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Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay

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SUMMARY

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 in 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, SCD and SCSA, whereas 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 spermatozoa 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 foetal development (Evenson *et al.*, 1999; Carrell *et al.*, 2003; Lewis & Simon, 2010). Moreover, high SDF 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 remodelling, 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 & 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 labelling 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 microscope (Dominguez-Fandos *et al.*, 2007), by

decompaction of the DNA with DTT (Mitchell *et al.*, 2011) or not including the apoptotic bodies on the final result (Marchiani *et al.*, 2007). Second, the Comet assay (Singh *et al.*, 1988) has the unique feature that it can distinguish between single and double stranded DNA breaks (ssSDF and dsSDF respectively) when it is performed under alkaline or neutral conditions. It is based on nuclear decompaction followed by electrophoresis and visualization of individual spermatozoa. Clinical cut-off values for male infertilty, assessing Comet tail DNA and percentage of fragmented spermatozoa have been published using the alkaline Comet assay for both total semen sample (Simon *et al.*, 2011; Ribas-Maynou *et al.*, 2012a) and also differentiating swim-up sperm cells (Simon *et al.*, 2011). Moreover, our group demonstrated a clinical association of dsSDF assessed by neutral Comet with recurrent miscarriage risk in couples without female factor (Ribas-Maynou *et al.*, 2012b), showing that differences in the DNA break type, ssSDF or dsSDF, has different implications for human reproduction.

Other methods such as Sperm Chromatin Structure Assay (SCSA) (Evenson *et al.*, 1980) and the Sperm Crhomatin Dispersion (SCD) test (Fernandez *et al.*, 2005) base their detection of SDF on the denaturing capacity of the sperm chromatin. The SCSA uses acridine orange staining to label the double stranded DNA with green and the single stranded DNA with red. The proportion of these two emissions, with a previous acid-denaturing step, has widely been demonstrated to determine the percentage of DNA fragmentation, and several reports for clinical usage have been published (Evenson & Jost, 2000; Evenson *et al.*, 2002; Bungum *et al.*, 2004; Virro *et al.*, 2004; Nijs *et al.*, 2009; Venkatesh *et al.*, 2011). Moreover, SCSA provides also an additional parameter named high DNA stainability (HDS). This parameter is a measure of the percentage of immature spermatozoa within the semen sample, which can also be taken into account on the male infertility assessment (Evenson *et al.*, 1999).

Finally, the SCD test assesses the capacity of the sperm chromatin to form dispersion halos, and allows differentiating the non-fragmented spermatozoa (with halo) from the fragmented spermatozoa (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; Nunez-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 such as in vitro fertilization (IVF) or intra-cytoplasmatic sperm injection (ICSI) (Esbert *et al.*, 2011; Bungum *et al.*, 2012; Simon *et al.*, 2013). This lack of the predictive quality of SCSA could be because of the presence of a female factor, such as differences between oocytes on their efficiency of DNA repair after fertilization (Payne *et al.*, 2005; Evenson & Wixon, 2006).

We have previously shown that extensive sperm ssSDF may prevent pregnancy, but that spermatozoa with dsSDF can fertilize ooctyes achieving pregnancy, but compromise the foetus 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 in sensitivity and specificity in detecting the total SDF in the semen sample between methods may be because of 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 objectives of this study were 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.

MATERIALS AND METHODS

Sample collection

Semen samples from 240 human males were collected in collaboration with reproduction centres 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 3 days and maximum of 7 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 SDF analysis. The total sample sizes that were analyzed 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 (Roche Diagnostic Gmbh, Penzberg, Germany) following the protocol previously described (Barroso *et al.*, 2000). The analysis of SDF was performed by flow cytometer analysis (FACSCalibur; Becton Dickinson, Franklin Lakes, 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

The SCSA methodology has been described elsewhere by Evenson *et al.* (1999). Briefly, each semen sample was diluted to reach a concentration of 2×10^6 spermatozoa/mL in TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5) in a total volume of 200 μL. Then, the sample was treated with an acid solution (150 mM NaCl, 0.1% Triton X-100, pH 1.2) and after 30 sec a staining was performed using acridine orange 6 μg/mL for 3 min. Finally, a total of 5000 sperm cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson). The percentage of spermatozoa with DNA fragmentation shows increased red fluorescence, unlike the non-fragmented population that shows a normal level of red fluorescence. The percentage of HDS spermatozoa had not been included in this SDF comparative study.

SCD test

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

Comet assay

The alkaline and neutral Comet assay was performed simultaneously in two different slides to assess single and double stranded DNA fragmentation respectively. The assay has been performed following the protocol reported before (Ribas-Maynou *et al.*, 2012a,b). Briefly, samples were washed and sperm concentration was adjusted to 10×10^6 spermatozoa/mL. Then incubations with two lysis solutions were performed and the samples were electrophoresed, using alkaline or neutral buffer depending on the assay, with a previous denaturation on alkaline Comet slide. Finally, both the slides were submerged on a neutralization solution and were dehydrated in ethanol series of 70, 90 and 100%. Samples were stained with DAPI SlowFade Gold antifade (Invitrogen, Eugene, OR, USA), and 400 spermatozoa were classified according fragmented or non-fragmented following the criteria reported before (Fig. 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, USA). 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 Receiver Operating Characteristic (ROC) analysis was performed 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

SDF regarding male infertility

For each assay, the percentage of spermatozoa in the sample that was positive for the test was calculated. The average percentage of SDF for fertile and infertile patients using the five different techniques is shown in Table 1, and a histogram for the same results is displayed in Fig. 1 to show their distribution.

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

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 test 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 were 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 2, and a graphical representation of ROC curves for all techniques is shown on Fig. 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 (Gandini *et al.*, 2000; Irvine *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 assay 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 (Fig. 1 and Table 1). 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 bebecause of the electrophoresis

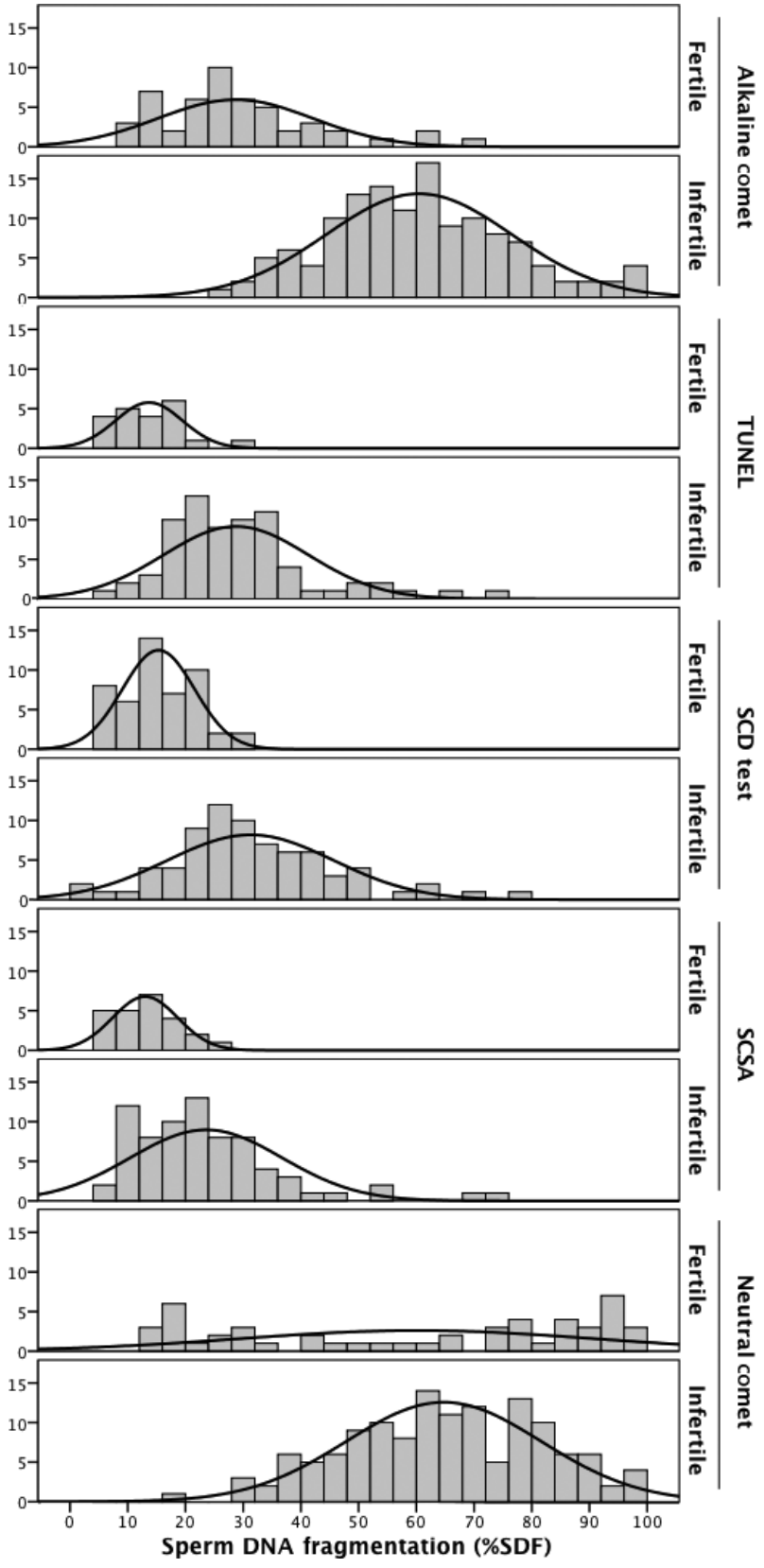


Figure 1 Fertile and infertile sperm DNA fragmentation distribution in the five different techniques. Curves show the approximation to a normal distribution.

step, which could be increasing the sensitivity of the detection of the DNA breaks with respect to other methodologies. Regarding infertile patients, values of SDF obtained by the alkaline Comet assay were statistically higher than SCD test, SCSA and TUNEL methodologies, showing that Comet assay seems to have higher sensitivity on detecting the sperm DNA breaks as Comet assay show values up to 100% of Spermatozoa with DNA fragmentation in some infertile patients, and the other methodologies do

Table 1 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)

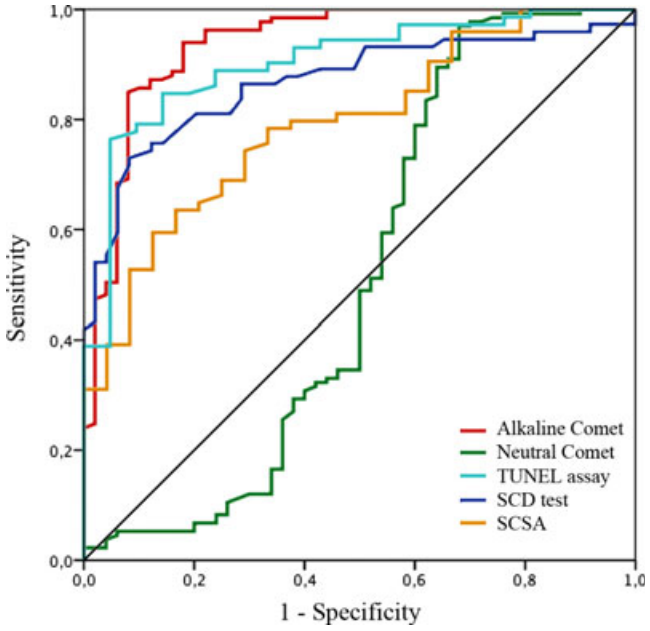
*Statistical differences with fertile donors ($p < 0.001$).

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



not reach this value (Fig. 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 SDF, as 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 the American Society for Reproductive Medicine, 2008; Henkel *et al.*, 2010).

Regarding 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; Villani *et al.*, 2010; Garcia-Peiró *et al.*, 2011). 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 as it is known that it shows variations in SDF detected depending on minor variations in the procedure (Dominguez-Fandos *et al.*, 2007; Muratori *et al.*, 2008; Mitchell *et al.*, 2011) or in 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 has been tested before, with similar results to the present work (Chohan *et al.*, 2006; Villani *et al.*, 2010; Garcia-Peiró *et al.*, 2011).

Similarly, the alkaline Comet assay showed a moderate correlation with the SCD test, the TUNEL assay and SCSA, as has been described before by different laboratories (Donnelly *et al.*, 2000; Villani *et al.*, 2010). This correlation was not as strong as the correlations found among the latter three techniques, which might be because of a possible higher sensitivity of the alkaline Comet assay with respect to the other methodologies.

In contrast, the neutral Comet assay does not show any correlation with the other four methodologies to assess SDF. As has been proposed before, the neutral Comet assay is related to the risk of having a miscarriage, as the dsDNA breaks could be a non-extensive type of DNA damage located only in a few points along the genome (Kaneko *et al.*, 2012), preferently in the matrix attachment regions between toroids (Ribas-Maynou *et al.*, 2012b) and might be occuring by an acute or fractionated exposition to radiation, as it has been demonstrated in tumour cells (Jayakumar *et al.*, 2012). Although it is known that techniques such as the TUNEL assay and SCSA are detecting both single and double stranded DNA damage (The Practice Committee of the American Society for Reproductive Medicine, 2008; Villani *et al.*, 2010), our data show a correlation between both TUNEL or SCSA and the alkaline Comet assay, which would be detecting mainly ssSDF. However, they do not show a correlation with the neutral Comet assay, which has been demonstrated to assess mostly dsDNA breaks (Van Kooij *et al.*, 2004; Ribas-Maynou *et al.*, 2012a). Moreover, the neutral and alkaline Comet assays showed a tendency to a moderate correlation in infertile patients, a fact that could be related to the possibility that the presence of many 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 2 and Fig. 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 with 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 this study (Table 2) do not differ from previously published works (Fernandez *et al.*, 2005; Velez de la Calle *et al.*, 2008; Nunez-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% (Evenson & Jost, 2000; Evenson *et al.*, 2002; Larson-Cook *et al.*, 2003; Bungum *et al.*, 2004; Payne *et al.*, 2005; Boe-Hansen *et al.*, 2006; Venkatesh *et al.*, 2011; Evenson, 2013). Our results show a threshold value of 18.9% of SDF, which is at the low end of the published range. Despite 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, as 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, as 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 because of a male factor (Ribas-Maynou *et al.*, 2012b).

For 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 SDF 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.

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AUTHOR'S CONTRIBUTION

J.R-M. contributed to experimental procedures, statistical analysis, graphics and table elaboration and document writing. A.G-P. contributed to experimental design, results discussion, statistical analysis and document writing and revising. A.F-E contributed to experimental procedures. M.J.A. and C.A contributed to recruitment of patients, sample collection, storage and semen parameter analysis. J.N. and J.B. contributed to experimental design and direction and coordination of the work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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.

Barroso G, Morshedi M & Oehninger S. (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* 15, 1338–1344.

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

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.

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

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

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

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

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

Erenpreiss J, Jepson K, Giwercman A, Tsarev I, Erenpreisa J & Spano M. (2004) Toluidine blue cytometry test for sperm DNA conformation: comparison with the flow 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. (2013) Sperm chromatin structure assay (SCSA(R)). *Methods Mol Biol* 927, 147–164.

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

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

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 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.

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.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Muratori M, Forti G & Baldi E. (2008) Comparing flow cytometry and fluorescence microscopy for analyzing human sperm DNA fragmentation by TUNEL labeling. *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.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

