Article

A two-tailed Comet assay for assessing DNA damage in spermatozoa

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Abstract

DNA fragmentation is considered an important parameter of semen quality, and of significant value as a predictor of male fertility. Poor quality chromatin is closely associated with, and highly indicative of, some fertility problems. Many methodologies to assess DNA fragmentation in spermatozoa are available, but they are all unable to differentiate between single-stranded DNA breaks (SSB) and double-stranded DNA breaks (DSB) in the same sperm cell. The two-tailed Comet assay (2T-Comet) protocol overcomes this limitation. A modification of the original Comet assay was developed for the simultaneous evaluation of DNA SSB and DSB in human spermatozoa. The 2T-Comet assay is a fast, sensitive, and reliable procedure for the quantification and characterization of DNA damage in spermatozoa. It is an innovative method for assessing sperm DNA integrity, which has important implications for human fertility and andrological pathology.

Keywords: *DNA fragmentation, double-stranded DNA breaks, single cell gel electrophoresis (SCGE), single-stranded DNA breaks, spermatozoa*

Introduction

Single-cell gel electrophoresis (SCGE) was developed in 1984 (Ostling and Johanson, 1984), and is known for its ability to detect DNA damage at the single cell level. In this assay, cells embedded in an inert agarose matrix on a slide are lysed by detergents and high salts (1–2.5 mol/l sodium chloride), resulting in deproteinized nuclei, i.e. nucleoids. Afterwards, DNA is electrophoresed, a process in which broken DNA strands migrate towards the anode, resulting in the formation of a Comet tail emerging from the nucleoid. The formation of a tail has implications: the larger the tail and/or the higher the DNA density in the tail, the greater the extent of DNA damage. There are two types of SCGE techniques: (i) the neutral Comet (Olive *et al.*, 1991), in which DNA migrates under neutral conditions, for identification of double-stranded DNA breaks (DSB); and (ii) the alkaline Comet, in which DNA is mobilized under alkaline conditions for DNA denaturation. This technique detects both single-stranded DNA breaks (SSB) and DSB, without distinguishing between the two (Singh *et al.*, 1988).

An evaluation of DNA integrity in spermatozoa shows the frequency of sperm cells containing fragmented DNA to be consistently higher in infertile males compared with fertile males (Zini *et al.*, 2001). Men with poor standard semen parameters were more likely to show a higher proportion of spermatozoa with fragmented DNA than were men with normal semen parameters, as were males with certain andrological pathologies such as infections (Gallegos *et al.*, 2007) or varicocele (Enciso *et al.*, 2006). Many studies suggest that sperm DNA fragmentation could influence fertilization, embryo quality and development, blastocyst achievement, and pregnancy rates and losses (Benchaib *et al.*, 2003; Seli *et al.*, 2004). There are also a few studies showing the lack of association between sperm DNA damage and embryo quality or pregnancy rate (Zini *et al.*, 2005). As they report, this may be the result of the selection of only morphologically normal spermatozoa and good quality embryos for transfer.

There are disparate viewpoints regarding the role of sperm DNA fragmentation in fertility. One possible reason for the discrepancies could be the imprecision of the term 'DNA fragmentation', leading to, for example, a lack of discrimination between SSB and DSB. Both types of DNA breaks could be present in the same cell in different proportions, which would impact the clinical implications. An appreciation of the differences between SSB and DSB is important, since DSB repair by the oocyte to maintain DNA base fidelity or the proper gene or chromosome arrangement should be more difficult than would be the repair of SSB. This rationale would also apply to large-scale SSB. SCGE using the alkaline or neutral technique could potentially distinguish both types of DNA breaks. However, due to the limitations of each technique, independent experiments are necessary under each condition. Consequently, the simultaneous assessment of SSB and DSB has been unattainable. The aim of the experiments reported here was to validate a two-dimensional or two-tailed Comet assay (2T-Comet) as a reliable method for the simultaneous characterization of SSB and DSB in the same human spermatozoa.

Materials and methods

2T-Comet assay

The study was carried out using human mature spermatozoa from five healthy donors. Sperm cells were diluted to a concentration of 10×10^6 spermatozoa/ml in phosphate-buffered saline (PBS). Next, 25 μl of the cell dilution was mixed at 37ºC with 50 μl of freshly prepared 1% low melting point agarose (LMP; type VII; Sigma, St Louis, MO, USA.) in distilled water. An aliquot of 15 μl of the mixture was placed on a pretreated slide for gel adhesion (Chromacell SL, Madrid, Spain) covered with a coverslip and transferred to an ice-cold plate to promote fast gelling. As soon as the gel solidified, coverslips were smoothly removed and the slides were submerged sequentially in two lysing solutions: lysing solution 1 [0.4 mol/l Tris–HCl, 0.8 mol/l dithiothreitol (DTT), 1% sodium dodecyl sulphate (SDS), pH 7.5] for 30 min, followed by lysing solution 2 (0.4 mol/l Tris–HCl, 2 mol/l NaCl, 1% SDS, 0.05 mol/l EDTA, pH 7.5) for 30 min. Then, slides were rinsed in TBE buffer (0.09 mol/l Tris–borate, 0.002 mol/l EDTA, pH 7.5) for 10 min, transferred to an electrophoresis tank and immersed in fresh TBE electrophoresis buffer. Electrophoresis was performed at 20 V (1 V/cm), 12 mA for 12.5 min. After washing in 0.9% NaCl, nucleoids were unwound in an alkaline solution (0.03 mol/l NaOH, NaCl 1 mol/l) for 2.5 min, transferred to an electrophoresis chamber, and oriented 90º to the first electrophoresis. The second electrophoresis was performed at 20 V (1 V/cm), 12 mA for 4 min in 0.03 mol/l NaOH. Then, slides were rinsed once in a neutralization buffer (0.4 mol/l Tris-HCl, pH 7.5) for 5 min, briefly washed in TBE buffer, dehydrated in increasing concentrations of ethanol, and air dried.

Finally, DNA was stained with SYBR Green I (Molecular Probes, Leiden, The Netherlands) at a 1:3000 dilution in Vectashield (Vector Laboratories, Burlingame, CA, USA). Samples could be immediately analysed or stored at room temperature in the dark until needed. The Comets could be assessed by visual scoring or digitalization and image processing. The frequency of sperm cells with fragmented DNA, i.e. the DNA fragmentation index (DFI), was established by measuring at least 500 sperm cells per slide. Cells were classified as undamaged (no DNA migration) or damaged (migrated DNA) cells. The type of damage could also be assessed; sperm cells were classified as containing SSB (up down migration), DSB (right left migration) or both.

Fluorescence in-situ hybridization (FISH)

Single-stranded DNA (ssDNA) detection

A digoxigenin-labelled whole human genome probe [5 ng/ml in 50% formamide/2 × saline sodium citrate (SSC) pH 7.0, 10% dextran sulphate, and 100 mmol/l calcium phosphate, pH 7.0], was denatured and hybridized overnight at 37ºC onto 2T-Comet assay processed slides. They were then washed in 50% formamide/2× SSC, pH 7.0, for 5 min; twice in 2× SSC, pH 7, for 3 min and finally, once in 4× SSC, 0.1% Tween 20, pH 7.0 for 2 min, at room temperature. The hybridized probe was detected with antidigoxigenin–fluorescein isothiocyanate (FITC) (1:200) (Sigma), and spermatozoa were counterstained with propidium iodide. Images were viewed with a fluorescence microscope.

Acridine orange (AO) staining

2T-Comet assay processed slides were air-dried and stained with freshly prepared AO stain (0.5×10^{-4} mol/l; Polysciences, Warrington, PA., USA). A 15 μl aliquot of this solution was placed on a slide, covered with a coverslip and immediately evaluated under a fluorescence microscope at the excitation wavelength of 450–490 nm.

Induction of SSB

Hydrogen peroxide (H_2O_2)

A human mature sperm sample diluted in PBS to a concentration of 10×10^6 spermatozoa/ml was divided into four aliquots of 200 μl each, allowing a control group to be compared with the treated cells. To induce SSB (Yamamoto, 1969), spermatozoa were incubated with 0.03, 0.15 and 0.3% H_2O_2 (Sigma Chemical Company, Poole, Dorset, UK) for 30 min at room temperature. Next, the 2T-Comet protocol was performed as described above. SSB DNA fragmentation index (*SSB-DFI*) was established by measuring at least 500 sperm cells per slide. Cells were classified as previously described.

Induction of DSB

Restriction enzyme digestion

A restriction enzyme (*Alu*I) was used to induce DSB in sperm nucleoids (Roberts, 1976; Brooks, 1987). After 1 h lysis treatment of the Comet assay protocol as described above, slides were extensively rinsed with TBE buffer for 20 min and incubated for 10 min in 50 μl of restriction enzyme reaction buffer. After that, 15 μl of reaction buffer containing 15 units (IU) of enzyme was placed on the slide, covered with a plastic coverslip and incubated in a moist chamber at room temperature. On the same slide, three samples were analysed: sperm cells incubated without enzyme (control), and sperm cells incubated with 15 IU of enzyme for 15 and 25 min. After washing in TBE for 10 min, slides were placed in an electrophoresis tank and the 2T-Comet assay protocol described above was continued. Finally, DSB DNA

fragmentation index (*DSB-DFI*) was established by measuring at least 500 sperm cells per slide. The cells were classified as described.

Digital image analysis (DIA)

Leica Qwin software was used (Leica Microsystems, Wetzlar, Germany). The aim was to apply DIA for classification of cells from a sample processed by 2T-Comet assay into the visually established Comet types. This analysis comprised the following steps.

Image capture

Grey-level images were captured with a charge-coupled device camera (Cool Snap; Roper Scientific, Tucson, AZ, USA) and stored in TIFF format employing Cool Snap software.

Processing of grey images

The original grey-level image was transformed into a binary one. A threshold of grey level was fixed. All pixels of the image that adjusted to the defined threshold were transferred to a binary image. Background subtraction was performed to fix a basal level of background with grey values 0. The rest of the image information was considered as DNA. For visual checking enhancement, a no-true-colour assignment, based on grey image levels and area after background subtraction was performed.

Measurements

Several parameters were considered for the quantitative measurement of each whole Comet: *Area* = number of pixels measured, *Sum grey* = sum of the grey levels from pixels, *Mean grey* = average of the grey levels from pixels, *Std dve* = standard deviation of the levels of greys from pixels, *Max grey* = maximum grey level from pixels, *Min grey* = minimum grey level from pixels, *Median* = medium of the grey levels from pixels, and *Mode* = mode of the grey levels from pixels.

Statistical analysis

A chi-squared test was used to detect significant differences in the frequencies of each Comet type between the control and each one of the treatments (H_2O_2 and *Alu*I). In order to identify the Comet types responsible for the significant differences, an adjusted standardized residuals analysis was performed. To determine significant differences in the values of each of the calculated parameters by the DIA software from the different 2T-Comet types, an analysis of variance was used. Finally, a Mann–Whitney *U*-test was performed to detect significant differences in the DNA Fragmentation Index (DFI) calculated between the groups of infertile and fertile subjects. Frequency of sperm cells with fragmented DNA due to SSB: (*SSB-DFI*) and frequency of sperm cells with fragmented DNA due to DSB (*DSB-DFI*) estimation and statistical tests were performed using the Statistics Package for Social Sciences (SPSS) v.14.0 (SPSS Inc., USA).

Results

The 2T-Comet assay presented was tested on five normozoospermic human sperm samples. First, a neutral lysis and electrophoresis was performed, the Comet tail being positioned in the X-axis. Second, an alkaline lysis and electrophoresis perpendicular to the first neutral run was performed (Y-axis). The Comet types found in the analysed semen samples are shown in **Figure 1**.

To identify the type of damage present in the tails produced by the combination of both lysis and electrophoresis, FISH with a human whole genome probe was performed in 2T-Comet-processed sperm cells. The DNA probe hybridized mainly in the head and tail placed on the Y-axis, showing that the Comet tail formed from this electrophoresis run contains mainly ssDNA (**Figure 2a**).

To further corroborate these results, another experimental approach was performed using the fluorochrome AO, a

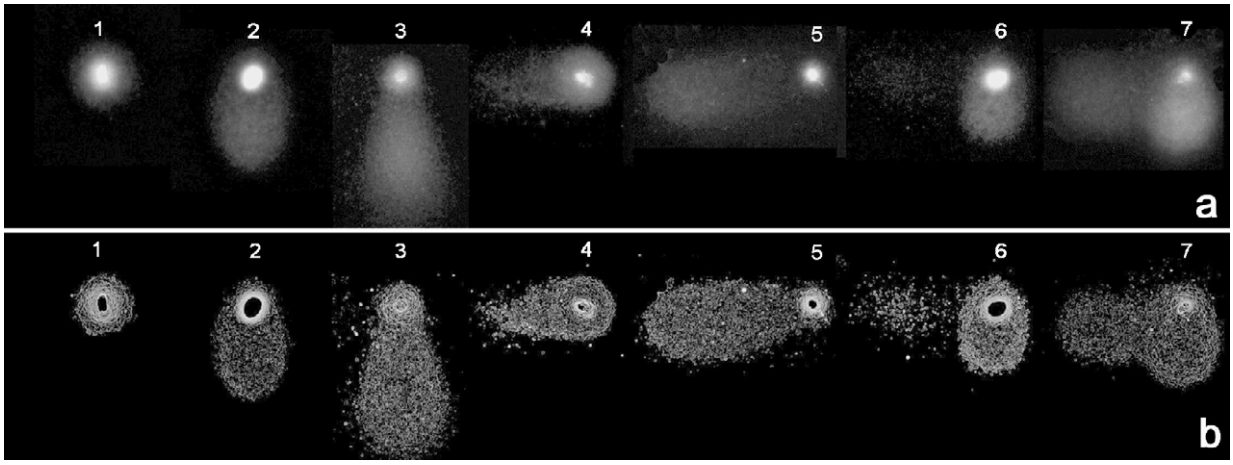

Figure 1. Two-tailed (2T)-Comet assay protocol detects seven Comet types: 1, undamaged; 2, low level of single-stranded DNA breaks (SSB); 3, high level of SSB; 4, low level of double-stranded DNA breaks (DSB); 5, high level of DSB; 6, low level of SSB and low level of DSB; 7, high level of SSB and high level of DSB. (**a**) 2T-Comet types stained with SYBR Green I found in a normal human semen sample first electrophoresed under neutral conditions (from right to left; X-axis) and then 90° electrophoresed (from upper to lower; Y-axis) under alkaline conditions. (**b**) Same images after application of a common electronic filter.

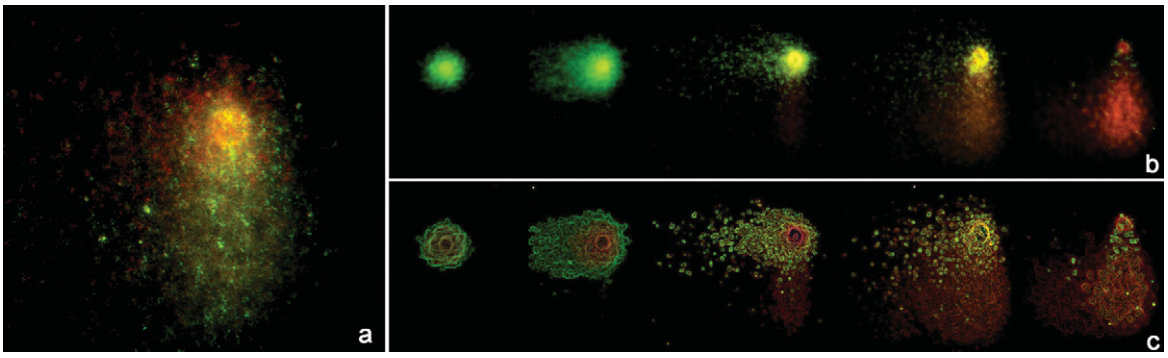

Figure 2. Characterization of single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) by fluorescence in-situ hybridization (FISH) and acridine orange staining. (**a**) ssDNA detection by FISH with a digoxigenin-labelled whole human genome probe and fluorescein isothiocyanate-detected (green) on two-tailed (2T)-Comets obtained from human spermatozoa, counterstained with propidium iodide (red). (**b**) 2T-Comet types found in a normal semen sample first electrophoresed under neutral conditions (from right to left; X-axis) and then 90° electrophoresed under alkaline conditions (from upper to lower; Y-axis), stained with 0.5×10^{-4} mol/l acridine orange. (**c**) Same images after application of a common electronic filter.

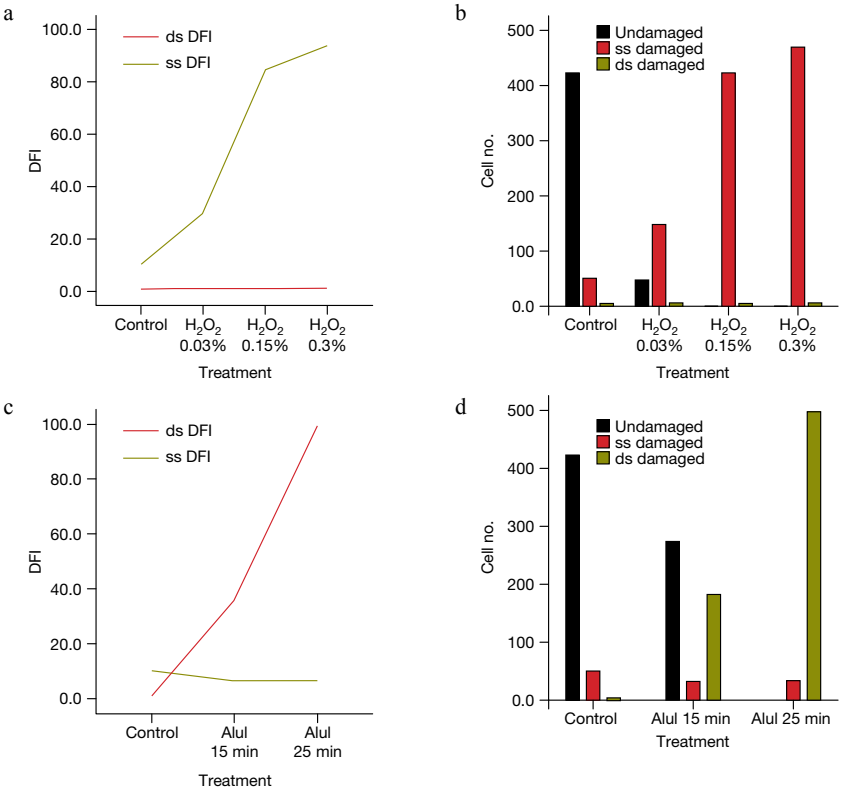

Figure 3. DNA damage detection. (**a**) Single-stranded breaks DNA fragmentation index (*SSB-DFI*) and double-stranded breaks DNA fragmentation index (*DSB-DFI*) from a normal semen sample treated with increasing concentrations of H_2O_2. (**b**) Histograms showing the number of undamaged, damaged cells with SSB and damaged cells with DSB in the sperm sample exposed to varying concentrations of H_2O_2. Significant differences (chi-squared test, $P < 0.05$) were found in the number of damaged cells with SSB between treated samples and control. (**c**) *SSB-DFI* and *DSB-DFI* of the same semen sample, *Alu*I-digested. (**d**) Histograms showing the number of undamaged, damaged cells with SSB and damaged cells with DSB in the sperm sample *Alu*I-digested. Significant differences (chi-squared test, $P < 0.05$) were found in the number of damaged cells with DSB between digested samples and control.

Figure 4. Binary image detection. 1, Undamaged spermatozoa; 2, spermatozoa with low level of SSB and without detectable DSB; 3, spermatozoa with high level of SSB and without detectable DSB; 4, spermatozoa with low level of SSB and high level of DSB; 5, spermatozoa with high level of SSB and high level of DSB.

selective metachromatic dye that interacts with DNA by intercalation or electrostatic attractions. When AO binds to dsDNA motifs, it intercalates in the DNA structure as a monomer, reaching a maximum emission at 525 nm (green). When AO binds to ssDNA, it forms non-ordered aggregates where the maximum emission shifts to 650 nm (red). AO staining reveals the presence of ssDNA in the Comet tail placed on the Y-axis and dsDNA in the Comet tail placed in the X-axis (**Figure 2b**,**c**). Therefore, the type of DNA damage can be identified by analysing the orientation of the DNA tail produced.

According to these results and the assumed principle that the larger the tail and/or the higher the DNA density in the tail, the greater the extent of DNA damage (Schmid *et al.*, 2003), the 2T-Comet types represented in **Figure 1** can be classified into seven types: 1, undamaged; 2, low level of SSB; 3, high level of SSB; 4, low level of DSB; 5, high level of DSB; 6, low level of SSB and low level of DSB; 7, high level of SSB and high level of DSB.

Moreover, a normozoospermic human semen sample was treated independently with active producers of SSB and DSB, hydrogen peroxide (H_2O_2) and the restriction endonuclease *Alu*I respectively. The results were expressed as the frequency of sperm cells with fragmented DNA, i.e. DNA fragmentation index (DFI), discriminating *SSB-DFI* when DNA breaks corresponded to SSB, and *DSB-DFI* when DNA breakage corresponded to DSB.

In samples treated with H_2O_2, the frequency of sperm cells with the Comet tail placed on the Y-axis, i.e. with SSB *(SSB-DFI)* significantly increased (chi-squared test, $P < 0.05$) (**Table 1**) with concentration, while no change in the frequency of sperm cells with the Comet tail placed in the X-axis, i.e. with DSB (*DSB-DFI)* was found (**Figure 3**).

In the samples treated with *Alu*I, the results were reversed, and a statistically significant increase in the frequency of sperm cells with DSB was observed (**Figure 3**) (chi-squared test, $P < 0.05$) (**Table 1**). Similar results were obtained when the same experimental design was performed on human lymphocytes (data not shown).

These results confirm that the first neutral lysis and electrophoresis is measuring DSB and the second alkaline lysis and electrophoresis is detecting SSB. Therefore, the 2T-Comet assay is able to detect and discriminate SSB and DSB simultaneously in the same cell.

Neutral and alkaline Comet assays were run in parallel to compare the *SS* and *DS*DNA damage levels obtained. In the samples treated with H_2O_2, significant differences ($P < 0.05$) were found between the *SSB-DFI* calculated using the alkaline Comet assay and the 2T Comet assay; this result was expected, since the alkaline version is able to measure both SSB and DSB (Singh *et al.*, 1988; Collins *et al.,* 1997). In the samples treated with *Alu*I, no significant differences were found between the *DSB-DFI* calculated using the neutral Comet assay and the 2T-Comet assay (data not shown).

In addition to visual scoring, digital image analysis was performed to produce a prototype for automatic measure of the DNA damage level. After image capture, QWin software (Leica Microsystems, Wetzlar, Germany) was used to quantify the DNA migration registered on each spermatozoon (**Figure 4**). An analysis of the variance determined significant differences ($P < 0.05$) in the values of all of the calculated parameters from the different 2T-Comet types except *Max grey*. Some of the parameters measured, in particular, *Mean grey* and *Min grey* together, presented significantly different values in all analysed Comet types allowing the quantitative characterization of 2T-Comet types previously established visually (**Table 2**). This allowed every sperm cell to be classified as a Comet type if specific thresholds values were previously fixed.

Inter-assay variability was evaluated by measuring the same normozoospermic human sperm sample in five different assays (A, B, C, D, E). Intra-assay variability was determined by assessing the same sample five times in the same assay (A1, A2, A3, A4, A5). Results are shown in **Table 3**. A chi-squared test ($P < 0.05$) found no significant differences in the presence of each Comet type between the slides.

Finally, the frequency of sperm cells with SSB *(SSB-DFI)* and DSB *(DSB-DFI)* was studied in a group of 10 infertile patients with abnormal standard semen parameters (volume, concentration, motility, viability and normal morphology based on WHO criteria) and compared with those obtained in a group of 10 normozoospermic fertile men. The infertile patients had a significantly higher percentage of spermatozoa with DSB, (Mann–Whitney *U*-test, $P < 0.05$) compared with the group of fertile subjects. Nevertheless, no significant differences were found in the percentage of spermatozoa with SSB between the infertile patients and the fertile men (**Table 4**).

Discussion

The Comet assay has been used for years to assess DNA damage. In this assay, only a small number of cells are required for analysis, and data are collected at the individual cell level, providing information on the intracellular distribution of DNA damage (Collins *et al.*, 1997). The one-dimensional Comet assay is highly reproducible, easy to perform, and capable of identifying specific types of DNA damage with a high level of confidence.

Only two main reports included data about results and possible interpretation using two-dimensional gel electrophoresis. The first report used either neutral or alkaline conditions in both electrophoreses to demonstrate the presence of DNA fragments that were free to be mobilized by the second run (Klaude *et al.*, 1996). The second report suggested the correlation of the two-dimensional tails with the presence of SSB and DSB, but was not able to demonstrate the methodology or its evaluation (Fernandez *et al.*, 2003).

The methodology presented here suggests an additional step to achieve a clear distinction between SSB and DSB that could relate male-factor infertility to the type of DNA damage. DSB are generally considered to be more relevant than SSB. Some authors comment that the SSB observed in the alkaline version are not the most interesting of DNA lesions, as they are quickly repaired and are not regarded as a cause of chromosome

Table 1. Absolute frequencies of two-tailed Comet types found in a normozoospermic semen sample (control) that was then treated with H_2O_2 or digested with *Alu*I (n = 500 spermatozoa).

Treatment	*Undamaged*	*DSB low*	*DSB high*	*SSB low*	*SSB high*	*SSB low, DSB low*	*SSB low, DSB high*	*SSB high, DSB low*	*SSB high, DSB high*
Control	423	4	0	18	48	2	2	1	2
H_2O_2 (%)									
0.03%	48[a]	2	1	296[a]	144[a]	1	3	3	2
0.15%	2[a]	2	2	68[a]	421[a]	1	2	1	1
0.30%	0[a]	1	2	24[a]	467[a]	1	2	1	2
AluI digestion (min)									
15	274[a]	25[a]	154[a]	1[a]	12[a]	6	8[a]	0	20[a]
25	0[a]	2	143[a]	0[a]	0[a]	0	322[a]	0	33[a]

DSB = double-stranded breaks; SSB = single-stranded breaks.
[a]Within each column, these values are significantly different from the control ($P < 0.05$; chi-squared test).

Table 2. Multiple comparisons analysis.

Cell type (I)	*Cell type (J)*	P-*value* *Mean grey*	*Min grey*
Normal	SSB Low	<0.001	0.017
	SSB High	<0.001	0.001
	DSB High SSB Low	<0.001	<0.001
	DSB High SSB High	<0.001	NS
SSB Low	Normal	<0.001	0.017
	SSB High	<0.001	NS
	DSB High SSB Low	<0.001	<0.001
	DSB High SSB High	<0.001	NS
SSB High	Normal	<0.001	0.001
	SSB Low	<0.001	NS
	DSB High SSB Low	<0.001	0.005
	DSB High SSB High	<0.001	NS
DSB High SSB Low	Normal	<0.001	<0.001
	SSB Low	<0.001	<0.001
	SSB High	<0.001	0.005
	DSB High SSB High	NS	0.010
DSB High SSB High	Normal	<0.001	NS
	SSB Low	<0.001	NS
	SSB High	<0.001	NS
	DSB High SSB Low	NS	0.010

The mean difference is significant at $P = 0.05$. DSB = double-stranded breaks; NS = not statistically significant; SSB = single-stranded breaks.
The multiple comparisons analysis indicated that the variables Mean grey (average of the grey levels from pixels) and Min grey (minimum grey level from pixels) together present significantly different values in all analysed Comet types. These two variables would be able to classify the cells analysed in one of the visually established Comet types.

aberrations and loss of genetic material (Collins *et al.* 1997; Speit *et al.*, 1998). More studies are required to investigate the biological meaning of the DNA damage observed by all of the reported versions of the assay conditions, since the nature of Comets, and the physicochemical events underlying their formation, are not fully understood or agreed upon. The 2T-Comet potentially could resolve some of the scientific debate presently surrounding the actual influence of sperm DNA fragmentation on pregnancy and other fertility parameters.

In addition to the one-dimensional Comet assay, other methodologies to assess sperm DNA fragmentation are available. The most popular are sperm chromatin structure assay (SCSA) (Evenson and Jost, 1994), in-situ nick translation

Table 3. Inter- and intra-assay variability of two-tailed (2T)-Comet assay.

Assay	*Undamaged*	*SSB low*	*SSB high*	*DSB low*	*DSB high*	*SSB low, DSB low*	*SSB high, DSB high*	*SSB-DFI*	*DSB-DFI*
A	81.1	3.0	9.0	4.0	1.5	0.6	0.8	13.4	6.9
B	80.4	3.6	9.6	3.6	2.0	0.4	0.4	14.0	6.4
C	82.4	3.2	8.0	4.2	1.0	0.6	0.6	12.4	6.4
D	78.4	3.6	9.6	4.6	2.2	0.8	0.8	14.8	8.4
E	77.1	4.0	9.4	5.0	2.5	1.0	1.0	15.4	9.5
Inter-assay variability (mean ± SD)	79.88 ± 1.90	3.48 ± 0.35	9.12 ± 0.60	4.28 ± 0.48	1.84 ± 0.53	0.68 ± 0.20	0.72 ± 0.20	14 ± 1.05	7.52 ± 1.23
Assay A									
1	76.7	3.9	10.0	5.1	1.2	1.6	1.5	17.0	9.4
2	79.0	3.8	9.0	5.2	1.0	1.0	1.0	14.8	8.2
3	76.9	4.1	9.2	6.0	1.4	1.2	1.2	15.7	9.8
4	77.8	3.7	8.9	4.8	1.2	1.8	1.8	16.2	9.6
5	75.2	4.5	10.0	5.0	2.0	1.3	2.0	17.8	10.3
Intra assay variability (mean ± SD)	77.12 ± 1.26	4 ± 0.28	9.42 ± 0.48	5.22 ± 0.41	1.36 ± 0.41	1.38 ± 0.29	1.5 ± 0.37	16.3 ± 1.04	9.46 ± 0.70

Frequencies of the 2T-Comet types found in five different assays (A, B, C, D, E) and the estimated inter-assay variability. Frequencies of the 2T-Comet types found in five assessments of the same assay (A, 1–5) and the calculated intra-assay variability. DFI = DNA fragmentation index; DSB = double-stranded breaks; SSB = single-stranded breaks.

Table 4. Double-stranded breaks DNA fragmentation index (*DSB-DFI*) and single-stranded breaks DNA fragmentation index (*SSB-DFI*) calculated (mean ± SE) from semen samples from infertile patients with abnormal semen parameters and fertile control subjects.

Semen sample taken from	DSB-DFI %	SSB-DFI %
Infertile patients with abnormal semen parameters	33.60 ± 7.56	27.80 ± 8.74
Fertile controls	6.60 ± 1.80	26 ± 11.94

(Manicardi *et al*., 1995) and TdT (terminal deoxynucleotidyl transferase)-mediated nick-end labelling assays (Gorczyca *et al*., 1993). Other alternatives are DNA breakage detection–fluorescence in-situ hybridization (Fernandez *et al*., 2000) and the sperm chromatin dispersion test (Fernandez *et al*., 2003). A limitation common to all these procedures is their inability to differentiate between SSB and DSB in the same sperm cell.

The 2T-Comet overcomes this limitation. This technique allows identification of the total level of sperm DNA fragmentation revealing both SSB and DSB in individual cells, allowing for a more precise and extensive analysis of the damage. The image analysis facilities provide an advanced, quantitative, unbiased approach to measuring sperm DNA damage. This tool could be further enhanced and linked with an automated microscopy platform, allowing an integrated and autonomous system to score large numbers of cells.

SCGE is accepted as one of the most sensitive techniques for measuring DNA strand breaks. The two-tailed Comet variant presented here offers a reliable method to assess sperm nuclear damage to provide a more comprehensive analysis of sperm DNA quality.

In fact, in the study of sperm samples from fertile and infertile populations presented, whereas no differences in the percentage of sperm cells with SSB were evident with respect to fertile subjects, infertile patients exhibit a higher level of sperm cells with DSB. It seems that DSB could be responsible for some fertility problems, especially since DSB repair by the oocyte is more difficult than is the repair of SSB. Nevertheless, this issue needs further investigation.

In any case, these results illustrate the value of assessing different types of DNA damage within each sperm cell and the particular ability of the 2T-Comet to reveal them. This technique also may be adapted to assess different DNA break types in other cell types.

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