results. In collaboration with Dr Monica Antinori and the Ginemed Clinic (Sevilla, Spain), we are investigating the correlation between SDF and the presence of sperm vacuolization in the same sperm cell. The preliminary results suggest that high sperm vacuolization and abnormal sperm morphology may be associated with increased SDF (Fig. 10.3).

The SCD test, due to its technical simplicity, reliability and lack of requirement of technical equipment, is quite adequate to accomplish large epidemiological studies or screening of specific male populations exposed to presumed toxic agents or environmental contaminants. This is true not only for humans but also for different domestic, farming or endangered animal species. The SCD methodology has been used outdoors in the field, where electric-powered facilities such as freezers, microscopes or heaters are not available. With only minor modifications to the standard protocol, the SCD test can be performed readily in the field, offering reliable information on SDF. An LED-equipped microscope attached to a laptop, a gas heater and a CO_2 spray for cooling are sufficient to assess the quality of sperm DNA. The results obtained after assessing ram semen samples under different conditions (30°C in the laboratory and at 17 and 4°C in the field) showed that, except when processing at 4°C, the technique was highly reproducible [59]. This opens up the possibility to study the fertility potential of sperm samples post-mortem since mature spermatozoa collected from the *caudal epididymis* have been used successfully for artificial insemination [60]. A decision can be made on site based on DNA quality to inseminate, cryopreserve or reject the sample. This decision can be made within 30 min of sperm recovery.

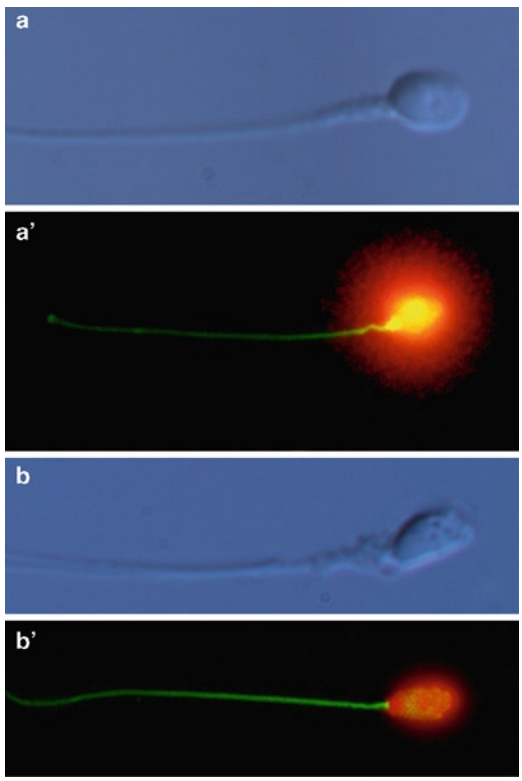

Fig. 10.3 High magnification selected sperm (**a**, **b**) and the characterization of SDF in the same sperm (**a´**, **b´**). The SCD test allows the direct assessment of the DNA status and the sperm morphology

The SCD Test in the Assisted Reproductive Technology (ART) Laboratory

The SCD test produces results that correlate highly with those obtained with other methodologies [23, 40]; however, relatively few studies have been published with this technique. Therefore, when discussing the clinical applications of SDF, we have considered studies performed using other techniques as well, making particular mention of those that use the SCD test.

Fertility Assessment

Infertile men possess significantly more SDF than their fertile counterparts [61]. It therefore follows that DNA damage may adversely affect reproductive outcomes. Numerous groups have suggested that there may be a threshold level of DNA damage above which pregnancy is impaired [4]. Indeed, the percentage of sperm cells with fragmented DNA has been suggested as a complementary parameter to the standard semen

quality parameters (sperm concentration, motility and morphology) in predicting the success of natural conception. In a recent study [61] using 127 men from infertile couples with no known female factor, it was demonstrated that in men with normal standard semen parameters the odds ratio (OR) for infertility was significantly higher than in control patients when the percentage of sperm cells with DNA fragmentation was above 20% (OR 5.1, 95% confidence interval [CI] 1.2–23). Moreover, if one of the standard semen parameters was abnormal, the OR for infertility was significant above 10% (OR 16, 95% CI: 4.2–60). Such findings have been corroborated by similar studies that suggest that SDF above approximately 30% is associated with low success for natural conception and prolonged time to pregnancy [62].

Elevated values of SDF have also been associated with decreased success rates in intrauterine insemination (IUI). Sperm samples with SDF values over approximately 30% have been shown to reduced the efficacy of IUI from 16 to 4% [63] or lower [64]. Probably, one of the most robust studies investigating the influence of SDF on IUI outcome was conducted by Bungum and colleagues [65]. Using data collected from 387 cycles of insemination, the authors demonstrated that there was a significant decrease in the percentage of biochemical pregnancies, clinical pregnancies and deliveries (19.0–1.5%, OR 9.9, 95% CI 2.37–41.51, $p<0.001$) when SDF was above the 30% threshold.

The influence of SDF on the outcome of IVF and ICSI may perhaps have received the most attention [65–72]. A detailed study performed using the SCD test on 85 couples subjected to IVF and ICSI demonstrated that the percentage of sperm cells with fragmented DNA was inversely correlated with the fertilization rate of the oocyte (r −0.245, $p<0.05$). Higher DNA fragmentation was associated to type IV zygotes with asynchronous nucleolar precursor bodies (73.8 vs. 28.8%, $p<0.001$). Moreover, high SDF was correlated with slower embryo development and day-6 embryos classified as lower quality by morphological assessment (47.7 vs. 29.4%, $p<0.05$). Lastly, high DNA fragmentation was negatively correlated with implantation rate (r −0.250, $p<0.05$) [70]. This study was later expanded to 622 couples, collected from five clinics in France [72], and the results obtained were in line with those from the previous report.

Interestingly, despite the clear impact of SDF on fertilization and the development of the embryo, neither study found a significant correlation with pregnancy outcome in IVF or ICSI. Along the same lines, a systematic review and meta-analysis of nine IVF studies suggests that sperm DNA damage is only weakly associated with lower IVF pregnancy rates (combined OR 1.57, 95% CI 1.18–2.07, $p<0.05$ [4]). The same meta-analysis reviewing 11 ICSI studies revealed that sperm DNA damage is not associated with ICSI pregnancy rates (combined OR 1.14, 95% CI 0.86–1.54, $p=0.65$). The explanation for this apparent contradiction is that there exist several processes in these techniques that mitigate the effect of SDF: (1) Sperm selection by swim-up before IVF or ICSI reduces the percentage of sperm cells with DNA damage [73]; (2) The selection of sperm cells for ICSI based on morphology is likely to result in the selection of a sperm cell with minimal DNA fragmentation, as abnormal morphology has been shown to correlate with DNA damage and the presence of aneuploidies [74, 75]; (3) Since embryos with poor morphology and slower development are associated with SDF, it is likely that the embryos selected for transfer have resulted from fertilization by sperm cells with less DNA damage [70, 72]; (4) As we shall discuss below, SDF is a dynamic process that increases over time such that a semen sample assessed for SDF immediately after ejaculation will have a lower percentage of damaged sperm cells than when assessed following a few hours. In this way, the effect of SDF is much more pronounced in IUI where the time to fertilize the oocyte is much longer than IVF or ICSI [76, 77].

Thus, assessment of SDF may serve to evaluate the most appropriate assisted reproduction technique given that SDF is highly correlated with pregnancy outcome in IUI but not in IVF

Fig. 10.4 Number of deliveries after the first cycle, taking into account SDF in the choice of assisted reproduction technique. Selecting IUI for all patients (*left panel*) yields a total of 62 live births, whereas only subjecting couples with high SDF to IVF and couples with low SDF to IUI (*right panel*) yields a total of 78 live births (adapted from Bungum et al. [65], with permission)

or ICSI. Couples presenting values of SDF above the 30% threshold should undergo IVF or ICSI in their first cycle, avoiding unnecessary IUI cycles. If one considers the results obtained by Bungum et al. [65] by selecting IVF rather than IUI in the first cycle for couples presenting SDF values over 30%, there is a significant increase from 62 to 78 deliveries, that is, a 25.8% increase in the efficacy of the first cycle of ART (Fig. 10.4).

Much debate remains and it is clear that the clinical applications of measuring SDF require more study. The long-term effects of SDF on techniques that bypass the natural selection barriers to fertilization such as ICSI are as yet unknown. A recent experimental study in mice has shown that ICSI performed using semen with a high percentage of cells with fragmented DNA resulted in reduced pre-implantation embryo development and less offspring [78]. Most interestingly, this study demonstrated that offspring from animals produced from semen with high SDF performed less well in a battery of behavioural tests than control animals. These animals also presented tumours and aged prematurely, suggesting that despite the ability of the oocyte to repair sperm DNA damage [79], incomplete repair may lead to long-term pathologies. In line with this, a recent study has demonstrated that a 10% increase in SDF increased the probabilities of not achieving pregnancy by an order of 1.31 times, but this effect was absent when using donor oocytes (Meseguer personal communication and submitted). This points to the fact that oocyte quality is a conditioning factor to be taken into account, as the capacity of oocytes to repair DNA lesions in both quantity and fidelity, may be compromised, especially in oocytes from older women or with certain fertility problems. The concurrence of undetected female factor may influence the results from the different reports measuring SDF and pregnancy outcome.

Lastly, as alluded previously, the majority of studies fail to take into account the progressive increase in sperm cells with fragmented DNA over time after ejaculation or thawing. The rate of SDF and shape of the curve of dynamic progression of SDF over time has a unique pattern, but remarkable differences may exist among individuals [80–82] and species [83]. Thus, the sperm DNA longevity may be quite different when different individuals are compared, and individuals with a similar baseline level of SDF may exhibit large differences when SDF is assessed some hours after ejaculation. A differential amount of iatrogenic SDF may therefore be embedded into the results cited in these studies depending on the time taken handling the sperm sample in the laboratory. This factor may partially explain the controversial correlations obtained in different reports when trying to

establish correlations between sperm DNA damage and fertility or pregnancy outcome. Owing to its outstanding implications, the dynamic approach of SDF is further developed in a subsequent section.

The SCD Test in the Andrology Laboratory

Varicocele

Varicocele is the dilation of the pampiniform venous plexus above and around the testicle. It occurs in approximately 15–20% of the general male population, mainly in adolescents. Moreover, 19–41% of men seeking infertility treatment and around 80% of men with secondary infertility experience this pathology. Thus, this anatomical abnormality is perhaps one of the most common causes of poor sperm production and decreased semen quality. When the SCD test was applied to sperm samples collected from a group of infertile males with varicocele, it was found that 32.4 ± 2.3% of the spermatozoa had fragmented DNA [42]. These values are more than double those measured in control fertile subjects. Such values are similar to those obtained from infertile men with other pathologies. However, varicocele patients exhibit a higher proportion of degraded sperm cells (1 in every 4.2 cells) compared to fertile (1 in 8.2) or infertile patients with other pathologies [42]. The effect of increased SDF has been claimed to be a consequence of an increase in ROS production and a decrease in the antioxidant capacity [84–86]. Moreover, the dilated veins may produce high levels of nitric oxide and peroxinitrite, which also attack sperm DNA [87, 88].

Thus, varicocele promotes SDF in such a manner that nuclear injury tends to be very intense. Given that in certain cases varicocelectomy decreases the frequency of sperm cells with fragmented DNA and increases pregnancy rate [71, 89], while in other cases the difference between preoperative and postoperative values is not so evident [90], it should be of great interest to evaluate the presence of this degraded sperm class after surgery.

Genitourinary Infections

Chlamydia trachomatis is the most prevalent sexually transmitted bacterium with nearly 90 million cases detected worldwide annually. This infection is the main cause of subfertility in both males and females [91] and is frequently associated with other pathogens such as *Mycoplasma*. In males, *Chlamydia* is responsible for 50% of non-gonococcal urethritis and the majority of post-gonococcal urethritis. Furthermore, it may be associated with epididymitis, prostatitis and orchitis, as well as stenosis of the ducts. The standard semen parameters are only very subtly altered, so this cannot account for subfertility in infected males. In vitro studies of co-incubation of *Chlamydia* or its lipopolysaccharide with sperm cells demonstrated an induction of phosphatidylserine membrane translocation and DNA fragmentation [92, 93]. To gain information about the situation in vivo, 143 patients infected with *Chlamydia trachomatis* and *Mycoplasma* were evaluated for standard semen parameters and SDF using the SCD test [94]. While the traditional semen parameters were only slightly affected, infected males displayed a percentage of sperm cells with DNA fragmentation of 35.2 ± 13.5%; that is, 3.2 times higher than in the control fertile group (10.8 ± 5.6%). A group of 95 patients was then further evaluated after antibiotic therapy, and the mean frequency of sperm cells with fragmented DNA significantly decreased from 37.7 ± 13.6 to 24.2 ± 11.2% [94]. This improvement was most pronounced after the first 3 months of treatment. These results suggest that the improvement in the DNA integrity of sperm cells after therapy could underlie an improvement in pregnancy rates. The mechanism of DNA fragmentation in vivo following infection may be complex. The bacterium's own components or toxins may induce the DNA fragmentation. Moreover, the accompanying acute or chronic inflammatory reaction in the genital tract may result in oxidative stress by overproduction of ROS by the epithelium or activated leukocytes. Local heat and systemic fever may also have an influence. If this is true, other genitourinary infections originated by different bacteria [95], viruses, fungi such as *Candida*

albicans [96] or protozoa could also affect sperm DNA integrity. As demonstrated in the *Chlamydia* infection, the SCD test may be useful to evaluate the possible affectation of sperm DNA integrity and its recovery after therapy.

Sperm DNA Damage and Cancer

Induction of DNA damage is the main mechanism of cell death produced by most drugs or local radiotherapy used for cancer treatment. It is known that cancer itself is linked to disruption of spermatogenesis [97] and that chemotherapy usually results in temporary or permanent azoospermia. The determination of SDF may be useful to monitor the toxicogenetic effect of cancer therapy on sperm cells and to evaluate their recovery in terms of DNA integrity [98]. Sperm cryopreservation before radio/chemo-treatment remains the best option for cancer patients to preserve their fertility. With the introduction of IVF and ICSI, even the poorer sperm samples might be frozen with good expectations of success [99]. In spite of this, the quality of sperm DNA may be affected in tumorous cancers (non-seminoma type), seminoma and others. The mean SDF in these patients was 35.8%, which is comparable to what has been reported in infertile patients, and higher than that of fertile donors. The percentage of SDF was 46.2% in leukaemia and 48.8% for other types, but was lowest in Hodgkin lymphoma (28.08%). A recent study with the SCSA has also reported similar results [100]. In conclusion, the presence of cancer, regardless of its origin, affects sperm DNA quality and could perhaps be an underlying cause of temporary infertility. SDF should therefore be evaluated in the sperm samples to be frozen before therapy, in order to choose those samples with the best DNA quality.

Azoospermia

Azoospermia may be due to testicular failure or due to duct obstruction. In any case, foci of spermatogenesis may still exist within the testicle, and so, sperm cells may be obtained from testicular biopsies. The SCD technique is especially adequate to analyze samples with low amounts of spermatozoa and much debris. Testicular sperm samples from 62 patients were analyzed with the SCD test. The patients with obstructive azoospermia ($n=40$) showed $35.9 \pm 2.6\%$ of sperm cells with fragmented DNA, whereas those with non-obstructive azoospermia ($n=22$) revealed $46.9 \pm 4.5\%$ of cells with SDF [101]. Thus, the incidence of DNA damage in testicular sperm populations from infertile men with azoospermia is much lower in normal and active spermatogenic testis than in testis with incomplete sperm production. A recent study by Smit et al. [102] has also confirmed that SDF is higher in patients with poor spermatogenesis than in those with normal spermatogenesis. It is possible that defective spermatozoa are sensed by a genomic screening mechanism that triggers DNA fragmentation to genetically inactivate sperm cells with a defective genomic makeup. In fact, sperm cells containing aneuploidies are more prone to contain fragmented DNA [43]. A study by Greco et al. [103] showed that the incidence of DNA fragmentation was lower in testicular spermatozoa compared with ejaculated spermatozoa, proposing its use in ICSI for patients with high levels of SDF in the ejaculate. Both studies clearly show that sperm DNA damage may be detected just after finishing telophase II at the onset of spermiogenesis or can occur during the epididymal sperm passage.

Toxicogenetics

Reproductive toxicology is a discipline of remarkable interest, with strong implications on the potential adverse reproductive health effects of exposure to internal or environmental toxic agents. SDF is an ideal parameter to monitor, as it is a very sensitive marker of reproductive toxicants. Many agents that affect germs cells at different stages of meiosis or spermiogenesis induce genome modifications that will later be translated as DNA fragmentation in the sperm cell [104]. For example, exposure to anticancer chemotherapy [98], air pollution [105], pesticides such as

DDT [106], mobile phone radiation [107], and treatment with the serotonine reuptake inhibitor paroxetine [108], have all been shown to induce SDF. Interestingly, in many cases, DNA fragmentation is observed without any significant effect on standard seminal parameters. In a study by Viloria et al. [109], 99 males provided semen samples that were analyzed by the SCD test before and after swim-up treatment. The results were correlated with the patient's cigarette smoking habits. Although no differences were detected before swim-up, in the capacitated samples, smokers and especially heavy smokers (≥20 cigarettes per day) showed significantly impaired DNA quality compared to non-smokers. The fact that differences are observed after swim-up but not in the ejaculate may be due to the fact that the incubation time necessary for the swim-up technique allows cryptic DNA damage to be expressed. This highlights the potential interest in a dynamic evaluation of DNA fragmentation as a more sensitive assay for reproductive toxicology.

The effect of vaccination on SDF was assessed in rams vaccinated with Miloxan (*Clostridium perfringens* type C, D and *C. oedematiens* type B), using the SCD test [110]. Miloxan increased the percentage of sperm cells with fragmented DNA by tenfold on average (from 6.5 ± 7.9 to 63.4 ± 24.2%). However, the negative impact of vaccination on SDF was reversible, decreasing to 21.7 ± 10.6% 40 days after vaccination. The effects of vaccination on sperm quality and particularly on sperm DNA integrity probably consist of many factors and effectors, such as the genetic background, and the capacity to respond to oxidative stress or temperature variations. This result has important implications in the use of semen samples from vaccinated animals and the same implications for post-vaccination in humans.

Sperm DNA Fragmentation Dynamics

Semen parameters such as motility, viability, etc., are usually evaluated once at different periods in time after sperm collection. However, these values may change during the useful lifespan of a sperm sample. Measurements are therefore of value when performed (1) at the time of ejaculation and (2) at the time of insemination, IVF or intracytoplasmic injection. Usually, ART logistics generate a time lapse between both periods and a clear reference to the time of assessment is generally not precisely stated in the literature. Similarly, when values for SDF are quoted, clear references about the time of assessment following ejaculation are seldom included.

When SDF is assessed immediately after ejaculation, using the SCD test, donors with proven fertility show significantly less fragmentation than infertile patients (Fig. 10.5a, b). One would assume that sperm DNA is unstable when maintained in a para-biological environment such as those used to store a semen sample after ejaculation. The conditions of sperm storage influence the sperm DNA longevity and a certain amount of iatrogenic DNA damage is to be expected. Some reports indicate that when the kinetics of sperm DNA damage are analyzed, DNA degrades progressively when incubated in identical conditions to those used for IVF. The use of semen samples incubated at 37°C during a period of 24 h produce a cumulative increase in the level of the DNA in the order of 2–8% during the first 4 h of incubation [77] depending on the individual analyzed. In donors of proven fertility, the rate of SDF behaves independently of other sperm parameters such as the dynamic loss of sperm viability, although a certain degree of negative correlation exists [80].

This dynamic loss of sperm DNA quality has also been observed in other animal species such as stallion [27], ram [29], boar [34, 111], donkey [28], rhinoceros [41], koala [30, 31], echidnas [32] and fish [35]. In all species analyzed to date, two important factors must be taken into account (1) the existence of a large variation in the species-specific rate for SDF and (2) the variability in the inter-individual rate for SDF. Thus, while in fish the increase in SDF is triggered after a few minutes of sperm activation, in boar, the increase SDF is triggered after days incubated at 37°C in the appropriate semen extender. In humans, there exists large

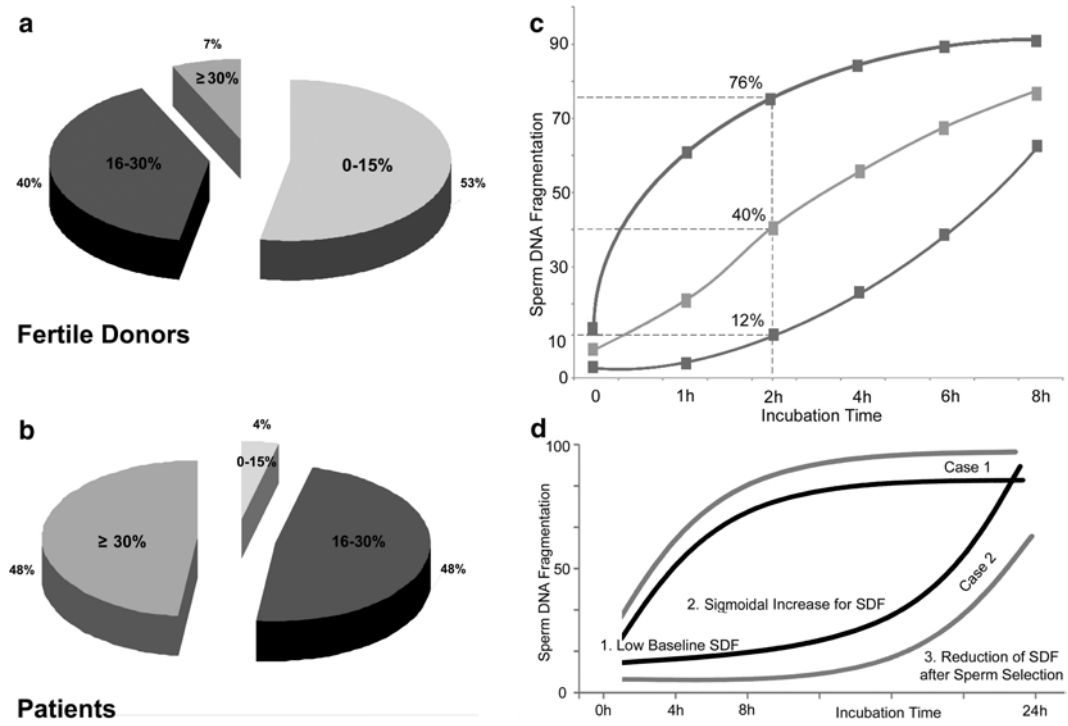

Fig. 10.5 Distribution of SDF a time 0 (baseline SDF) in fertile donors (**a**; $n=55$) and patients (**b**; $n=75$). Distribution ranges were fixed to <15, 16–30 and >31% of SDF (**c**) Different values for SDF obtained at different incubation times in three individuals showing different dynamics for SDF. Note that large differences are obtained at different incubation times (values at 2 h are represented). (**d**) Recommended criteria to discriminate between a "good" and a "bad" sperm sample considering the dynamic behaviour of the SDF after sperm selection

variability in the rate of SDF from individual to individual. Thus, sperm samples with a similar level of SDF as measured immediately after ejaculation will behave differently when incubated at 37°C. As shown in Fig. 10.5c, the SDF level obtained after 4 h of incubation is 15% in one individual and 70% in the other. The general figure depicts three main patterns for SDF increase that can be adjusted to a logarithmic, linear or sigmoidal curve (Fig. 10.5d). Individuals presenting a sigmoidal tendency for the increase in SDF would have a lower percentage of sperm cells with damaged DNA at any given incubation time. Thus, as depicted in Fig. 10.5d, the best donor would be one that (1) presented the lowest level of baseline sperm DNA damage, (2) exhibited a sigmoidal tendency for increase in SDF and (3) showed a decrease in the level of SDF but maintained the sigmoidal tendency after sperm selection (swim-up or gradient). The analysis of the rate of SDF increase may provide useful information when used for IVF or IUI. Although this requires further inspection, there exists the possibility that the dynamic increase of SDF serves as a possible explanation to some of the discrepancies observed in the literature about the role of SDF and ART outcome.

The first clue about the impact of the dynamics of sperm DNA damage was offered by Young et al. [112]. The authors of this study demonstrated that semen collection away from the laboratory with overnight mail delivery could lead to sperm DNA damage and this had subsequent implications on fertilization. In particular, the longevity of the DNA molecule could be highly compromised in cases such as the use of samples from testicular sperm extraction or aspiration. In testicular sperm from men with obstructive azoospermia, DNA

fragmentation after cryopreservation is increased by 4 and 24-h incubations, and this effect is intensified by post-thaw incubation. In such circumstances, it is recommended that testicular sperm samples for ICSI should be used with the minimum delay in sperm capacitation [113, 114]. Bungum et al. [115] found that co-incubation of 777 sibling oocytes from 81 women undergoing IVF produced good fertilization rates using co-incubation for either 30 s or for 90 min and significantly lower rates of polyspermy. All these inputs indicate that there may be beneficial effects for short sperm/oocyte co-incubation in IVF. Although more relevant studies are needed, taking into account the dynamic increase of SDF, the probability of fertilization with a damaged sperm would diminish using short incubation periods.

The debate over whether cryopreservation induces direct damage on the DNA molecule is still open. A comparison of the dynamics of SDF in fresh and cryopreserved semen samples from the same donor showed that sperm DNA tends to degrade very quickly after thawing. In practice, sperm DNA degradation could be detected at the onset of thawing and temperature recovery to 37°C. However, large differences in the level of SDF were not observed when the semen sample was assessed for SDF just after thawing [82]. This indicates that cryopreservation does not change the baseline rate of SDF when analyzed just after thawing but may change the dynamics of SDF [29, 80].

In conclusion, the dynamic behaviour of SDF indicates that when the semen sample is used for IUI or IVF, the level of SDF may be higher at the time of fertilization than when assessed in the clinical practice. In natural reproduction and IUI, only a small fraction of the sperm cells will enter the cervix, pass into the uterus, and progress to the uterotubal junctions to reach the Fallopian tubes. In this environment, the selected sperm fraction is maintained in a fully functional state by connecting with endosalpingeal epithelium [116, 117]. To reduce the delay in fertilization and mitigate the effect of a rapid rate of SDF, full synchronization of the oestrus and time of insemination is required, reducing the handling of semen ex vivo. The role of semen plasma in connection with the female tract and its implications in sperm protection for SDF is largely unknown. There are indications that semen plasma proteins are absent in the oviduct. This indicates that their presence is probably restricted to uterine environments and not to other female reproductive regions closer to the oocyte [118]. These considerations should be taken into account when making extrapolations about the stability of sperm DNA ex vivo and in vivo.

Finally, we want to draw attention to the fact that the comparison of results for SDF from different laboratories or even those obtained within the same laboratory may be biased if clear references to the time of measurement are not precisely given. This could be aggravated if details of the storage or thawing conditions are not clearly communicated.

Conclusion: Value of the SCD Test

Sperm DNA damage has been connected, among other things, with an increased incidence of miscarriage and enhanced risk of disease in the offspring. However, its occurrence is multifaceted, and many of the variable consequences it has for fertility are as yet not fully understood [119–121]. Fertility is a multifactorial phenomenon that usually involves both members of the couple, and assessment of sperm DNA integrity is only one piece of a complex puzzle. Tests that assess sperm quality should identify not only the ability of spermatozoa to reach the oocyte with an intact DNA molecule but also their ability to fertilize the oocyte and activate embryo growth. To paraphrase Makhlouf and Niederberger [122] when referring to the sperm as a whole functional cell, it is not just the carrier but also the content that is important. With the appearance of ICSI, however, the content seems to have taken a preponderant role. SDF should therefore be considered a parameter of sperm quality. Its determination may provide beneficial information in andrological pathology, complementary to that obtained from standard semen parameters. SDF must be evaluated concurrently and examined within the clinical context of each patient or couple.

Compared to other methods of assessing DNA fragmentation, the SCD test can be conducted promptly and without the need for complex and expensive laboratory equipment. The SCD test is a powerful and versatile approach for investigating DNA fragmentation, allowing the assessment of damaged DNA over a diverse range of clinical situations. The technique can be easily adapted to incorporate new research directions, and the analysis of sperm DNA can be performed on wide range of species. The SCD test has the unique ability to allow direct observations to be made of the spermatozoa and the corresponding DNA damage; this technical advantage allows direct correlations between DNA fragmentation and DNA sequence variations, nucleotide modification and/or protein status. The SCD is a procedure that allows researchers the flexibility to use their creative imagination when designing and conducting experiments to disentangle the obscure topic of sperm DNA damage.

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