Received: 13 February 2019 | Revised: 15 April 2019 | Accepted: 20 June 2019

DOI: 10.1111/and.13375

ORIGINAL ARTICLE

First International Journal of Andrology
ANDROLOGIA
WILEY

TUNEL assay: Establishing a sperm DNA fragmentation cut-off value for Egyptian infertile men

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Abstract

Male factor infertility is responsible for half of all infertility cases. Conventional semen analysis is inadequate to evaluate male fertility. Sperm DNA fragmentation (SDF) test can be done by: direct methods such as Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) and Comet assay, or indirect like Sperm Chromatin Structure Assay (SCSA) and Sperm Chromatin Dispersion (SCD). TUNEL assay measures both single- and double-strand breaks and is technically less demanding, while SCSA tests for the susceptibility for nuclear DNA denaturation and samples should be sent to the reference lab. Studies showed that a single cut-off value does not fit all. Therefore, this study aimed at establishing a cut-off value to discriminate between fertile and infertile Egyptian men. We enrolled 354 infertile men and 40 proven fertile volunteers.TUNEL assay was performed using Apo-Direct kit and bench top flow cytometer.The calculated SDF cut-off value was 20.3% with a sensitivity of 96.6% and specificity of 87.5%, and the overall accuracy of the test was 95.7%. Sperm DNA fragmentation Test using TUNEL assay is valuable tool for male infertility evaluation, and it assists in offering the best treatment options based on it's results.

KEYWORDS

male infertility, SDF cut-off value, sperm DNA fragmentation, TUNEL assay

1 | INTRODUCTION

Male factor infertility is responsible for approximately half of all infertility cases (Agarwal, Gupta, & Sharma, 2016, Agarwal, et al., 2015). Since standard seminal parameters have a high natural biological variability, several studies confirmed that conventional semen analysis is insufficient to evaluate the fertility potential of men (Agarwal et al., 2016), or even predict the clinical outcomes after any assisted reproduction procedure (Cissen et al., 2016). Yet, a sperm DNA fragmentation test combined with the traditional semen fluid analysis can provide a more accurate prediction of male fertility potential (Malhotra, 2017).

Sperm DNA presents in a compacted state bound to protamines protecting it from damage during transport (Agarwal et al., 2016). During spermatogenesis in the mitotic and meiotic phases, sperm DNA repair ability is high, but is lost in post-meiotic spermatids (Marchetti, Bishop, Gingerich, & Wyrobek, 2015). With the reduction of the cytoplasmic content and the replacement of histones by protamines which is resulting in the compaction of the sperm nuclei, access of the DNA repair machinery to the nuclear material is limited or not possible (Marchetti et al., 2015). Male germ cells lack DNA repair mechanisms; the oocyte can repair this damage and restore biologically stable genome needed for fertilisation and embryo development (Fernández-Díez, González-Rojo, Lombó, & Herráez, 2016). Intact sperm DNA and chromatin structure are crucial for a correct transmission of the paternal genetic material (Bungum et al., 2007) and activation of embryonic genome (Teperek et al., 2016). Paternal DNA is crucial at a time when the embryonic genome is activated and transcriptional activity has begun. At this time, the paternal genome plays a significant role in embryo function towards

implantation (Alvarez Sedó et al., 2017). Sperm DNA fragmentation may affect fertility by hindering fertilisation, early embryo development, implantation and pregnancy (Alvarez Sedó et al., 2017).

The most commonly used techniques to test sperm DNA fragmentation (SDF) are the TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labelling), the sperm chromatin structure assay (SCSA), sperm chromatin dispersion test (SCD) and the Comet assay (Majzoub, Esteves, & Gosálvez, 2016). These assays can be categorised into two groups: those that can directly measure the extent of DNA fragmentation by using probes or dyes, and those that measure the susceptibility of DNA to denaturation, which occurs more commonly in fragmented DNA (Cho, Agarwal, Majzoub, & Esteves, 2017a, 2017b; Henkel, 2017). Among these test systems, the TUNEL assay and the SCSA are most commonly used.

While the TUNEL assay measures both single- and double-strand DNA breaks by identifying its definite end point (presence of free 3-hydroxyl groups), the SCSA detects potential DNA damage after exposure of the spermatozoa to denaturing conditions. Unlike the SCSA, where semen samples are batched and shipped to the primary SCSA testing laboratory and analysed for DNA damage using a dedicated flow cytometer, the TUNEL assay appears to be technically less demanding, though the procedure takes more time.

The TUNEL assay can be performed by flow cytometry or fluorescence microscopy. Microscopic determination of TUNEL-positive spermatozoa is affected by the subjectivity and the duration of the observations caused by fluorescence bleaching; these factors are difficult to standardise (Domínguez-Fandos, Camejo, Ballesca, & Oliva, 2007). Therefore, performing the TUNEL assay by flow cytometry using tight gate settings to exclude counting TUNEL-positive cells other than spermatozoa is the preferred option (Domínguez-Fandos et al., 2007).

Currently, there is lack of sufficient clinical data to support the routine use of SDF in male factor evaluation (Johnson & Sandlow, 2017). In addition, a single cut-off value for SDF testing does not fit all in terms of methodology as well as the kind of samples analysed, due to using different direct or indirect techniques, lack of standardised protocols, and presence of different reported cut-off values (Cho, Agarwal, Majzoub, & Esteves, 2017a, 2017b). The cut-off value has its importance in scientific studies of SDF, but it is crucial to the clinician to make decisions regarding ART options and its expected outcomes (Cho, Agarwal, Majzoub, & Esteves, 2017a, 2017b).

Therefore, goal of this study was to set a discriminating SDF threshold value for the Egyptian population by comparing values measured by TUNEL assay with flow cytometric assessment, in men of proven fertility (fertile control) and men from couples consulting for infertility (infertile patients). In this study, we examined a larger sample size for both the fertile and infertile groups than any published ROC analysis for TUNEL assay by flow cytometer in an Egyptian population (Chenlo et al., 2014; Sergerie, Laforest, Bujan, Bissonnette, & Bleau, 2005; Sharma, Ahmad, Esteves, & Agarwal, 2016; Sharma, Forte, et al., 2016; Sharma et al., 2010).

2 | MATERIALS AND METHODS

2.1 | Study participants

A total of 354 infertile men, aged between 23 and 40 years, attending Ganin Fertility Center, Cairo, Egypt, seeking medical advice for male factor infertility were enrolled in this study. In addition, 40 proven fertile volunteers were included, 11 of them established pregnancy in the last year, and 29 of them fathered a child in the last year. The fertile volunteers' age ranged from 20 to 40 years. All healthy volunteers and infertile patients accepted participating in the study and signed fully informed consents.

2.2 | Semen collection

Semen samples were collected by masturbation after 1–2 days of abstinence, and a 10 µl aliquot semen was loaded on a Makler sperm counting chamber (SEFI-Medical Instruments) for sperm count and motility assessment. Normal sperm morphology was assessed according to WHO (2010) criteria.

An aliquot of liquefied semen containing 3–5 million spermatozoa was washed in phosphate-buffered saline (Lonza), and sample fixation is done by re-suspension in 3.7% paraformaldehyde (Sigma Aldrich) followed by storage at 4°C until analysis using flow cytometry.

2.3 | TUNEL assay and flow cytometric analysis

Paraformaldehyde was removed by centrifugation at 300 *g* for 4 min at room temperature, supernatant was discarded, and pellet was permeablised by adding 1 ml of ice cold 70% ethanol (CHEM-LAB) for at least 30 min at −20°C.

Sperm DNA fragmentation was measured by TUNEL assay with the Apodirect Kit (BD Pharmingen), using BD Accuri C6 benchtop flow cytometer (BD Pharmingen). The designed gates were used to exclude any cells that may interfere with the spermatozoa. Further virtual gain analysis was done to calculate an accurate SDF percentage using C flow plus software version 1.0.264.15 (BD Pharmingen). Positive and negative kit controls provided by the manufacturer and internal controls (semen samples with known DNA fragmentation) were included in each run.

All samples were washed twice with washing buffer containing 0.05% sodium azide followed by adding 50 µl of freshly prepared staining solution containing (reaction buffer, FITC-dUTP, and TdT enzyme) and then incubated for 1 hr at 37°C. Excess staining was removed by adding rinsing buffer containing 0.05% sodium azide followed by centrifugation and discarding the supernatant. A volume of 0.5 ml Propidium Iodide (PI)/RNase Staining Buffer was added to the pellet and incubated for 30 min at room temperature for flow cytometric analysis.

Ten thousand spermatozoa were counted and evaluated for DNA fragmentation. Green fluorescence produced from FITC with excitation wavelength (480–530 nm) was detected on FL1 channel.

Red fluorescence produced from PI with excitation wavelength (580–630 nm) was detected on the FL-2 channel. The sperm DNA fragmentation index was calculated by using the flow cytometer software Accuri C flow plus (BD Pharmingen).

2.4 | SDF distribution

Sperm DNA fragmentation values of all tested samples were distributed into categories 0%–10%, 10%–20%, 20%–30%, 30%–40%, and >40% (Sharma et al., 2010), and statistically described in terms of frequencies and percentages when appropriate. It was tested using Kolmogorov–Smirnov normality test.

2.5 | Inter-observer and intra-observer variation

Fifty-eight samples from the infertile group were evaluated for SDF values by two observers to calculate the intra-observer and inter-observer variability using interclass correlation coefficient (ICC) with its 95% CI.

2.6 | Statistical analysis

All statistical calculations and analyses were performed by using IBM SPSS (Statistical Package for Social Science; IBM Corp) release 22 for Microsoft Windows. Accuracy was represented using the terms sensitivity and specificity. Receiver operator characteristic (ROC) analysis was used to determine the optimum cut-off value for DFI. *p*-Values <.05 were considered significant.

3 | RESULTS

3.1 | SDF distribution in fertile and infertile men

A total of 394 subjects were included in the study. The mean value SDF (%) in infertile group was 27.6% ± 7.5%, while the fertile group SDF mean value was 15.8% ± 3.4%. In infertile men, the mean SDF value was significantly higher compared to the fertile men ($p < .001$).

SDF values were distributed into categories 0%–10%, 10%–20%, 20%–30%, 30%–40%, and >40% (Sharma et al., 2010). Descriptive analysis showed that the SDF values were not normally distributed among the fertile and infertile groups. Among the fertile group, 5% of the donors had SDF values between 0% and 10%, while 77.5% had SDF values between 10% and 20%, and 17.5% between 20% and 30%. None of the donors showed SDF values in the higher categories. In contrast, the infertile group showed a different distribution pattern. While none of the patients had SDF values between 0%–10% and 10%–20%, 71.46% had SDF values between 20% and 30%, 22.03% between 30% and 40%, and 6.49% higher than 40%. The distribution of SDF values among all the samples is shown in Figure 1.

The variability of the SDF values of both fertile and infertile groups, the highest and lowest observed SDF values and the calculated median SDF are depicted in Figure 2.

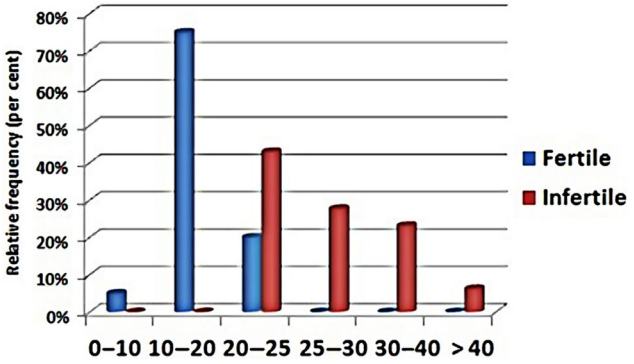

FIGURE 1 Distribution of DFI categories between fertile and infertile study groups

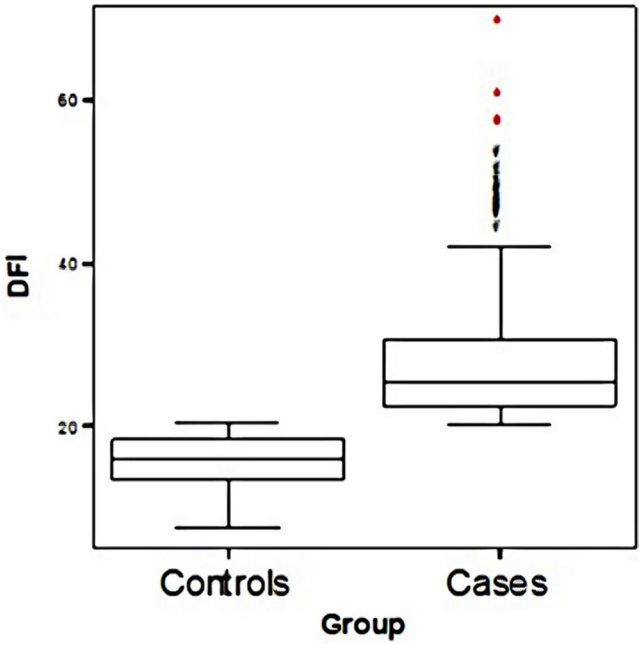

FIGURE 2 Box plot of the DFI values in fertile and infertile study groups

3.2 | Intra- and Inter-observer variability

Intra- and inter-observer variabilities were calculated using 58 valid samples from the infertile group, and for the inter-observer variability, the calculated interclass correlation coefficient (ICC) for average measures was 0.987 with a 95% confidence interval of 0.978 and 0.992.

For the intra-observer variability, the calculated interclass correlation coefficient (ICC) for average measures was 0.988. The 95% confidence interval was 0.980 and 0.993.

3.3 | ROC analysis

The discriminating power of sperm DNA fragmentation, measured by TUNEL assay using a flow cytometer, was calculated by ROC

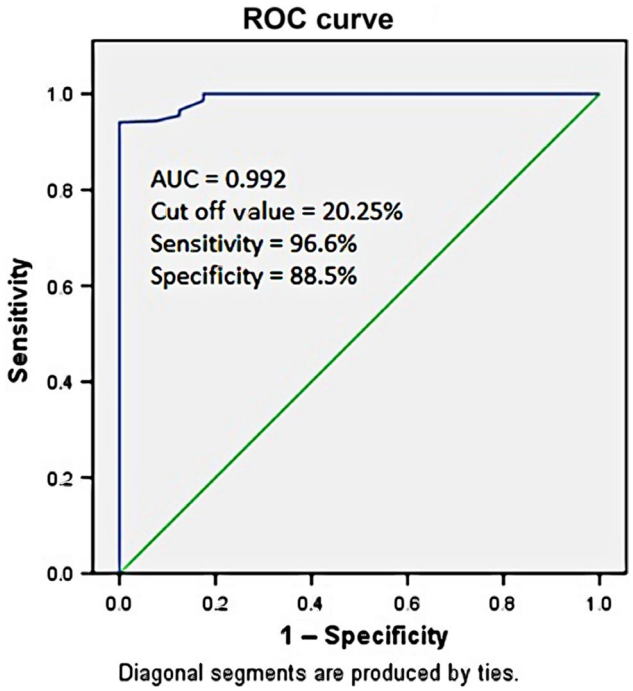

FIGURE 3 Receiver operating characteristic curve showing the area under the curve, cut-off value, sensitivity and specificity

curve analysis. The area under the ROC curve was 0.992. The calculated threshold value that discriminates between fertile and infertile men is 20.3% with a specificity of 88.5% and sensitivity of 96.6% as shown in Figure 3.

The overall accuracy of the test was calculated 95.7%, and also, the positive predictive value was calculated 98.6% while the negative predictive value was 74.5%.

4 | DISCUSSION

There are different techniques for measuring SDF, each of which has its own measuring mechanism, limitations, clinical value and its cut-off value. There is a lack of consensus in-between the techniques used, generating different cut-off values that makes it difficult to interpret data from different studies (Sakkas & Colaco, 2018). Among all the SDF techniques, TUNEL assay seems to have the highest clinical value because it can directly measure both single- and double-strand breaks with high accuracy and sensitivity especially with the use of flow cytometric analysis. In addition, TUNEL assay had a standardised protocol using Apodirect kit and benchtop flow cytometer; the correlation of TUNEL results between two different laboratories was significant, besides reporting the high reproducibility of TUNEL assay within and across laboratories (Sharma, Ahmad, et al., 2016; Sharma, Forte, et al., 2016).

Every population has its own demographics that can affect semen quality and particularly sperm DNA fragmentation (Salas-Huetos, Bulló, & Salas-Salvadó, 2017). These factors include biological like male age and body mass index (Salas-Huetos et al., 2017), the use of physical agents like mobile phone radiations (Desai, Kesari, & Agarwal, 2009), environmental factors like air pollution (Lafuente, Blàquez, Jacquemin, & Checa, 2016), and lifestyle changes like everyday stress or smoking (Cui, Jing, Wu, Wang, & Li, 2016, Taha, 2012). Redmon et al found significant differences in semen parameters among men of different ethnicities based on variants in the population genetics of a certain ancestry (Redmon et al., 2013). A systematic review by Salas-Huetos et al suggested an association between semen quality and male diet or nutrient intake. This review has also indicated that healthy diets (rich in antioxidants, high in omega 3, low in saturated fatty acids) are correlated with good semen quality including SDF (Salas-Huetos et al., 2017). In Egypt, the malnutrition is a result of high fat intake, high consumption of fast food, and high legumes intake. All these diversified factors are different in-between regions of the world that raises the question about the clinical validity of establishing an overall SDF cut-off value, and even hints to the need for a specific cut-off value for every geographical region. In this study, a cut-off value for SDF in an Egyptian population was established.

Looking back into literature, we did not find any previous published studies that established a cut-off value of SDF using TUNEL assay by flow cytometer or by any other SDF measuring technique on the Egyptian population. Therefore, we conducted this study on a large group of Egyptian infertile patients, and controls of proven fertility over the last year to establish a cut-off value and to check whether the previously published SDF cut-off values can apply for the Egyptian population or not. For SDF testing using TUNEL assay and flow cytometric analysis considered of a clinical value, it should have high specificity and high positive predictive value. We found that the cut-off point of 20.3% maximised the specificity to 88.5%, the sensitivity to 96.6%, and has high overall accuracy of 95.7%, and also, the calculated positive predictive value of 98.6% is considered high.

In our study, the SDF values (mean ± *SD*) in infertile group were 27.6% ± 7.5% versus 15.8% ± 3.4% in the fertile group; however, there is no significant difference between them ($p = .1094$). Upon analysing the SDF values, in the fertile group, 35/40 (87.5%) were below the calculated cut-off 20.3% and only 5/40 (12.5%) samples had SDF values higher than 20.3% ($p < .0001$). However in the infertile group, 12/354 (3.4%) showed SDF values lower than 20.3% compared to 342/354 (96.6%) patients showed SDF values higher than 20.3% ($p < .0001$).

Compared to other studies using TUNEL assay with a flow cytometer, in Sharma et al. (2010) studied 25 fertile and 194 infertile men, his calculated cut-off value of 19.25% maximised the sensitivity 64.9% and specificity 100% of the assay, while the calculated overall accuracy was 70%, his mean DNA damage was significantly small in the donors 11.9% ± 6.8%, compared to patients 29.5% ± 18.7%, with ($p \leq .01$). Also 100% of donors and 64.9% of patients had DNA damage below the cut-off value of 19.25. In Sharma, Ahmad, et al. (2016), Sharma, Forte, et al. (2016) published a new cut-off value of SDF after increasing his numbers to 95 fertile and 261 infertile men, so his cut-off value decreased to 16.8% with a sensitivity of 32.6%, specificity of 91.6% and overall accuracy of 48.3%; regarding the SDF distribution, he reported

different data, where only 8.4% of the control samples had SDF values higher than their calculated cut-off (16.8%), compared to 32.6% in the infertile group with ($p \leq .001$). Despite using the same (sample preparation method, Apodirect kit, BD C6 benchtop flow cytometer and flow cytometric analysis) as the American Center for Reproductive Medicine, Cleveland Clinic, we got different cut-off value and subsequently different sensitivity, specificity and overall accuracy. The difference in the cut-off values may be because of the sample size of the patient and control groups, and the difference in the demography of the populations studied, and it is worth mentioning that Sharma et al. (2010) and Sharma, Ahmad, et al. (2016), Sharma, Forte, et al. (2016) studies used donors of proven and unproven fertility while we used donors of proven fertility only.

Another study by Sergerie et al. (2005), he compared TUNEL results of 47 proven fertile and 66 infertile patients. The calculated cut-off value to distinguish between fertile controls and infertile men was 20%, and at this cut-off, specificity was 89.4% and sensitivity was 96.9%. This study reported approximately the same cut-off value we reported 20.3% with specificity of 88.5% and sensitivity of 96.6%. Our results match that of Sergerie but we studied a larger population of fertile and infertile men.

In Chenlo et al. (2014) reported a cut-off value of 26% with a sensitivity and specificity of 85% and 89%. This value is higher than our calculated value of 20.3% and has mildly lower specificity and lower sensitivity, which makes our cut-off value stronger and more reliable where clinicians can depend on while taking their decisions regarding what is the best assisted reproductive technique for them and its predicted reproductive outcomes.

5 | CONCLUSION

Measuring sperm DNA fragmentation by TUNEL assay using a bench top flow cytometer is an accurate method for discrimination between Egyptian fertile and infertile men at a cut-off point of 20.3%. The test has high overall accuracy, high sensitivity and specificity that make it very useful in identifying whether the sperm DNA fragmentation is a contributory factor in the patient infertility. This test will benefit fertility clinics and laboratories to characterise infertile patients and offer them suitable assisted reproductive techniques as needed.

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How to cite this article: Hassanen E, Elqusi K, Zaki H, Henkel R, Agarwal A. TUNEL assay: Establishing a sperm DNA fragmentation cut-off value for Egyptian infertile men. *Andrologia*. 2019;51:e13375. https://doi.org/10.1111/and.13375