Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using bench top flow cytometer for evaluation of sperm DNA fragmentation in fertility laboratories: protocol, reference values, and quality control

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Received: 8 October 2015 / Accepted: 7 December 2015 / Published online: 16 January 2016
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Abstract

Purpose The purpose of this study is to provide a detailed protocol and quality control steps for measuring sperm DNA fragmentation (SDF) by terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay using a new bench top flow cytometer, determine the reference value of SDF, and assess sensitivity, specificity, and distribution of SDF in infertile men and controls with proven and unproven fertility.

Methods Semen specimens from 95 controls and 261 infertile men referred to a male infertility testing laboratory were tested for SDF by TUNEL assay using Apo-Direct kit and a bench top flow cytometer. Percentage of cells positive for TUNEL was calculated. Inter- and intraobserver variability was examined. TUNEL cutoff value, sensitivity, specificity, and

distribution of different cutoff values in controls and infertile patients were calculated.

Results The reference value of SDF by TUNEL assay was 16.8 % with a specificity of 91.6 % and sensitivity of 32.6 %. The positive and negative predictive values were 91.4 and 33.1 %, respectively. The upper limit of DNA damage in infertile men was significantly higher (68.9 %) than that in the controls (19.6 %).

Conclusions TUNEL assay using flow cytometry is a reproducible and easy method to determine SDF. At a cutoff point of 16.8 %, the test showed high specificity and positive predictive value. The results of this test could identify infertile men whose sperm DNA fragmentation does not contribute to their infertility and confirm that a man who tests positive is likely to be infertile due to elevated sperm DNA fragmentation.

Keywords Sperm DNA fragmentation · TUNEL assay · Male infertility · Sensitivity · Specificity

Capsule Measurement of sperm DNA fragmentation by bench top flow cytometer.

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Introduction

A male factor is the primary cause of infertility in almost 20 % of cases while an additional 30 to 40 % of cases are caused by both male and female factors [1]. Conventional semen analysis is the first step in the laboratory evaluation of male factor infertility. In approximately 15 % of infertile men however, semen analysis parameters are within reference ranges [2] and therefore may not reveal obvious abnormalities. Additionally, classic sperm parameters such as count, motility, and morphology may not be the best predictors of successful implantation and pregnancy [3].

The transmission of genetic information to the next generation depends on sperm DNA integrity [4, 5]. Several etiological factors have been implicated in the impairment of sperm DNA integrity. Among such factors, cigarette smoking,

irradiation, chemotherapy, leukocytospermia, varicocele, cancer, obesity, and advanced age have been shown to compromise sperm DNA quality [1, 6–10].

Reduced fertility rates have been reported in cases of high sperm DNA damage [11]. Impaired embryo development [12], compromised integrity of the embryonic genome [13], and increased rates of miscarriage [14] have also been associated with high sperm DNA damage [15]. In this context, the evaluation of sperm DNA fragmentation (SDF) would be ideal, especially in cases of unexplained infertility, repeated ART failure, and recurrent miscarriage, as suggested by several investigators [16, 17].

Several tests have been introduced to measure the sperm DNA damage [1, 18–25]. The most commonly used tests are the sperm chromatin structure assay (SCSA), sperm chromatin dispersion test (SCD), and terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) [14, 26]. The TUNEL assay is advantageous because it can measure “true” single and double-stranded DNA fragmentation by directly incorporating modified nucleotides into the site of damage. A few studies have suggested that TUNEL is better at predicting the potential of embryo implantation than the other tests [14, 16]. Unlike other sperm DNA fragmentation (SDF) testing, namely, SCSA, TUNEL can be performed in house with no need to ship semen samples to a central laboratory for analysis [27]. Therefore, the turnaround time of TUNEL testing is shorter, allowing for quicker clinical decisions.

Despite these advantages, there have been difficulties in standardizing and validating the TUNEL assay [28]. Many factors involved in the processing, fixation, and permeabilization of the specimen may affect results, thus limiting its widespread clinical use [29]. Additionally, the reference values of SDF assessed by TUNEL assay have not been clearly established [28].

The goals of our study were to (1) provide a detailed TUNEL protocol for the assessment of SDF using a new bench top flow cytometer that can serve as a reference protocol for laboratories conducting SDF testing, (2) establish reference values for SDF in semen, and (3) and assess the sensitivity, specificity, and distribution of SDF according to reference values in both infertile men and controls with proven and unproven fertility.

Materials and methods

Subjects

The study was approved by the Institutional Review Board (IRB) of Cleveland Clinic. Semen samples were used from 95 controls with proven and unproven fertility and 261 infertile men referred to our andrology lab from June 2014 through April 2015. The infertile men attending our male infertility clinic had confirmed male factor infertility. All patients were evaluated by a male infertility specialist. All infertile patients were seeking treatment for male factor infertility (idiopathic infertility,

varicocele, infection, and other known etiologies). Patients with infection, azoospermia, severe oligozoospermia, or retrograde ejaculation were not included in the study. None of these patients were obese or had a history of chemotherapy or radiation.

The female partners of these patients underwent a complete gynecological investigation and were found to be healthy.

The controls consisted of healthy male volunteers with normal semen analysis according to the WHO fifth edition guidelines [30]. Our control group consisted of men with proven and unproven fertility.

Sperm DNA fragmentation analysis

Semen samples were tested for sperm DNA fragmentation by TUNEL assay using Apo-Direct kit (BD Pharmingen, CA, USA) through Accuri C6 flow cytometer (BD Biosciences, MI, USA). Each sample was run in duplicate along with negative and positive controls. Samples were run through a flow cytometer, and cells positive for TUNEL were defined as those containing fragmented DNA. Results were expressed as the percentage of sperm with DNA fragmentation (% DNA fragmentation) using the flow cytometer software. A minimum of 10,000 events were recorded. The step-by-step approach to measurement of sperm DNA fragmentation is described below.

TUNEL assay principle

TUNEL utilizes a template-independent DNA polymerase called terminal deoxynucleotidyl transferase (TdT) that non-preferentially adds deoxyribonucleotides to 3' hydroxyl (OH) single and double-stranded DNA. dUTP is the substrate that is added by the TdT enzyme to the free 3'-OH break-ends of DNA (Fig. 1).

Two laser detectors were used: FL1 (488) with a standard 533/30 nm Band Pass (BP) that detects green fluorescence fluorescein isothiocyanate (FITC) and FL2 with standard 675/25 nm BP that detects red or propidium iodide (PI) fluorochrome.

I Specimen collection

Samples were collected after 2–3 days of ejaculatory abstinence. The name of the patient, abstinence time, date, time, and place of collection were recorded on the form accompanying each semen analysis.

II Reagents

A APO-DIRECT™ Kit containing the following:

- (a) PI/RNase staining buffer (4 °C)
- (b) Rinsing buffer (4 °C)
- (c) Wash buffer (4 °C)
- (d) Reaction buffer (−20 °C)

Fig. 1 Schematic of the DNA staining procedure using the TUNEL assay

(e) FITC-dUTP (−20 °C)

(f) TdT enzyme (−20 °C)

(g) Negative kit control (−20 °C)

(h) Positive kit control (−20 °C)

B Serological pipettes (2 and 5 mL)

C Eppendorf pipette and tips (20, 100, and 1000 µL)

D MicroCell counting chamber

E Paraformaldehyde (3.7 % in PBS at 4 °C)

F Microfuge Eppendorf tubes

G Ethanol (70 % at 4 °C)

H Flow cytometer

I Instrument Quality Control (8 and 6-Peak Validation Beads)

III Sperm preparation for TUNEL assay

A Following complete liquefaction, semen specimens were evaluated for volume, round cell concentration, sperm concentration, total sperm count, motility, and morphology.

B Using a cryomarker, 5-mL (12 mm×75 mm) tubes were labeled with the specimen information, i.e., TUNEL, patient name, medical record number, and date.

C An aliquot containing 5×10^6 /mL sperm (control and patients) was pipetted into each tube. The tubes were centrifuged at 300g for 7 min, and the seminal plasma was removed.

D 1.0 mL of paraformaldehyde (3.7 %) was added to each tube and vortexed. The samples were batched and stored at 4 °C until use.

E Paraformaldehyde was removed by centrifuging the samples at 300g for 7 min. Supernatants were discarded and the pellets resuspended with 1 mL of ice-cold ethanol (70 % vol./vol.). The tubes were kept at −20 °C for at least 30 min.

IV Staining method

Preparation of assay kit controls and sperm samples (control and infertile patients)

A. Negative and positive assay control vials provided in the kit were brought to room temperature.

B. 2 mL of both negative and positive assay controls were aliquoted in duplicate.

C. Negative and positive assay control and sperm samples were centrifuged at 300g for 7 min. Supernatant was carefully removed by aspiration without disturbing the cell pellet.

D. 1.0 mL of “Wash Buffer” was added to each tube, vortexed, and centrifuged. Clear supernatant was discarded. This step was repeated.

E. The tubes were numbered consecutively.

F. Based on the number of tubes to be run plus additional five to seven tubes, the staining solution was prepared by mixing the reagents provided in the assay kit.

G. Reaction buffer was taken out from 4 °C and TdT and FITC-dUTP from −20 °C and placed at room temperature for 15 min.

H. The staining solution was prepared according to the number of assays to be run by adding each reagent following the sequence provided in Table 1.

I. 50 µL of the staining solution was added to all the tubes.

J. The tubes were covered with aluminum foil and incubated for 60 min at 37 °C. At the end of the incubation time, 1.0 mL of “Rinse buffer” was added to each tube and the mixture centrifuged at 300g for 7 min. The supernatant was discarded, and this step was repeated.

Table 1 Preparation of the staining solution for TUNEL assay

Staining solution	1 assay (µL)	6 assays (µL)	12 assays (µL)
Reaction buffer	10.00	60.00	120.00
TdT enzyme	0.75	4.50	9.00
FITC-dUTP	8.00	48.00	96.00
Distilled H ₂ O	32.25	193.50	387.00
Total volume	51.00	306.00	612.00

- K. 0.5 mL of the PI/RNase staining buffer was added to each tube, and the tubes incubated in the dark for 30 min at room temperature.
- L. The tubes were analyzed for DNA fragmentation using the BD Accuri C6 flow cytometer (Fig. 2).

- Note 1 The preparation of the staining solution and all subsequent steps were performed in the dark.
- Note 2 Negative and positive controls were included in each run. Negative controls contained all the above reagents except TdT. Positive control spermatozoa were treated with H₂O₂ (0.02 % for 50 min), permeabilized and processed similar to other samples before the stain was added.
- Note 3 The staining solution remains active for approximately 24 h at 4 °C.
- Note 4 The cells must be analyzed within 3 h of staining. Cells may begin to deteriorate if left overnight without analysis.

V Running assay kit controls

- Note 1 Assay kit controls were run under the “collect” tab.

All data was saved in the following format:
 “Assay Kit Controls-DATE-TECH INITIALS”
 The steps were as below.

- A Double-click “Assay Kit Control Template” on the computer drive.
- B Select well “A1” (move to adjacent cell if occupied).
- C In field “A01” type “Assay kit negative-date-tech initials.”
- D Set the run parameters as follows:
 1. “Run with limits”: 10,000 events
 2. “Fluidics” speed: slow medium.
 3. Threshold: 80,000 on (Forward Scatter-Height; FSC-H)
- E Remove tube of filtered, deionized water from the Sample Introduction Probe (SIP) (Fig. 2).

- F Vortex and place negative control on the SIP.
- G Click “RUN” button.
- H Run will finish after collecting 10,000 events.
- I Data will populate in plots 1 and 2.

Note: Plot 1: FL2-area on X-axis, FL2-height on Y-axis.
 Plot 2: FL1-height on X-axis, count on Y-axis.

- J Select well “A2” and repeat steps C-I.
- K Remove negative kit control from SIP/stage.
- L Clean SIP with lint-free wipe.
- M Insert positive control tube on SIP.
- N Click well “A3.”
- O Name “A03” as “kit positive-date-tech initials.”
- P Repeat steps D-I.
- Q Select well “A4” and repeat steps for positive control run (steps O & P).

VI Data acquisition Assay kit controls

- A. Under the collect tab, click on well A1 for the first negative kit control.
- B. Observe the graph for the negative control.
- C. The assay kit control template automatically separates the negative (left side of graph) and positive (right side of graph) regions of the graph with a vertical marker. The right side of the vertical marker represents the percentage of FITC-positive (DNA fragmented) cells.
- D. Record the result in the following template: kit control result folder
- E. Click on well A2 and follow steps B-D for the second negative kit control.
- F. Click on well A3 for the first positive kit control.
- G. Observe the graph for the positive kit control.
- H. Follow steps C-D for the positive kit control.
- I. Click on well A4 for the second positive kit control and repeat steps C-D.

VII Running semen samples

Note: Semen samples are run under the “collect” tab,

Fig. 2 Set up of the BD Accuri C6 bench top flow cytometer

utilizing patient/control template created on the computer.

All data should be saved in this folder with the following format: “TUNEL-DATE-TECH INITIALS”

- A. Double-click on the “Assay Template.”
- B. Wait for the software to load.
- C. Check each well to ensure that it is blank and no data exists.
- D. If necessary, click “delete events” for any wells which contain data.
- E. Select well “A5.”
- F. Begin with first sperm sample (tube #5; sample).
- G. Remove deionized water from the SIP.
- H. Vortex the tube (test sample) and place on the SIP.
- I. In the name field, name the field as “TUNEL-SAMPLE#-DATE-TECH INITIALS.”
- J. Set the run parameters as follows:
 1. “Run with limits”: 10,000 events; ungated sample; 2 min.
 2. “Fluidics” speed: slow (flow rate 14 $\mu\text{L}/\text{min}$)
 3. Threshold: 80,000 on FSC-H
- K. Click the “RUN” button to start the collection.
- L. After 10,000 events, the run will finish.
- M. Remove tube from the SIP and clean the SIP with a lint-free wipe.
- N. Vortex and place the subsequent tube on the SIP.
- O. Select the next well (A6, and so on) for the new sample.
- P. Repeat steps I–O until all samples have been processed.

VIII Data acquisition for test samples

- A. Under the collect tab, click on well A5 for the first test sample.
- B. Observe the upper-right graph for the sample.
- C. The assay template automatically separates the negative (left side of graph) and positive (right side of graph) regions of the graph with a vertical marker. Observe only the positive (FITC+) value.
- D. Record the result in the following template: TUNEL assay Results.xlsx folder.
- E. Repeat these steps A–D for each subsequent well A6, A7, A8, etc.

IX Reporting the percentage of TUNEL-positive cells

The percentage of TUNEL-positive cells in a given sample was noted from the percentage of cells to the right of the vertical marker in the FL1-A vs. count plot.

X Method for calculating test reproducibility

Two experienced observers independently tested 10 samples for interobserver and intraobserver variability.

The interobserver variability was calculated by analyzing the readings obtained by two observers while the intraobserver variability was analyzed by calculating the difference between two readings obtained by the same observer.

XI Instrument quality control

Quality control was done each time the TUNEL assay was run as per the manufacturer’s instructions. It consisted of testing the 8-peak validation beads and the 6-peak validation beads. The 8-peak validation beads are used for monitoring the FL1, FL2, and FL3 (670 Long Pass filter) channels and are excited by a blue laser. The beads are 3.2 μm particles that emit light at eight different wavelengths. The 6-peak beads are used for monitoring the FL4 channel (640 laser; 675/ 25BP) and emit six different wavelengths.

The performance validation process consists of measuring the coefficient of variation (CVs) of 8- and 6-peak validation readings. After all the samples are analyzed, shut down the instrument by following these steps:

- A. Place a tube with 2 mL of bleach (diluted decontamination solution) on the SIP.
- B. Select an empty “data well” in the collect tab.
- C. Set a time limit of “2 min” and set fluidics speed to “fast.”
- D. Click the “RUN” button.
- E. Once the run is finished, remove the tube from the SIP.
- F. Place a tube with 0.22 μm -filtered DI water on the SIP and select another empty “data well” in the software.
- G. Repeat steps #3 and #4, above.
- H. When the run is finished, leave the tube on the SIP.
- I. Press the power button to start the shutdown cycle.
- J. The cycle will take 15 min to complete at which time the cytometer should automatically shut down.

Statistical analysis

The Wilcoxon rank-sum test was used for group comparison with respect to quantitative variables and chi-square test to categorical variables. For intra- and interobserver variability, absolute and percent differences between individual measurement and final recorded value with the cumulative percentages were calculated. The difference in the distribution of DNA fragmentation levels between the two groups (control and patient) was assessed. A receiver operating characteristic (ROC) curve was used to assess the ability of DNA fragmentation as a means of discriminating patients with controls. A cutoff value that maximized the sum of estimated sensitivity and specificity was chosen as the reference value (SPSS, version 20, IL).

A p value of <0.05 was considered statistically significant. A test with high sensitivity has a low type II error rate. On a ROC curve, maximizing sensitivity corresponds to a large Y value on the ROC curve. A test with high specificity has a low type I error rate. On a ROC curve, maximizing specificity corresponds to a small X value on the ROC curve. In the ROC curve, the accuracy of the test is area under the curve.

Results

ROC curve analysis

Test sensitivity, specificity, positive and negative predictive values, and TUNEL cutoff values were calculated using the ROC curve (Fig. 3a). Different cutoff values in respect to the corresponding sensitivity, specificity, unadjusted positive and negative predictive values (PPV, NPV) are shown in Table 2. The SDF was significantly higher (<0.001) in the infertile men than in the controls. Sperm DNA fragmentation at a cutoff value of 16.8 % was associated with the best specificity of 91.6 % and a positive predictive value of 91 % to discriminate infertile men from the controls. At this given cutoff, 91.6 % of the control population was below this level compared to 67.4 % of infertile men. The upper limit of SDF in controls was 19.6 % compared to 68.9 % in infertile men.

Inter- and intragroup distribution of TUNEL-positive samples

Distribution of the percentage DNA fragmentation (TUNEL-positive) between the controls and infertile men

(intergroup) is given in Fig. 3b. The percent population falling in different range of sperm DNA fragmentation (intragroup) is given in Table 3. Nearly 12 % of the infertile men had a SDF >20.1 % whereas all controls were below this value.

Interobserver variation

The inter- and intraobserver calculations were carried out in a blind fashion by two experienced observers. More than 40 readings were conducted to calculate the differences within and between the observers. The differences were within the acceptable range.

We calculated absolute as well as percent differences relative to the assigned TUNEL values. The average TUNEL measurement from a given observer was within the two observers' average measurements with an absolute difference of 1.73 % or less in 80 % of cases (Fig. 4a).

The average TUNEL measurement from a given observer was within the two observers' average measurements by a percent difference of 6.68 % or less in 80 % of cases (Fig. 4b).

Intraobserver variation

Both absolute and percent differences were calculated to analyze the intraobserver variability. A single TUNEL measurement from a given observer was within that observer's average measurement by an absolute difference of 3 % or less in 90 % of cases (Fig. 4c). A single TUNEL measurement from a given observer was within that observer's average measurement by a percent difference of 9.68 % or less in 80 % of cases (Fig. 4d).

Fig. 3 **a** Receiver operator characteristic (ROC) curve showing TUNEL assay cutoff and the area under the curve. Values within the parentheses represent the 95 % confidence interval. **b** Distribution of TUNEL test values between controls and infertile men

Table 2 Sensitivity, specificity and unadjusted negative and positive predictive values for various TUNEL assay cutoff levels

Cutoff	Sensitivity	Specificity	PPV	NPV
16.0	34.0	85.2	86.4	32.0
16.1	33.7	87.3	88.0	32.4
16.2	33.7	87.3	88.0	32.4
16.3	33.3	88.4	88.7	32.5
16.3	33.3	88.4	88.7	32.5
16.4	32.9	89.4	89.5	32.6
16.5	32.9	90.5	90.5	32.9
16.6	32.5	90.5	90.4	32.8
16.8	32.5	91.6	91.4	33.1
16.9	32.1	91.6	91.3	32.9
16.9	32.1	91.6	91.3	32.9
17.0	31.4	91.6	91.1	32.7
17.0	31.4	91.6	91.1	32.7
17.0	30.2	91.6	90.8	32.3
17.1	30.2	91.6	90.8	32.3
17.2	29.1	91.6	90.4	31.9
17.2	29.1	91.6	90.4	31.9
17.3	28.3	91.6	90.2	31.8
17.5	27.9	91.6	90.1	31.6
17.6	27.5	91.6	90.0	31.5
17.8	27.2	91.6	89.8	31.4
17.8	27.2	91.6	89.8	31.4
17.9	26.4	91.6	89.6	31.1

Discussion

Our aims were to standardize the TUNEL assay using a new bench flow cytometer and conduct a study on a large cohort of patients as well as controls with proven and unproven fertility. We calculated the sensitivity, specificity, accuracy, and predictive values of different cutoff points to determine the optimum reference value of TUNEL assay for discriminating infertile men from controls with proven and unproven fertility. We used receiver operator characteristic (ROC) curve model to calculate the cutoff, sensitivity, and specificity of the test. At a cutoff point of 16.8 %, the test yielded the highest specificity (91.6 %) and positive predictive value (91 %). Of the 95 control samples, 85 (91.6 %) were below this cutoff value while 176 (67.4 %) of

the 261 patients were below this cutoff value ($p<0.001$). On the other hand, only 8/95 (8.4 %) control samples showed DNA fragmentation higher than this cutoff value compared to 85/261 (32.6 %; $p<0.001$) in the infertile group.

In contrast, both sensitivity and accuracy of TUNEL assay were low, thus indicating the assay has limited value in correctly identifying infertile men in an unselected male population. This shortcoming of the TUNEL assay has been demonstrated previously and is related to the difficulty in sperm chromatin permeabilization due to its highly dense compaction and tightly packed protamines, which may prevent TdT from directly interacting with DNA strand breaks [31].

For a diagnostic test to be considered valid for clinical use, it should combine high sensitivity, specificity, accuracy, and positive and negative predictive values. Provided a given test has high sensitivity, it will correctly identify subjects with the condition of interest. In this case, the number of false-negatives will be low, which means that subjects who tested negative are unlikely to harbor the condition. In contrast, highly specific tests correctly identify subjects who do not have the condition of interest. In this case, the number of false-positives will be low, which means that patients who tested positive are unlikely not to have the condition of interest.

However, the most important characteristic of a screening test is its predictive value. Although predictive values are influenced by sensitivity and specificity, they depend on the prevalence of the condition of interest in the general population. For instance, if the prevalence of the condition is low (e.g., infertility in young adults), the PPV (the probability that a man who tests positive truly has infertility) will be low despite a high sensitivity and specificity. If the prevalence of a given condition is high (e.g., infertility in patients with clinical varicocele and oligozoospermia), the PPV will be high if a highly specific test cutoff point is chosen. Therefore, it is obvious that SDF testing will be clinically useful in identifying infertility in men at risk for infertility.

Our test methods had a very high specificity (91.6 %) and positive predictive value (90 %) at a cutoff point of 16.8 %. The high specificity of the TUNEL assay, as described in this study, will be useful in correctly identifying infertile patients who do not have sperm DNA fragmentation as a contributory factor for their infertility condition. And due to its high positive predictive value, the assay will be able to confirm that a man who

Table 3 Intragroup distribution of sperm DNA fragmentation in overall, controls and patient samples

DNA fragmentation (%)	Overall ($n=356$)	Controls ($n=95$)	Patients ($n=261$)
0-10	152 (42.7 %)	40 (42.1 %)	112 (42.9 %)
10.1-20	152 (42.7 %)	55 (57.9 %)	97 (37.2 %)
20.1-30	31 (8.7 %)	0	31 (11.9 %)
30.1-40	15 (4.2 %)	0	15 (5.7 %)
>40	6 (1.7 %)	0	6 (2.3 %)

Fig. 4 Panel showing interobserver variability for **a** absolute, **b** percent difference, and intraobserver variability for **c** absolute **d** percent difference between individual measurements and final values

tests positive is likely to be infertile due to elevated sperm DNA fragmentation. In a recent study, a similar specificity (91 %) of TUNEL assay was reported [32]. The calculated cutoff value would be ideal as any value above this threshold will be strongly associated with infertility.

Male factor infertility is believed to be multifactorial yet poorly understood, and approximately half of the cases are deemed unexplained or idiopathic [33]. A number of studies have investigated the role sperm DNA fragmentation plays in the pathophysiology and etiology of male infertility. Several studies show that sperm DNA fragmentation is higher in infertile men with poor semen parameters [34, 35]. Reduced pregnancy rates have been reported in couples undergoing intrauterine insemination (IUI) and in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) with high sperm DNA fragmentation as assessed by the TUNEL assay [24]. In one study involving couples undergoing IVF and ICSI, no pregnancy was reported in cases of sperm DNA fragmentation greater than 20 % [36]. Likewise, a threshold of DNA fragmentation of 24.3 % measured by TUNEL in ICSI cycles was

associated with low chances of achieving a successful pregnancy [37]. In cases of intrauterine insemination (IUI), no pregnancy was reported when the sperm DNA fragmentation analyzed by TUNEL assay was >12 % [38]. In contrast, the literature is scarce in studies investigating the SDF in men consulting for unexplained infertility or with normal sperm parameters. [26, 39–41]. Therefore, the clinical utility of the SDF test in such men deserves further investigation.

A useful approach to describe interobserver and intraobserver variability is to determine the extent to which an individual measurement differs from the designated final measurement, and results can be expressed as absolute or percent differences. In the interobserver variability, we found that the absolute difference between an observer's assigned value and the calculated average from two observers was 1.73 % in 80 % of the cases and less than 4 % in 100 % cases. As far as percent differences are concerned, in 80 % of the cases, the relative difference between an individual observers' designated value and the calculated average from two observers was less or equal to 6.68 % and was less than 14 % in 100 % of the cases. For intraobserver

variability, in 90 % of the measurements, the absolute difference was <3 % of the designated value. Similarly, 80 % of the TUNEL measurements showed <9.68 % difference in the designated value. These results confirm the consistency and reproducibility of the tests for inter- and intraobserver variability.

Two previous studies have assessed TUNEL cutoff values to discriminate between fertile and infertile men [28, 42]. In a previous study from our group, TUNEL references values were calculated to be 19.2 % [28]. Sergerie et al. [42] reported that a TUNEL value of 20 % was associated with infertility. However, both studies included smaller cohorts than our present report. One limitation of our present study was that our control group consisted of a mixed cohort of men with proven and unproven fertility. Using only men with proven fertility will further increase the specificity and sensitivity of this assay.

We standardized the new Accuri C6 by analyzing the samples on the Accuri bench top flow cytometer and FACSscan flow cytometer (both from BD Biosciences). We found the results from both machines to be very similar (unpublished work).

In this study, we have demonstrated for the first time the novelty of using a compact, cost-effective, user-friendly bench top Accuri C6 flow cytometer, which does not involve the use of software such as FlowJo to further analyze the data for the assessment of SDF by TUNEL. This bench top contains the easy-to-use software Zoom tool, which allows precise control when setting gates.

Conclusions

We report a detailed method for measuring sperm DNA fragmentation by TUNEL assay using a bench top flow cytometer and determined test cutoff points in a large population of infertile men. At a cutoff point of 16.8 %, the test was both highly specific and associated with a high positive predictive value. The high specificity of the TUNEL assay, as described in this study, will be useful in correctly identifying infertile patients who do not have sperm DNA fragmentation as a contributory factor. Due to its high positive predictive value, the assay will be able to confirm that a man who tests positive is likely to be infertile due to elevated sperm DNA fragmentation. This protocol will benefit fertility testing laboratories interested in introducing the TUNEL assay and establish their own reference values.

Acknowledgment The study was supported by funds from the American Center for Reproductive Medicine. Dr. Gulfam Ahmad was supported by the Fulbright fellowship. The authors are grateful to the Andrology Center technologists for scheduling the study subjects and to Jeff Hammel, Biostatistician, for his contribution to the data analysis.

Compliance with ethical standards The study was approved by the Institutional Review Board (IRB) of Cleveland Clinic

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