Sperm DNA damage output parameters measured by the alkaline Comet assay and their importance

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Keywords
Alkaline Comet assay—ART outcomes—Comet distribution plot—olive tail moment—sperm DNA damage

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Summary
The alkaline Comet assay has shown high diagnostic value to determine male reproductive health and prognostic ability to predict ART success. Here, spermatozoon was analysed in 47 fertile donors and 238 patients, including 132 couples undergoing ART [semen was collected: Group I – within 3 months of their treatment (n = 79); and Group II – 3 months prior to their treatment (n = 53)]. We introduce four Comet distribution plots (A, B1, B2 and C) by plotting the level of DNA damage (x-axis) and percentage of comets (y-axis). Fertile donors had low mean DNA damage, olive tail moment and per cent of spermatozoa with damage and increased type A plots. Comet parameters were associated with clinical pregnancies in Group I. About 66% of couples with type A distribution plot were successful after ART, whereas couples with type B1, B2 and C distribution plots achieved 56%, 44% and 33% pregnancies respectively. The efficiency of the Comet assay was due to complete decondensation process, where the compact sperm nuclear DNA (28.2 ± 0.2 µm³) is decondensed to ~63 µm³ (before lysis) and ~1018 µm³ (after lysis). A combinational analysis of all the Comet output parameters may provide a comprehensive evaluation of patient’s reproductive health as these parameters measure different aspects of DNA damage within the spermatozoa.

Introduction
In a living organism, free radicals and other reactive oxygen species (ROS) are generated as a by-product of normal cell respiration (Kirkinezos & Moraes, 2001); as a result, ROS cause damage to proteins, lipids and nucleic acids and thereby compromise cell function (Cabiscoi et al., 2000; Ko et al., 2014). Nucleic acid is probably the most significant target of oxidative attack, which results in oxidative DNA damage that leads to a number of biological processes such as apoptosis, ageing, cancers and infertility (Totter, 1980; Ames et al., 1993; Simon et al., 2011a; Noblanc et al., 2013; Wright et al., 2014). In semen, there are two principal sources of free radicals production: leucocytes and spermatozoa themselves (Aitken et al., 1994, 2003). The removal of cytoplasm from spermatozoa during spermatogenesis leaves the spermatozoa particularly vulnerable to oxidative stress-mediated damage (Aitken & Baker, 1995). Although the spermatozoa lacks any protective biomolecules and repair mechanisms, it is protected from oxidative damage through two known approaches. First, the sperm DNA is tightly packed with protamines to form a crystalline structure, protecting it from ROS-mediated attack (Oliva, 2006). Second, the seminal plasma contains high level of enzymatic and nonenzymatic antioxidants, which reduces the level of ROS in the semen (Fraga et al., 1991; Twigg et al., 1998). A number of reports suggest that abnormal protamination (Aoki et al., 2005, 2006; Nasr-Esfahani et al., 2008) and reduced seminal antioxidant activity (Smith et al., 1996; Lewis et al., 1997; Mostafa et al., 2001; Song et al., 2006) in infertile men lead to an increase in DNA damage (Aitken et al., 1998; Oger et al., 2003; Henkel et al., 2004, 2005; Kao et al., 2008).

Numerous studies have considered sperm DNA damage as a useful biomarker for male fertility, and a high level of sperm DNA damage has been associated with an increased time to conception, lower fertilisation rates, impaired embryo cleavage, higher miscarriage rates and recurrent pregnancy loss after ART (Evenson et al., 1999;
Spero et al., 2000; Carrell et al., 2003; Zini et al., 2008; Simon et al., 2011a, 2014a,b; Breznik et al., 2013; Ni et al., 2014; Zhao et al., 2014; Garolla et al., 2015; Osman et al., 2015). To measure sperm DNA damage, four assays are commonly used, namely sperm chromatin structure assay (SCSA; Evenson et al., 1980, 2002), terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL; Gorczyca et al., 1993), sperm chromatin dispersion (SCD) assay (Fernandez et al., 2003) and the Comet assay (Hughes et al., 1997). Controversies still exist onto which method has higher diagnostic and prognostic value (Simon et al., 2014a). Of the four assays, the alkaline Comet assay and TUNEL assay measure the actual level of DNA damage in spermatozoa, while the SCSA and SCD assay indirectly measure the susceptibility of DNA to damage (Lewis et al., 2008). In this study, we identify the unique features of the alkaline Comet assay and show how they are useful to differentiate fertile from infertile men and their ability to predict successful pregnancy after ART.

Materials and methods

Study population

Forty-seven fertile donors (recently fathered a child within 1 year of sperm donation) and 238 infertile couples presenting at the University of Utah IVF Laboratory, Salt Lake City, UT, USA, for infertility diagnosis between April 2008 and July 2014, including 132 couples with ART, participated in this study. The patients undergoing ART were divided into two groups based on the time duration between semen collection and their ART [Sperm DNA damage was assessed in Group I – the time of semen collection was within 3 months of their treatment (n = 79); and in Group II – the time of semen collection was 3 months prior to their treatment (n = 53).] The Institutional Review Board governed by the University of Utah approved this study. Sperm samples for research were obtained after each couple gave written consent. Sperm from fertile donors and from patients undergoing fertility diagnosis was cryopreserved. In patients undergoing ART, semen samples were collected prior to their treatment and stored in liquid nitrogen (−196 °C) using a rapid freezing protocol (Sherman, 1990). Briefly, equal volume of semen and cryoprotectant (sperm-freezing media; Irvine Scientific, Santa Ana, CA, USA) was mixed together and aliquoted into 1-ml cryogenic vials. The vials were suspended above the level of liquid nitrogen for 10–15 min (−80 °C) and immersed in liquid nitrogen. Semen samples were collected by masturbation with 2–5 days of recommended abstinence. After liquefaction, routine semen analyses were performed according to the WHO guidelines (WHO, 1999). In patients undergoing ART, female partners with age greater than 39 years were excluded from the study.

Alkaline Comet assay

Sperm DNA damage was assessed using single-cell gel electrophoresis (Comet) assay as modified previously by Hughes et al. (1997) and Donnelly et al. (1999). A step-wise protocol of the alkaline Comet assay is published in Simon & Carrell (2013). Briefly, the Comet protocol contains the following steps:

1 Embedding of spermatozoa in agarose gel: Fully frosted microscope slides (Surgipath Europe, Peterborough, UK) were coated with 200 μl of 1% normal-melting-point agarose (Sigma Chemical Co, St Louis, MO, USA) in phosphate-buffered saline (Sigma Chemical Co), kept at 47.0 °C and immediately covered with a glass coverslip (22 × 50 mm). Slides were left at ambient temperature (18.0 °C) to allow the agarose to solidify. The coverslips were removed, and 10 μl of semen or prepared spermatozoa (6 × 10⁶ m ml⁻¹) was mixed with 75 ml of 0.5% low-melting-point agarose (Sigma Chemical Co) at 37.0 °C. This cell suspension was pipetted over the first layer of gel, covered with a glass coverslip and allowed to solidify at ambient temperature.

2 Lysing of cells and decondensation of DNA: Coverslips were removed and the slides immersed in a Coplin jar containing 23.75 ml of fresh lysis solution [2.5 M NaCl, 100 mM EDTA and 10 mM Tris (pH 10), with 1% Triton X-100 (Sigma Chemical Co) added just prior to use], for 1 h at 4.0 °C. Subsequently, 0.25 ml of 1 M dithiothreitol [DTT] (Sigma Chemical Co) was added to achieve a final concentration of 10 mM for a further 30 min at 4.0 °C. This was followed by addition of 1 ml of 100 mM lithium diiodosalicylate [LIS] (Sigma Chemical Co) to achieve a final concentration of 4 mM, which was then incubated at ambient temperature for 90 min.

3 Unwinding of DNA: Slides were removed from the lysis solution and drained of any residual fluid. Fresh alkaline electrophoresis solution was prepared (300 mM NaOH, 1 mM EDTA, pH 13; Sigma Chemical Co) and poured into a horizontal gel electrophoresis tank. The agarose-coated slides were placed side-by-side in the tank, for 20 min, allowing the exposed DNA to unwind. Separation of DNA fragments was performed by electrophoresis. Electrophoresis was carried out for 10 min at 25 V, with the current adjusted to 300 mA, by the addition or removal of buffer from the tank. Following this, slides were removed from the tank, drained and flooded with three changes of neutralisation buffer (0.4 mM Tris, pH 7.5; Sigma Chemical Co), removing any residual alkali or
detergents that may interfere with staining. Slides were stained with 50 µl of 20 mg ml⁻¹ ethidium bromide (Sigma Chemical Co) and analysed immediately.

4 Image analysis: Slides were viewed on a Nikon E600 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with an excitation filter of 515–560 nm from a 100-W mercury lamp and a barrier filter of 590 nm. The degree of sperm DNA fragmentation was determined using an image analysis system (Komet 6.5; Kinetic Imaging, Nottingham, UK) to analyse 50–100 spermatozoa per slide.

Statistical analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS 22) for MAC (SPSS, Inc., Chicago, IL, USA). Data are presented as mean ± SE. The %damage (per cent damage is defined as the percentage of spermatozoa with DNA damage; a spermatozoon is considered to have DNA damage when >25% of its nuclear DNA is migrated to the Comet tail), meanDD (mean DNA damage is defined as the mean of all Comets – tail DNA) and mOTM (mean olive tail moment is defined as the product of the tail length and the fraction of total DNA in the tail) were obtained for each sample. Shapiro–Wilk test for normality was used to identify normal distribution of the test parameters. Spearman’s rho correlation coefficient was used to analyse the relationship between Comet parameters. The size of spermatozoa after decondensation with or without lysis (stained with ethidium bromide, 2 mg ml⁻¹) was measured using a field-view microscopic scale and expressed as mm. Volume of sperm chromatin was calculated and expressed as mm³. Comets with no visible tail and feebly tail (≤25% of Comet tail fluorescence compared to their Comet head) were considered as Comets with normal DNA. A Comet distribution plot graph was obtained for each sample by plotting the level of DNA damage (x-axis) and percentage of comets (y-axis; Fig. 1). Chi-square statistics on all Comet output parameters was used to differentiate fertile donors (n = 47) from the patient group (n = 238) and couples who were successful (n = 38) and unsuccessful (n = 30) after ART. Using the two-by-two table, the diagnostic potential of each output parameter and of combinations of the output parameters was determined. Comet parameters were compared between native semen and density gradient centrifugation (DGC)-prepared spermatozoa (n = 127) using a paired-sample t-test. Sperm DNA damage was assessed in couples undergoing ART who were assigned to two groups: Group I (n = 79), semen collection within 3 months prior to their ART; and Group II (n = 53), semen collection more than 3 months prior to their ART.

Results

Decondensation of sperm nucleus

The alkaline Comet assay was able to detect a wide range of DNA damage in spermatozoa of patients (0–100%) and fertile donors (0–44%). This effect was due to a complete decondensation of sperm nuclear DNA using DTT and LIS during the procedure. The size of a normal fresh sperm head was 4.79 ± 0.26 µm in length and 2.82 ± 0.23 µm in width (Maree et al., 2010), and when the DNA is decondensed using DTT and LIS (without lysis of cell membrane), the size increases to 4.94 ± 0.34 µm in diameter. The size of sperm nuclear

Fig. 1 Types of Comet distribution plots.
DNA after lysis of cell membrane and decondensation (before Comet assay) ranged from 10 to 14 μm in diameter, with a mean of 12.48 ± 0.82 μm (Fig. 2).

**Comet distribution plots**

For the first time we categorise the distribution of sperm DNA damage on a graph with three distinct patterns of scattering (Fig. 1). All fertile donors \((n = 47)\) exhibited type A distribution, while type A, B and C distributions were observed in 44.96%, 40.76% and 14.29% of the patients respectively. Type B distribution was further categorised into two types: B1 when the peak is between 26% and 50% damage (37.11%) and B2 when the peak is between 51% and 75% damage (62.89%). A typical representation of the Comet distribution plots is presented in Fig. S1.

**Correlation between Comet output parameters**

The %damage was positively correlated with meanDD \((r^2 = 0.931; \ P < 0.001)\), while mOTM was positively associated with meanDD \((r^2 = 0.379; \ P < 0.001)\) and %damage \((r^2 = 0.318; \ P < 0.001)\). Sperm preparation by DGC resulted in selection of spermatozoa with reduced DNA damage when compared with raw semen (Table 1). Correspondingly, the Comet distribution plot type changed after DGC (Table 2).

**Comparison of Comet output parameters between fertile donors and infertile men**

Fertile donors \((n = 47)\) had low meanDD \((14.56 ± 0.77\) versus \(40.01 ± 1.49; \ P < 0.001)\), low mOTM \((2.01 ± 0.17\) versus \(5.51 ± 0.36; \ P < 0.001)\) and low %

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**Fig. 2** The effect of decondensation of sperm nucleus, stained by ethidium bromide. (a) Spermatozoa with normal nuclei, (b) decondensed sperm nuclei without lysis of cell membrane and (c) decondensed sperm nuclei following lysis of cell membrane.

**Table 1** Comparison of Comet parameters in the native semen and following DGC-prepared spermatozoa in patients \((n = 238)\)

<table>
<thead>
<tr>
<th>Comet parameters</th>
<th>Native semen</th>
<th>DGC-prepared spermatozoa</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean DNA damage</td>
<td>49.39 ± 2.19</td>
<td>36.12 ± 2.04</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Olive tail moment</td>
<td>12.60 ± 0.67</td>
<td>8.38 ± 0.59</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>% of spermatozoa with damage</td>
<td>85.87 ± 1.21</td>
<td>77.67 ± 1.35</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

DGC, density gradient centrifugation.

Paired-sample t-test was used to compare sperm DNA damage between native semen and DGC-prepared spermatozoa. \(P\) value < 0.05 is statistically significant. Values expressed in mean and standard error.

**Table 2** Change in Comet distribution plot before and after density gradient centrifugation

<table>
<thead>
<tr>
<th>Semen ((n))</th>
<th>Spermatozoa selected after density gradient centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type A</td>
</tr>
<tr>
<td>Type A (42)</td>
<td>100 (42)</td>
</tr>
<tr>
<td>Type B1 (16)</td>
<td>88 (14)</td>
</tr>
<tr>
<td>Type B2 (36)</td>
<td>47 (17)</td>
</tr>
<tr>
<td>Type C (32)</td>
<td>15 (5)</td>
</tr>
</tbody>
</table>

Values expressed in percentage and \(n\).
damage (20.77 ± 1.56 versus 61.50 ± 1.86; \( P < 0.001 \)) compared with the patients (\( n = 238 \)). All fertile donors had Comet distribution plot type A, whereas 45% of the patients showed type A distribution. The predictive value of the Comet output parameters to determine male infertility is presented in Table 3.

Comparison of Comet output parameters between successful and unsuccessful couples following ART

There was no difference in semen volume, total sperm count, progressive motile count, normal morphology, men’s age (33.46 ± 0.50 versus 34.44 ± 0.49; \( P = \text{NS} \)) and women’s age (31.63 ± 0.39 versus 32.70 ± 0.39; \( P = \text{NS} \)) between pregnant and nonpregnant groups. However, sperm concentration (million ml\(^{-1}\)) was higher in the nonpregnant group compared to the pregnant group (81.12 ± 5.32 versus 102.67 ± 8.94; \( P = 0.031 \)). Comet parameters were associated with clinical pregnancies in Group I (\( n = 79 \)). Successful couples (\( n = 38 \)) had low meanDD, mOTM and %damage compared with unsuccessful couples (\( n = 30 \); Fig. 3). In Group I, men having plot type A and B1 distribution showed an increase in clinical pregnancy (82%) compared to unsuccessful couples (63%). Sixty-six per cent of couples with type A distribution plot were successful after ART, whereas couples with type B1, B2 and C distribution plots achieved 56%, 44% and 33% clinical pregnancy respectively. Such correlation was absent in Group II (\( n = 53 \)). The predictive value of Comet output parameters to determine a successful pregnancy is presented in Table 4. The predictive value of Comet assay was enhanced when the Comet output parameters were analysed in combinations of 2, 3 or 4 (Table S1).

Discussion

In this study, we measured sperm DNA damage using the alkaline Comet assay and identified various Comet output parameters that are associated with male reproductive health and clinical pregnancy following ART. For many years, the mean DNA damage of all Comets was thought to be the most prognostic factor (Hughes et al., 1997; Donnelly et al., 1999; Simon et al., 2010, 2011b), along with the mean olive tail moment (Irvine et al., 2000; McVicar et al., 2004; Baumgartner et al., 2012; Kumar et al., 2013). Recently, we showed that the percentage of spermatozoa with damage is another prognostic output for Comet assay (Simon et al., 2014a,b). Here in this study, we show the Comet distribution plot as another promising parameter to measure sperm DNA damage.

A sperm functional test should have higher predictive value to discriminate fertile from infertile men and have

#### Table 3
A comparison between sperm DNA damage parameters to determine male infertility

<table>
<thead>
<tr>
<th>Comet parameters</th>
<th>Threshold value</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Odds ratio (95% CI)</th>
<th>z-statistics (P-value)</th>
<th>Relative risk (95% CI)</th>
<th>ROC curve (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per cent of spermatozoa with damage</td>
<td>60%</td>
<td>72.27</td>
<td>97.87</td>
<td>99.42</td>
<td>41.07</td>
<td>119.88 (16.20–887.04)</td>
<td>4.687 (&lt;0.001)</td>
<td>1.69 (1.45–1.97)</td>
<td>0.889 (0.852–0.927)</td>
</tr>
<tr>
<td>DNA damage: mean of all Comets</td>
<td>25%</td>
<td>68.91</td>
<td>93.62</td>
<td>98.20</td>
<td>37.29</td>
<td>119.88 (16.20–887.04)</td>
<td>5.799 (&lt;0.001)</td>
<td>1.57 (1.36–1.80)</td>
<td>0.883 (0.844–0.921)</td>
</tr>
<tr>
<td>Mean olive tail movement</td>
<td>4.68%</td>
<td>50.45</td>
<td>97.87</td>
<td>98.25</td>
<td>45.54</td>
<td>119.88 (16.20–887.04)</td>
<td>3.740 (&lt;0.001)</td>
<td>1.80 (1.50–2.15)</td>
<td>0.834 (0.772–0.896)</td>
</tr>
<tr>
<td>Comet distribution plot</td>
<td>Type A</td>
<td>55.04</td>
<td>100.00</td>
<td>100.00</td>
<td>30.52</td>
<td>116.21 (7.08–1907.28)</td>
<td>3.333 (&lt;0.001)</td>
<td>1.44 (1.30–1.60)</td>
<td>0.775 (0.720–0.832)</td>
</tr>
</tbody>
</table>

PPV, positive predictive value; NPV, negative predictive value; ROC curve, receiver operating characteristic curve. P-value < 0.05 is statistically significant. Chi-square statistics was used to compare between fertile donors (\( n = 47 \)) and patient groups (\( n = 238 \)).

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the potential to help decide clinical options (Sigman, 2012; Krausz & Carrell, 2014; Oehninger et al., 2014). The conventional semen analysis is recommended for male partners with subfertile couple, and it is the first step to identify male factor infertility (WHO, 2010). However, sperm parameters do not measure the fertilising potential of the spermatozoa, and hence, a need for newer sperm tests to determine male infertility has been emphasised (Barratt & Mansell, 2013; Ruvo et al., 2013; Wang & Swerdloff, 2014). In recent years, sperm DNA test has become a useful clinical biomarker to determine male reproductive health (Bungum, 2012; Feijo & Esteves, 2014; Palermo et al., 2014; Muratori et al., 2015), while others have questioned the predictive ability of these assays on various ART outcomes (Tarlatzis & Goulis, 2010; Beshay & Bukulmez, 2012).

The existing literatures associating the effects of sperm DNA tests on ART outcome are controversial (Collins et al., 2008; Zini et al., 2008; Zhao et al., 2014). These controversies are probably due to the lack of understanding in a number of key aspects related to sperm DNA damage (Ramalho-Santos, 2014) and its measurement (Zini & Sigman, 2009; Barratt & De Jonge, 2010; Barratt et al., 2010). To date, there are four commonly used methods to measure sperm DNA damage, and each assay is known to measure different aspects of DNA damage (Henkel et al., 2010; Schulte et al., 2010). Of these assays, the sensitivity and predictive value of the alkaline Comet assay are shown to be higher (Simon et al., 2014a), as it measures both single- and double-strand breaks (Morris et al., 2002).

The decondensation step is a unique feature of the alkali Comet assay and is absent in other sperm DNA assays. The sperm nucleus contains one haploid copy of the genome organised in a unique pattern (Ward & Zalensky, 1996; Wykes & Krawetz, 2003) tightly packed with the help of protamines (Oliva, 2006). The sperm chromatin is almost crystalline in nature and thought to be six times more condensed than the somatic cell (Fuentes-Mascorro et al., 2000). The reduction of disulphide bonds connecting protamines (Liu et al., 2013) and removal of protamines under alkali conditions (Gosalvez et al., 2014) help relaxation of sperm chromatin. We show that the volume of sperm nuclei (28.2 ± 0.2 μm³; Gilmore et al., 1995) is almost doubled (~63 μm³) by the process of decondensation within the intact spermatozoa (Fig. 2a,b). The process of lysis and decondensation during the Comet protocol results in 34-fold increase in volume (~1018 μm³) of the sperm chromatin (Fig. 2c), which exposes the chromatin, and allows the migration of broken DNA strands during electrophoresis. The separation of double-stranded DNA into single strand at alkali conditions (pH 13) is an added feature of the Comet assay that helps simultaneous measurement of single- and double-strand breaks (Olive & Banath, 2006; Simon & Carrell, 2013).

In this study, for the first time we categorise ‘Comet distribution plot’ as another measurement for Comet, in addition to mOTM, meanDD and %damage. Morris et al. (2002) showed a marked difference in distribution pattern between a fertile and an infertile patient. Spermatozoon is known to have a variable level of DNA damage, and in semen of infertile men, it ranges from 0% to 100%, as observed here and elsewhere (Simon et al., 2014a). Such wide range helps to distribute the Comets into three main types (Fig. 1). Plot A consists of 45% of the population and includes fertile men (100%) and infertile men. Two-thirds of the couples having plot A had a successful pregnancy, whereas one-third of the couple having plot C had a successful pregnancy. Plot B

![Fig. 3](image-url) Bar chart showing association between various Comet parameters and clinical pregnancy outcome when sperm DNA damage was assessed within 3 months of ART (***p < 0.001).
could be categorised into two subtypes (Fig. 1), where B1 having higher probability of achieving a pregnancy than B2. Interestingly, spermatozoa from all semen samples could be categorised into one of the three types, making the distribution plot as a useful parameter to analyse sperm DNA damage.

In this study, we show that sperm DNA damage parameters (meanDD, mOTM and %damage) were reduced after sperm preparation, when compared to native semen. Similarly, changes in Comet distribution plots were observed following DGC (Table 2). Semen sample with plot A remained as the same after DGC, whereas changes in plot types were observed in samples with plot B and C types. Such changes in plot type are possible as a result of the removal of dead and abnormal spermatozoa including spermatozoa with DNA damage following DGC (Larson et al., 1999; Simon et al., 2010, 2011a; Brahem et al., 2011; Jayaraman et al., 2012; Bucar et al., 2015; Sa et al., 2015). In this study, we did not analyse sperm DNA damage in DGC population in patients undergoing ART. However, it could be hypothesised that change to plot type A or B1 following DGC in patients with plots B2 and C in semen may increase their chances of a ART pregnancy. In support of this hypothesis, sperm DNA damage measured in the prepared sperm population is reported to predict clinical pregnancies (Larson et al., 2000; Simon et al., 2010, 2011a,b; Liu & Liu, 2013); however, some studies have also questioned the predictive ability of DNA damage measured in prepared population (Duran et al., 2002; Gandini et al., 2004; Bungum et al., 2008).

Here, we show that all four Comet output parameters were predictive of male infertility and clinical pregnancies following ART. Previous studies using Comet have established the threshold values for the outputs such as meanDD (Simon et al., 2010, 2011a) and %damage (Simon et al., 2014a). Olive tail moment is another output of Comet (Irvine et al., 2000; McVicar et al., 2004; Baumgartner et al., 2012; Kumar et al., 2013) and is defined as the product of the tail length and the fraction of total DNA in the tail (Berthelot-Ricou et al., 2011). Here, we observed that mOTM reported the highest positive predictive value (PPV) to determine a successful pregnancy. This study reports that the PPV of the Comet parameters to determine a successful pregnancy is lower, when compared to the PPV to determine male infertility. The reason for such low PPV may be due to the inclusion of couples with female and idiopathic infertility factors, where the effect of sperm DNA damage could be masked by the presence of female factor infertility (Simon et al., 2011a).

We observed a correlation between sperm DNA damage and ART outcome in Group I (DNA damage was

<table>
<thead>
<tr>
<th>Comet parameters</th>
<th>Threshold value</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Odds ratio (95% CI)</th>
<th>Z-statistics (P value)</th>
<th>ROC curve (95% CI)</th>
<th>Relative risk (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per cent of sperm with damage</td>
<td>86%</td>
<td>67.35</td>
<td>62.00</td>
<td>36.67</td>
<td>52.57</td>
<td>62.00</td>
<td>62.00</td>
<td>62.00</td>
<td>62.00</td>
</tr>
<tr>
<td>Mean of all Comets</td>
<td>62.75</td>
<td>62.75</td>
<td>62.75</td>
<td>62.75</td>
<td>62.75</td>
<td>62.75</td>
<td>62.75</td>
<td>62.75</td>
<td>62.75</td>
</tr>
<tr>
<td>Comet distribution plot Type A1</td>
<td>61.11</td>
<td>61.11</td>
<td>61.11</td>
<td>61.11</td>
<td>61.11</td>
<td>61.11</td>
<td>61.11</td>
<td>61.11</td>
<td>61.11</td>
</tr>
</tbody>
</table>

PPV, positive predictive value; NPV, negative predictive value; ROC curve, receiver operating characteristic curve. p-value < 0.05 is statistically significant. Chi-square statistics was used to compare between pregnant (n = 57) and nonpregnant (n = 22) groups.

Table 4: A comparison between sperm DNA damage parameters to determine a successful clinical pregnancy following ART in Group I (n = 79)
measured in patients where semen collection was within 3 months prior to the couples’ treatment). Although DNA damage is a common property of the spermatozoon, the level of DNA damage may vary with time, with the use of medications, exposure to drugs or radiation, oxidative stress and environmental toxicants (Bian et al., 2004; Evenson & Wilson, 2005; Stahl et al., 2006; Barenys et al., 2009; Wright et al., 2014; Iommiello et al., 2015). Based on the results observed here and elsewhere, we recommend DNA damage should be assessed preferably on the day of ART (Simon et al., 2010, 2011a) and not more than 3 months prior to ART.

The predictive value of the alkaline Comet assay to determine a successful pregnancy was high by measuring %damage. Previous studies from our group had shown that %damage is also associated with other ART outcomes like fertilisation rate, embryo quality and implantation rate (Simon et al., 2014a,b). Here, for the first time we show that the combinations of the Comet output parameters provide a superior predictive value than each parameter individually. In addition, combining the parameters resulted in a high PPV (74%), whereas the PPV of each individual parameter was low (37–53%). Combining the output parameters may help identify subtle changes that are not observed by individual parameters, as these parameters measure different aspects of DNA damage within the spermatozoa.

The values of odds ratio and ROC curve to determine male infertility and ART success were higher by measuring %damage; however, all the Comet output parameters alone or in combination had greater predictive ability. Such high predictive value of the Comet assay could be attributed to the complete decondensation of sperm chromatin and measurement of both single- and double-strand breaks. The Comet assay is a simple, cost-efficient method to measure DNA damage of individual spermatozoon (Singh et al., 1989; Olive et al., 1998; Duty et al., 2002; Olive & Banath, 2006; Dhawan et al., 2009), but the assay is laborious and known to have inter-laboratory variations (Olive et al., 1992, 2001; Collins et al., 2014). The clinical importance of Comet assay in spermatozoon has been proven by a number of studies (Hughes et al., 1996; Morris et al., 2002; Lewis & Agbaje, 2008; Simon et al., 2010, 2011a, 2014a; Ribas-Maynou et al., 2012, 2014). The combinational analysis of all the Comet output parameters may provide a comprehensive evaluation of patient’s reproductive health.

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References


Supporting Information
Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1.** Typical representation of the Comet distribution plot.

**Table S1.** Combination of Comet sperm DNA damage parameters to determine a successful clinical pregnancy following ART in Group I (n = 79).