Comprehensive Analysis of Sperm DNA Fragmentation by Five Different Assays: TUNEL Assay, SCSA, SCD Test and Alkaline and Neutral Comet assay

Running Title: **SDF analysis by TUNEL assay, SCSA, SCD test and Comet assay**

Ribas-Maynou, J.[*,1]; García-Peiró, A.[*,1,2]; Fernández-Encinas, A.[1]; Abad, C. [3]; Amengual, MJ.[4]; Prada, E.[5]; Navarro, J.[1] and Benet, J.[1]

[*]*Authors contributed equally to this work*

[1] *Departament de Biologia Cel·lular, Fisiologia i Immunologia. Universitat Autònoma de Barcelona. 08193 Bellaterra, Spain.*

[2] *Centro de Infertilidad Masculina y Análisis de Barcelona (CIMAB). Edifici Eureka, PBM5. Parc de Recerca de la UAB (PRUAB). Campus de la UAB. 08193 Bellaterra, Spain.*

[3] *Servei d'Urologia. Corporació Sanitària Parc Taulí. Sabadell. Institut Universitari Parc Taulí – UAB. 08208 Sabadell, Spain.*

[4] *UDIAT, Centre Diagnòstic. Corporació Sanitària Parc Taulí. Sabadell. Institut Universitari Parc Taulí – UAB. 08208 Sabadell, Spain.*

[5] *Servei de Ginecologia. Hospital Universitari Mútua de Terrassa, 08221 Terrassa, Spain.*

Corresponding Author: J. Benet, PhD and J. Ribas-Maynou MSc
Departament de Biologia Cel·lular, Fisiologia i Immunologia. Facultad de Medicina,
Universitat Autònoma de Barcelona (UAB)
08193 Bellaterra, Spain
Phone: +34 935811773;
Fax: +34 935811025;
E-mail: jordi.benet@uab.cat and jordi.ribas@uab.cat

Conflict of interest: The authors declare no conflict of interest.

ABSTRACT

Assaying sperm DNA fragmentation (SDF) is becoming an important test to assess male infertility. Several different tests are available but no consensus has yet been reached as to which tests are most predictive of infertility. Few publications have reported a comprehensive analysis comparing these methods within the same population. The objective of this study was to analyze the differences between the five most common methodologies, to study their correlations, and to establish their cut-off values, sensitivity and specificity in predicting male infertility. We found differences of SDF between fertile donors and infertile patients in TUNEL, SCSA, SCD and alkaline Comet assays, but none with the neutral Comet assay. The alkaline COMET assay was the best in predicting male infertility followed by TUNEL, SCSA and SCD, while the neutral COMET assay had no predictive power. For our patient population threshold values for infertility were 20.05% for TUNEL assay, 18.90% for SCSA, 22.75% for the SCD test, 45.37% for alkaline Comet and 34.37% for neutral Comet. This work establishes in a comprehensive study that the all techniques except neutral Comet are useful to distinguish fertile and infertile men.

INTRODUCTION

In recent years, the sperm DNA fragmentation (SDF) has become a biomarker for male infertility, because it has been shown that fertilization of a sperm with fragmented DNA could cause defects in embryo development, giving rise to the risk of undergoing a pregnancy loss at early pregnancy stages, or problems with fetal development (Evenson et al., 1999; Carrell et al., 2003; Lewis and Simon, 2010). Moreover, high sperm DNA fragmentation has been associated with recurrent miscarriage, higher difficulty in achieving a pregnancy, and different childhood diseases (Cooke et al., 2003; Aitken et al., 2009; Brahem et al., 2011; Zini, 2011; Absalan et al., 2012). Etiological studies have concluded that oxidative stress is one of the most common factors associated with sperm DNA damage (Agarwal et al., 2008; Makker et al., 2009; Aitken & De Iuliis 2010). Other factors involved in sperm nuclear DNA fragmentation include incorrect chromatin remodeling, nuclease activity, or different external factors such as radiation (Maione et al., 1997; Sailer et al., 1997; Sotolongo et al., 2005; Aitken & De Iuliis, 2010; Sakkas and Alvarez, 2010).

Several methodologies have been developed to assess SDF, and most of them have been applied for clinical purposes by establishing their cut-off values for predicting pregnancy, and monitoring their sensitivity and specificity (Evenson et al., 2002; Sergerie et al., 2005; Velez de la calle et al., 2008; Nijs et al., 2009; Sharma et al., 2010; Simon et al., 2011; Venkatesh et al., 2011). First, the TUNEL assay (Gorczyca et al., 1993) uses a terminal TdT transferase to label the 3’ free ends of DNA, resulting in a higher labeling on spermatozoa with fragmented DNA. For this methodology, different cut-off values have been reported to assess the fertility status of the male (Sergerie et al., 2005; Sharma et al., 2010). It has been demonstrated that sensitivity and specificity can be increased by analyzing the results with a cytometer instead of an epifluorescence

78 microscope (Dominguez-Fandos et al., 2007), by decompaction of the DNA with DTT
79 (Mitchell et al., 2011), or not including the apoptotic bodies on the final result
80 (Marchiani et al., 2007). Second, the Comet assay (Singh et al., 1988), has the unique
81 feature that it can distinguish between single and double stranded DNA breaks (ssSDF
82 and dsSDF, respectively) when it is performed under alkaline or neutral conditions. It is
83 based on nuclear decompaction followed by electrophoresis and visualization of
84 individual sperm. Clinical cut-off values for male infertilty, assessing Comet tail DNA,
85 and percentage of fragmented spermatozoa have been published using the alkaline
86 Comet assay for both total semen sample (Simon et al., 2011; Ribas-Maynou et al.,
87 2012a), and also differentiating swim-up sperm cells (Simon et al., 2011). Moreover,
88 our group demonstrated a clinical association of dsSDF assessed by neutral Comet with
89 recurrent miscarriage risk in couples without female factor (Ribas-Maynou et al.,
90 2012b), showing that differences in the DNA break type, ssSDF or dsSDF, has different
91 implications for human reproduction.
92 Other methods such as SCSA (Evenson et al., 1980) and the SCD test (Fernandez et al.,
93 2005) base their detection of SDF on the denaturing capacity of the sperm chromatin.
94 The SCSA uses acridine orange staining to label the double stranded DNA with green
95 and the single stranded DNA with red. The proportion of these two emissions, with a
96 previous acid-denaturing step, has widely been demonstrated to determine the
97 percentage of DNA fragmentation, and several reports for clinical usage have been
98 published (Evenson and Jost, 2000; Evenson et al., 2002; Bungum et al., 2004; Virro et
99 al., 2004; Nijs et al., 2009; Venkatesh et al., 2011). Moreover, SCSA provides also an
100 additional parameter named High DNA Stainability (HDS). This parameter is a measure
101 of the percentage of immature sperm within the semen sample, which can also be taken
102 into account on the male infertility assessment (Evenson et al., 1999)

Finally, the SCD test, assesses the capacity of the chromatin to form dispersion halos, and allows differentiating the non-fragmented sperm (with halo) from the fragmented sperm (without halo). Like the other methods, studies showing the infertility cut-off value for the SCD test have been performed (Fernandez et al., 2005; Velez de la calle et al., 2008; Nuñez-Calonge et al., 2012; Ribas-Maynou et al., 2012b).

Although many studies reported different clinical values using these techniques, only a few studies have proved the correlation between TUNEL, SCSA and SCD (Chohan et al., 2006; Garcia-Peiró et al., 2011), however, these studies have not reported the sensitivity and specificity values for each technique.

On the other hand, although a study found a relationship between SDF and embryo quality using the SCSA (Niu et al., 2011), some studies failed in finding a relationship between the SDF predictive value and assisted reproduction techniques (ART) such as in vitro fertilization (IVF) or intra-cytoplasmatic sperm injection (ICSI) (Simon et al., 2013; Bungum et al., 2012; Esbert et al., 2012). This lack of the predictive quality of SCSA could be due to the presence of a female factor, such as differences between oocytes on their efficiency of DNA repair after fertilization (Payne et al., 2005; Evenson and Wixon, 2006).

We have previously shown that extensive sperm ssSDF may prevent pregnancy, but that sperm with dsSDF can fertilize ooctyes achieving pregnancy but compromise the fetus viability within the first trimester (Ribas-Maynou et al., 2012b). Moreover, the lack of relationship of most SDF assays with IVF or ICSI found by other authors might also be related to the method used to assess the sperm DNA damage, or to differences of sensitivity and specificity in detecting the total SDF in the semen sample between methods maybe due to a lack of method standardization. However, these two facts have not been exhaustively studied among methods, although it seems to be important

because there is still a limitation in the knowledge about the effects that DNA fragmentation could have on the embryo and the embryonic development.

The objective of the present study is to compare the five most commonly used techniques to assess DNA damage, to establish the correlations between them, and finally, to compare their sensitivity, specificity and threshold values attending male infertility.

MATERIAL AND METHODS

Sample collection

Semen samples from 240 human males were collected in collaboration with reproduction centers and hospitals from the Barcelona area. Samples from couples showing female factors have been excluded from the study. An informed consent was obtained from all donors and the appropriate ethics committee approved the study.

Samples were divided into fertile donors, who achieved a clinical pregnancy, and infertile patients, obtaining a group size of 50 and 190, respectively. Semen samples were obtained with a minimum of three days and maximum of seven days of sexual abstinence, and were cryopreserved in test-yolk buffer (14% glycerol, 30% egg yolk, 1.98% glucose, 1.72% sodium citrate) until the sperm DNA fragmentation analysis. The total sample size that was analysed for the different methods were: 183 for alkaline Comet, 183 for neutral Comet, 123 for SCD test, 93 for TUNEL assay, and 98 for SCSA.

TUNEL assay

The TUNEL assay was performed using the In Situ Cell Death Detection Kit from Roche (Roche Diagnostic Gmbh, Penzberg, Germany), following the protocol previously described (Barroso et al., 2000). The analysis of sperm DNA fragmentation was performed by flow cytometer analysis (FACSCalibur; Becton Dickinson, NJ, USA), and a total of 10.000 spermatozoa were analyzed at a flow rate of 200-300 spermatozoa/sec taking into account a negative control without the TdT enzyme. Data were processed using cellquest analysis software (Becton Dickinson) after gating out cell debris.

SCSA

159 The SCSA methodology has been described elsewhere by Evenson et al., (1999).
160 Briefly, each semen sample was diluted to reach a concentration of 2 x 10^6
161 spermatozoa/mL in TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, pH 7,5)
162 in a total volume of 200 µL. Then, the sample was treated with an acid solution
163 (150mM NaCl, 0.1% Triton X-100, pH 1.2) and after 30 sec. a staining was performed
164 using acridine orange 6 µg/mL for 3 minutes. Finally, a total of 5000 sperm cells were
165 analysed by flow cytometry (FACSCalibur; Becton Dickinson). The percentage of
166 spermatozoa with DNA fragmentation show increased red fluorescence, unlike the non-
167 fragmented population, that show a normal level of red fluorescence. The percentage of
168 HDS sperm had not been included in this SDF comparative study.

169 ***SCD test***

170 The SCD test was performed using the Halosperm kit (Halotech DNA; Madrid, Spain)
171 following the manufacturer's instructions. Samples were stained with propidium iodide
172 and 250 spermatozoa were assessed and classified as fragmented or non-fragmented
173 sperm, using a fluorescence microscope (Olympus AX70).

174 ***Comet assay***

175 The alkaline and neutral Comet assay was performed simultaneously in two different
176 slides to assess single and double stranded DNA fragmentation, respectively. The assay
177 has been performed following the protocol reported before (Ribas-Maynou et al., 2012a
178 and 2012b). Briefly, samples were washed and sperm concentration was adjusted to 10
179 x 10^6 spermatozoa/ml. Then incubations with two lysis solutions were performed and
180 the samples were electrophoresed, using alkaline or neutral buffer depending on the
181 assay, with a previous denaturation on alkaline Comet slide. Finally, both slides were
182 submerged on a neutralization solution, and were dehydrated in ethanol series of 70%,
183 90% and 100%. Samples were stained with DAPI SlowFade® Gold antifade

(Invitrogen; Eugene, OR, USA) and 400 spermatozoa were classified according fragmented and non-fragmented following the criteria reported before (Figure 1 at Ribas-Maynou et al., 2012a).

Statistical analysis

Statistical analysis was performed using the Statistics Package for the Social Sciences software, version 20 (SPSS Inc.; Chicago, IL). Comparisons of SDF between different groups were assessed using the Mann-Whitney U test. Correlations between techniques were assessed using the Spearman test, and the ROC analysis was performed in order to obtain the sensitivity, specificity and the cut-off value for each test. All statistical tests were performed taking into account the 95% of the confidence interval.

RESULTS

Sperm DNA fragmentation regarding male infertility

For each assay, the percentage of sperm in the sample that was positive for the test was calculated. The average percentage of sperm DNA fragmentation for fertile and infertile patients using the five different techniques is shown in Table I, and a histogram for the same results is shown in Figure 1 in order to show their distribution.

Statistical differences were found between fertile and infertile patients through TUNEL assay, SCSA, SCD test and alkaline Comet ($p< 0.001$), however, no differences were found when comparing fertile donors and infertile patients through neutral Comet ($p=0.862$).

Correlation between techniques

Correlation between all techniques was assessed using the Spearman test. High correlations were found between the SCD test and SCSA ($r=0.71$; $p<0.001$), between SCD and TUNEL assay ($r=0.70$; $p<0.001$), and between SCSA and the TUNEL assay ($r=0.79$; $p<0.001$), the latter being the highest correlation. .

Moderate correlations was found between the alkaline Comet assay and the SCD test ($r=0.61$; $p<0.001$), between the alkaline Comet and SCSA ($r=0.59$; $p<0.001$), and between the alkaline Comet and TUNEL assay ($r=0.72$; $p<0.001$).

Finally, no correlation was found between the neutral Comet assay and the other four methodologies.

ROC analysis, sensitivity, specificity, cut-off values

The sensitivity, specificity, the cut-off values for male factor infertility, and the area below the curve obtained by the ROC analysis are shown in Table II, and a graphic representation of ROC curves for all techniques is shown on Figure 2. The alkaline Comet showed the highest area below the curve (0.937 cm^2), and a cut-off value of

45.37% of SDF with a sensitivity and specificity of 0.850 and 0.920, respectively. TUNEL assay showed an area below the curve of 0.903 cm^2, and a cut-off value of 20.05% of SDF with a sensitivity and specificity of 0.764 and 0.952, respectively. The SCD test showed an area below the curve of 0.869 cm^2, and a cut-off value of 22.75% of SDF with a sensitivity and specificity of 0.730 and 0.918, respectively. The SCSA showed lower association with male infertility, with an area below the curve of 0.792 cm^2, and a cut-off value of 18.90% of SDF with a sensitivity of 0.595 and a specificity of 0.875. Finally, the neutral Comet assay showed no association with male infertility, with the lowest area below the curve (0.516 cm^2), a cut-off value of 34.37% of SDF with a sensitivity and specificity of 0.970 and 0.320, respectively.

DISCUSSION

Although the use of different methodologies to assess sperm DNA damage has been widely discussed, a few reports have compared the clinical utility and the correlation between the most common methods in a comprehensive manner (Erenpreiss et al., 2004; Chohan et al., 2006, Garcia-Peiró et al., 2011). Therefore, we performed this comparative analysis to test their correlation and to determine the different clinical cut-off values among the most used techniques.

The analysis of SDF showed statistical differences between fertile and infertile patients in the TUNEL assay, SCSA, the SCD test and the alkaline Comet assay, as different reports have previously found (Irvine et al., 2000; Gandini et al., 2000; Zini et al., 2001; Saleh et al., 2002; Chohan et al., 2006; Garcia-Peiró et al., 2012; Ribas-Maynou et al., 2012a). However, no differences were found between fertile donors and infertile patients with the neutral Comet assay. This was also found in a previous study from our group, demonstrating that neutral Comet is related to the miscarriage risk and it is not involved in the fertility status. Moreover, a bimodal distribution has also been found in fertile donors, showing the presence of two subgroups of fertile donors, as it has previously been described (Ribas-Maynou et al., 2012b). On the other hand, the neutral Comet assay showed a normal distribution on infertile samples, presenting mostly high values of dsSDF (Figure 1 and Table I). In fact, the distribution of infertile patients in the neutral Comet assay mirrored that of the alkaline Comet assay, suggesting that for infertile patients, at least, these two assays identify similar populations of patients.

When comparing the SDF and the SDF ranges among different methodologies, differences were found in fertile donors between the alkaline Comet assay and the SCD test, SCSA or TUNEL assay. These differences between the alkaline Comet assay and the other techniques might be due to the electrophoresis step, which could be increasing

the sensitivity of the detection of the DNA breaks in respect to other methodologies. Regarding infertile patients, values of SDF obtained by the alkaline Comet assay were statistically higher than SCD, SCSA and TUNEL methodologies, showing that Comet assay seems to have higher sensitivity on detecting the sperm DNA breaks since Comet assay show values up to 100% of SDF in some infertile patients, and the other methodologies do not reach this value (Figure 1). SCSA showed statistically lower values than SCD and TUNEL assay, which do not show statistical differences between their values. These data suggest that different methodologies might be detecting different aspects of the sperm DNA fragmentation, since SCD and SCSA might be detecting some aspects related to chromatin fragmentation, and Comet and TUNEL assays could be detecting DNA breaks, directly (The Practice Committee of American Society for Reproductive Medicine, 2008; Henkel et al., 2010).

Regarding to the correlations between the methods, the best correlation was found between the cytometric assays (TUNEL and SCSA), as has been previously reported (Chohan et al., 2006; Garcia-Peiró et al., 2011; Villani et al., 2010). This is interesting given that the two assays are thought to be measuring different aspects of SDF (Henkel et al., 2010). It also seems to be necessary to standardize the TUNEL methodology, since it is known that it shows variations on SDF detected depending on minor variations in the procedure (Domínguez-Fandós et al., 2007; Muratori et al., 2008; Mitchell et al., 2011) or on its analysis (Marchiani et al., 2007). Nevertheless, Despite the differences between the TUNEL assay and SCSA and the need for standardization of the former, both assays had very similar values for SDF. Moreover, they also present a good correlation with the SCD test, which is based on the capacity of the chromatin to form different dispersion halos depending on its SDF (Fernandez et al., 2005). The correlation between SCD and the two cytometric assays have been tested before, with

283 similar results to the present work (Chohan et al., 2006; Villani et al., 2010; Garcia-
284 Peiró, et al., 2011).
285 Similarly, the alkaline Comet assay showed a moderate correlation with the SCD test,
286 the TUNEL assay and SCSA, as has been described before by different laboratories
287 (Donnelly et al., 2000; Villani et al., 2010). This correlation was not as strong as the
288 correlations found among the latter three techniques, which might be due to a possible
289 higher sensitivity of the alkaline Comet assay in respect to the other methodologies.
290 In contrast, the neutral Comet assay does not show any correlation with the other four
291 methodologies to assess sperm DNA fragmentation. As has been proposed before, the
292 neutral Comet assay is related to the risk of having a miscarriage, since the dsDNA
293 breaks could be a non-extensive type of DNA damage located only in a few points
294 along the genome (Kaneko et al., 2012), preferently in the matrix attachment regions,
295 between toroids (Ribas-Maynou et al., 2012b) and might be occur by an acute or
296 fractionated exposition to radiation, as it has been demonstrated in tumor cells
297 (Jayakumar et al., 2012). Although it is known that techniques such as the TUNEL
298 assay and SCSA are detecting both single and double stranded DNA damage (Practice
299 Committee of American Society for Reproductive Medicine, 2008; Villani et al., 2010),
300 our data show a correlation between both TUNEL or SCSA and the alkaline Comet
301 assay, which would be detecting mainly ssSDF, however, they do not show a
302 correlation with the neutral Comet assay, which has been demonstrated to assess
303 dsDNA breaks (Van Kooij et al., 2004; Ribas-Maynou et al., 2012a). Moreover, the
304 neutral and alkaline Comet assays showed a tendency to a moderate correlation in
305 infertile patients, a fact that could be related to the possibility that the presence of many
306 single stranded DNA breaks could lead to double stranded DNA breaks.

To test the clinical utility of the different DNA damage tests on predicting male infertility, an analysis using ROC curves was performed. The higher area below the curve has been shown by alkaline Comet assay, followed by the TUNEL assay, the SCD test, SCSA and the neutral Comet assay (Table II and Figure 2).

First, the alkaline Comet assay showed a threshold value in predicting infertility of 45.37% of DNA fragmentation with an area below the curve of 0.937. This cut-off value shows a very high sensitivity and specificity, and is consistent with previous results from our group (Ribas-Maynou et al., 2012b). However, it is not comparable with previous studies, where the percentage of damaged DNA and not the percentage of fragmented sperm cells have been assessed (Simon et al., 2011).

The TUNEL assay showed a threshold value for male infertility of 20.05% of SDF, with very high values of area below the curve and specificity (0.903 and 0.952, respectively), however, a lower value of sensitivity in respect to alkaline Comet was obtained (0.764). These results were comparable to those obtained by Sharma et al., 2010, who obtained a cut-off value of 19.25%, with an area below the curve, sensitivity and a specificity of 0.890, 0.649 and 1.000, respectively. However, sensitivity found in this work slightly differs from those obtained by Sergerie et al., 2005, who obtained a higher value of 0.896.

The cut-off, sensitivity and specificity results obtained by the SCD test in the present study (Table II) do not differ from previously published works (Fernandez et al., 2005; Velez de la Calle et al., 2008; Nuñez-Calonge et al., 2012, Ribas-Maynou et al., 2012b), showing a good capacity of this technique to assess male infertility.

Reported values for SCSA threshold vary from 20% to 30% (Boe-Hansen et al., 2006; Larson-Cook et al., 2003; Payne et al., 2005; Evenson and Jost, 2000; Evenson et al., 2002; Evenson et al., 2013; Ventakesh et al, 2011; Bungum et al., 2004). Our results

show a threshold value of 18.9% of SDF, which is at the low end of the published range. Despite of being the lowest, it does not differ from studies that find threshold values about 20%. Moreover, it is very well known that SCSA is the most standardized technique between different laboratories (Evenson, 2013).

Finally, the neutral Comet assay showed a very weak association with male infertility, since fertile donors can show low or high values of dsDNA fragmentation analyzed with this method. However, infertile patients always show high values. Because of that, the threshold value established was 34.37% of SDF with a high sensitivity, but a very low specificity, since a bimodal distribution in fertile donors overlaps the infertile values, as it has also been shown before. This would mean that male infertility could be predictable, but always taking into account that high values are associated with the risk of suffering a miscarriage due to a male factor (Ribas-Maynou et al., 2012b).

To further assessment, as different techniques may measure different aspects of chromatin integrity, a double analysis using more than one SDF technique, would allow to confirm the diagnosis.

Conclusion

This work provides data from the five most used methodologies to assess the sperm DNA fragmentation on the same patient population. With this data, it can be concluded that the alkaline Comet assay, the SCD test, SCSA and the TUNEL assay are useful to distinguish fertile and infertile patients, with the alkaline Comet assay being the best predictor of male infertility. However, the neutral Comet shows no capacity on differentiating fertile donors and infertile patients. Moreover, threshold values have been compared in a comprehensive work to assess infertility. Finally, this work provides a comprehensive comparison in fertile donors and infertile patients, which could be useful to technique standardization.

ACKNOWLEDGEMENTS

We would like to thank Dr. Steve Ward for his exhaustive revision and useful comments on the final manuscript.

FUNDING

This work has been supported by FIS (PI11/00630), Generalitat de Catalunya (2009 SGR 1107), and J. Ribas-Maynou has a grant from Generalitat de Catalunya.

AUTHOR'S ROLES

Jordi Ribas-Maynou contributed in experimental procedures, statistical analysis, graphics and table elaboration and document writing.

Agustín García-Peiró contributed in experimental design, results discussion, statistical analysis and document writing and revising.

Alba Fernandez-Encinas contributed in experimental procedures.

María José Amengual and Carlos Abad contributed in recruitment of patients, samples collection, storage and semen parameters analysis.

Joaquima Navarro and Jordi Benet contributed in experimental design and direction and coordination of the work.

REFERENCES

Absalan F, Ghannadi A, Kazerooni M, Parifar R, Jamalzadeh F & Amiri S (2012) Value of sperm chromatin dispersion test in couples with unexplained recurrent abortion. *J Assist Reprod Genet* 29, 11-14.

Agarwal A, Makker K & Sharma R (2008) Clinical relevance of oxidative stress in male factor infertility: an update. *Am J Reprod Immunol* 59, 2-11.

Aitken RJ & De Iuliis GN (2010) On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod* 16, 3-13.

Aitken RJ, De Iuliis GN & McLachlan RI (2009) Biological and clinical significance of DNA damage in the male germ line. *Int J Androl* 32, 46-56.

Boe-Hansen GB, Fedder J, Ersboll AK & Christensen P (2006) The sperm chromatin structure assay as a diagnostic tool in the human fertility clinic. *Hum Reprod* 21, 1576-1582.

Brahem S, Mehdi M, Landolsi H, Mougou S, Elghezal H & Saad A (2011) Semen parameters and sperm DNA fragmentation as causes of recurrent pregnancy loss. *Urology* 78, 792-796.

Bungum M, Bungum L, Lynch KF, Wedlund L, Humaidan P & Giwercman A (2012) Spermatozoa DNA damage measured by sperm chromatin structure assay (SCSA) and birth characteristics in children conceived by IVF and ICSI. *Int J Androl* 35, 485-490.

396 Bungum M, Humaidan P, Spano M, Jepson K, Bungum L & Giwercman A (2004) The
397 predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome
398 of intrauterine insemination, IVF and ICSI. *Hum Reprod* 19, 1401-1408.

399 Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L *et al.* (2003)
400 Sperm DNA fragmentation is increased in couples with unexplained recurrent
401 pregnancy loss. *Arch Androl* 49, 49-55.

402 Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ & Carrell DT (2006) Comparison
403 of chromatin assays for DNA fragmentation evaluation in human sperm. *J Androl* 27,
404 53-59.

405 Cooke MS, Evans MD, Dizdaroglu M & Lunec J (2003) Oxidative DNA damage:
406 mechanisms, mutation, and disease. *FASEB J* 17, 1195-1214.

407 Dominguez-Fandos D, Camejo MI, Ballesca JL & Oliva R (2007) Human sperm DNA
408 fragmentation: correlation of TUNEL results as assessed by flow cytometry and optical
409 microscopy. *Cytometry A* 71, 1011-1018.

410 Donnelly ET, O'Connell M, McClure N & Lewis SE (2000) Differences in nuclear
411 DNA fragmentation and mitochondrial integrity of semen and prepared human
412 spermatozoa. *Hum Reprod* 15, 1552-1561.

413 Erenpreiss J, Jepson K, Giwercman A, Tsarev I, Erenpreisa J & Spano M (2004)
414 Toluidine blue cytometry test for sperm DNA conformation: comparison with the flow
415 cytometric sperm chromatin structure and TUNEL assays. *Hum Reprod* 19, 2277-2282.

Esbert M, Pacheco A, Vidal F, Florensa M, Riqueros M, Ballesteros A *et al.* (2011) Impact of sperm DNA fragmentation on the outcome of IVF with own or donated oocytes. *Reprod Biomed Online* 23, 704-710.

Evenson DP, Darzynkiewicz Z & Melamed MR (1980) Comparison of human and mouse sperm chromatin structure by flow cytometry. *Chromosoma* 78, 225-238.

Evenson DP, Jost LK, Zinaman MJ, Clegg E, Purvis K, de Angelis P *et al.* (1999) Utility of the sperm chromatin structure assay (SCSA) as a diagnostic and prognostic tool in the human fertility clinic. *Hum reprod* 14, 1039-1049.

Evenson D & Jost L (2000) Sperm chromatin structure assay is useful for fertility assessment. *Methods Cell Sci* 22, 169-189.

Evenson DP, Larson KL & Jost LK (2002) Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J Androl* 23, 25-43.

Evenson DP and Wixon R (2006) Predictive value of the sperm chromatin assay in different populations. *Fertil Steril* 85: 811-812.

Evenson DP (2013) Sperm chromatin structure assay (SCSA(R)). *Methods Mol Biol* 927, 147-164.

Fernandez JL, Muriel L, Goyanes V, Segrelles E, Gosalvez J, Enciso M *et al.* (2005) Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril* 84, 833-842.

436 Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C *et al.* (2000)
437 Study of apoptotic DNA fragmentation in human spermatozoa. *Hum Reprod* 15, 830-
438 839.

439 Garcia-Peiró A, Oliver-Bonet M, Navarro J, Abad C, Amengual MJ, Lopez-Fernandez
440 C *et al.* (2012) Differential clustering of sperm subpopulations in infertile males with
441 clinical varicocele and carriers of rearranged genomes. *J Androl* 33, 361-367.

442 Garcia-Peiró A, Oliver-Bonet M, Navarro J, Abad C, Guitart M, Amengual MJ *et al.*
443 (2011) Dynamics of sperm DNA fragmentation in patients carrying structurally
444 rearranged chromosomes. *Int J Androl* 34, e546-53.

445 Gorczyca W, Traganos F, Jesionowska H & Darzynkiewicz Z (1993) Presence of DNA
446 strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal
447 human sperm cells: analogy to apoptosis of somatic cells. *Exp Cell Res* 207, 202-205.

448 Henkel R, Hoogendijk CF, Bouic PJ & Kruger TF (2010) TUNEL assay and SCSA
449 determine different aspects of sperm DNA damage. *Andrologia* 42, 305-313.

450 Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA & Aitken RJ (2000) DNA
451 integrity in human spermatozoa: relationships with semen quality. *J Androl* 21, 33-44.

452 Jayakumar S, Bhilwade HN, Pandey BN, Sandur SK & Chaubey RC (2012) The
453 potential value of the neutral comet assay and the expression of genes associated with
454 DNA damage in assessing the radiosensitivity of tumor cells. *Mutat Res* 748, 52-59.

455 Kaneko S, Yoshida J, Ishikawa H & Takamatsu K (2012) Single-cell pulsed-field gel
456 electrophoresis to detect the early stage of DNA fragmentation in human sperm nuclei.
457 *PLoS One* 7, e42257.

458 Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET & Evenson
459 DP (2003) Relationship between the outcomes of assisted reproductive techniques and
460 sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil*
461 *Steril* 80, 895-902.

462 Lewis SE & Simon L (2010) Clinical implications of sperm DNA damage. *Hum Fertil*
463 *(Camb)* 13, 201-207.

464 Maione B, Pittoggi C, Achene L, Lorenzini R & Spadafora C (1997) Activation of
465 endogenous nucleases in mature sperm cells upon interaction with exogenous DNA.
466 *DNA Cell Biol* 16, 1087-1097.

467 Makker K, Agarwal A & Sharma R (2009) Oxidative stress & male infertility. *Indian J*
468 *Med Res* 129, 357-367.

469 Marchiani S, Tamburrino L, Forti G, Baldi E & Muratori M (2007) M540 bodies and
470 their impact on flow cytometric analyses of human spermatozoa. *Soc Reprod Fertil*
471 *Suppl* 65, 509-514.

472 Mitchell LA, De Iuliis GN & Aitken RJ (2011) The TUNEL assay consistently
473 underestimates DNA damage in human spermatozoa and is influenced by DNA
474 compaction and cell vitality: development of an improved methodology. *Int J Androl*
475 34, 2-13.

476 Muratori M, Forti G & Baldi E (2008) Comparing flow cytometry and fluorescence
477 microscopy for analyzing human sperm DNA fragmentation by TUNEL labeling.
478 *Cytometry A* 73, 785-787.

Nijs M, Creemers E, Cox A, Franssen K, Janssen M, Vanheusden E *et al.* (2009) Chromomycin A3 staining, sperm chromatin structure assay and hyaluronic acid binding assay as predictors for assisted reproductive outcome. *Reprod Biomed Online* 19, 671-684.

Niu ZH, Shi HJ, Zhang HQ, Zhang AJ, Sun YJ & Feng Y (2011) Sperm chromatin structure assay results after swim-up are related only to embryo quality but not to fertilization and pregnancy rates following IVF. *Asian J Androl* 13, 862-866.

Nunez-Calonge R, Caballero P, Lopez-Fernandez C, Guijarro JA, Fernandez JL, Johnston S *et al.* (2012) An improved experimental model for understanding the impact of sperm DNA fragmentation on human pregnancy following ICSI. *Reprod Sci* 19, 1163-1168.

Payne JF, Raburn DJ, Couchman GM, Price TM, Jamison MG & Walmer DK (2005) Redefining the relationship between sperm deoxyribonucleic acid fragmentation as measured by the sperm chromatin structure assay and outcomes of assisted reproductive techniques. *Fertil Steril* 84, 356-364.

Ribas-Maynou J, Garcia-Peiró A, Abad C, Amengual MJ, Navarro J & Benet J (2012a) Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. *Hum Reprod* 27, 652-658.

Ribas-Maynou J, Garcia-Peiró A, Fernandez-Encinas A, Amengual MJ, Prada E, Cortes P *et al.* (2012b) Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor. *PLoS One* 7, e44679.

501 Sailer BL, Sarkar LJ, Bjordahl JA, Jost LK & Evenson DP (1997) Effects of heat stress
502 on mouse testicular cells and sperm chromatin structure. *J Androl* 18, 294-301.

503 Sakkas D & Alvarez JG (2010) Sperm DNA fragmentation: mechanisms of origin,
504 impact on reproductive outcome, and analysis. *Fertil Steril* 93, 1027-1036.

505 Saleh RA, Agarwal A, Nelson DR, Nada EA, El-Tonsy MH, Alvarez JG *et al.* (2002)
506 Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective
507 study. *Fertil Steril* 78, 313-318.

508 Sergerie M, Laforest G, Bujan L, Bissonnette F & Bleau G (2005) Sperm DNA
509 fragmentation: threshold value in male fertility. *Hum Reprod* 20, 3446-3451.

510 Sharma RK, Sabanegh E, Mahfouz R, Gupta S, Thiyagarajan A & Agarwal A (2010)
511 TUNEL as a test for sperm DNA damage in the evaluation of male infertility. *Urology*
512 76, 1380-1386.

513 Simon L, Proutski I, Stevenson M, Jennings D, McManus J, Lutton D *et al.* (2013)
514 Sperm DNA damage has a negative association with live-birth rates after IVF. *Reprod*
515 *Biomed Online* 26, 68-78.

516 Simon L, Lutton D, McManus J & Lewis SE (2011) Sperm DNA damage measured by
517 the alkaline Comet assay as an independent predictor of male infertility and in vitro
518 fertilization success. *Fertil Steril* 95, 652-657.

519 Singh NP, McCoy MT, Tice RR & Schneider EL (1988) A simple technique for
520 quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175, 184-
521 191.

522 Sotolongo B, Huang TT, Isenberger E & Ward WS (2005) An endogenous nuclease in
523 hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments. *J*
524 *Androl* 26, 272-280.

525 The Practice Committee of the American Society for Reproductive Medicine (2008)
526 The clinical utility of sperm DNA integrity testing. *Fertil Steril* 90, S178–S180.

527 Van Kooij RJ, de Boer P, De Vreeden-Elbertse JM, Ganga NA, Singh N & Te Velde
528 ER (2004) The neutral comet assay detects double strand DNA damage in selected and
529 unselected human spermatozoa of normospermic donors. *Int J Androl* 27, 140-146.

530 Velez de la Calle JF, Muller A, Walschaerts M, Clavere JL, Jimenez C, Wittemer C *et*
531 *al.* (2008) Sperm deoxyribonucleic acid fragmentation as assessed by the sperm
532 chromatin dispersion test in assisted reproductive technology programs: results of a
533 large prospective multicenter study. *Fertil Steril* 90, 1792-1799.

534 Venkatesh S, Singh A, Shamsi MB, Thilagavathi J, Kumar R, Mitra DK *et al.* (2011)
535 Clinical significance of sperm DNA damage threshold value in the assessment of male
536 infertility. *Reprod Sci* 18, 1005-1013.

537 Villani P, Eleuteri P, Grollino MG, Rescia M, Altavista P, Spano M *et al.* (2010) Sperm
538 DNA fragmentation induced by DNAse I and hydrogen peroxide: an in vitro
539 comparative study among different mammalian species. *Reproduction* 140, 445-452.

540 Virro MR, Larson-Cook KL and Evenson DP (2004) Sperm chromatin structure assay
541 (SCSA) parameters are related to fertilization, blastocyst development, and ongoing
542 pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycle. *Fertil*
543 *Steril* 8, 1289-1295

544 Zini A (2011) Are sperm chromatin and DNA defects relevant in the clinic? *Syst Biol*
545 *Reprod Med* 57, 78-85.

546 Zini A, Bielecki R, Phang D & Zenzes MT (2001) Correlations between two markers of
547 sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile
548 men. *Fertil Steril* 75, 674-677.

549

TABLES

Table I. Sperm DNA fragmentation (%SDF) values for fertile donors and infertile patients in each assay

Technique	n	Fertile donors	Range	n	Infertile patients	Range
TUNEL assay	21	13.67±5.79	[6.6 - 29.3]	72	28.75±12.56 *	[7.1 - 74.1]
SCSA	24	13.01±5.64	[5.0 - 27.3]	74	23.58±13.17 *	[7.7 - 74.5]
SCD test	49	15.32±6.25	[4.1 - 31.5]	74	31.26±14.41 *	[6.5 - 78.0]
Alkaline Comet	50	28.64±13.40	[9.3 - 70.0]	133	60.48±16.03 *	[17.4 - 99.0]
Neutral Comet	50	60.09±30.57	[12.2 - 99.0]	133	64.74±16.90	[26.8 - 100.0]

* Statistical differences with fertile donors ($p < 0.001$).

Table II. Cut-off values with sensitivity and specificity obtained for each technique

Technique	n	Area*	Cut-off value	Sensitivity	Specificity
Alkaline Comet	183	0.937	45.37%	0.850	0.920
Neutral Comet	183	0.516	34.37%	0.970	0.320
SCD test	123	0.869	22.75%	0.730	0.918
SCSA	98	0.792	18.90%	0.595	0.875
TUNEL	93	0.903	20.05%	0.764	0.952

* Area below the ROC curve

FIGURE LEGENDS

Figure 1. Fertile and infertile sperm DNA fragmentation distribution in the five different techniques. Curves show the approximation to a normal distribution.

Figure 2. ROC curve comparing the five SDF techniques to assess male infertility.