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Evaluation of sperm genomic integrity of normozoospermic men: a prospective study

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Abstract: The objective of our study was to evaluate the incidence of spermatozoa with nuclear DNA strand breaks in patients with normal routine sperm parameters (26 subjects). Sperm DNA fragmentation was measured using TUNEL test assessed in flow cytometer. Variable percentages of sperm with damaged DNA (9.42±7.68%; range: 2-36) were found. Two categories of patients were distinguished: (1) patients (8 out of 26 subjects) with ≤4% of TUNEL-positive sperm and (2) patients (18 out of 26 subjects) with >4% of TUNEL-positive sperm. A significantly lower percentage of normal sperm forms was found in patients with >4% of TUNEL-positive sperm than in patients with ≤4% of TUNEL-positive sperm. Moreover, a significant negative correlation (r_s=-0.50) was noted only between a proportion of normal sperm forms and a proportion of TUNEL-positive spermatozoa. In electron microscope, a large number of spermatozoa with immature chromatin was observed more frequently in subjects with >4% of TUNEL-positive cells (11 out of 18 subjects). Our results suggest that in some patients with normal routine sperm parameters, DNA fragmentation may be associated with poor sperm morphology. The diminished sperm genomic integrity may result from molecular disturbances in nuclear remodeling process during spermiogenesis. TUNEL assay is a screening tool that may help to discriminate between fertile and infertile men and may help to predict successful *in vitro* fertilization. (www.cm-uj.krakow.pl/FHC)

Key words: Spermatozoa - TUNEL - Flow cytometry - Ultrastructure - Normozoospermia

Introduction

The development and application of Assisted Reproductive Techniques (ART), particularly Intracytoplasmic Sperm Injection (ICSI), require molecular diagnostics of sperm nuclear DNA. The ART may produce a risk of injecting a spermatozoon with damaged DNA into the ovum. The great number of ejaculated spermatozoa with diminished integrity of nuclear DNA may result in a failure of either fertilization or pregnancy and it may even stop development of embryo and finally contribute to an early abortion. The quality of sperm chromatin plays an important role in the interaction of sperm-oocyte, implantation and division of embryo blastomeres [2, 3, 6, 8, 9].

Men suffering from unexplained infertility (with normal routine sperm parameters) have significantly more DNA strand breaks in sperm nuclear DNA compared to the spermatozoa of fertile men. In the former case, the percentage of sperm with fragmented nuclear DNA may be comparable to the percentage of sperm with damaged DNA in patients with abnormal routine sperm parameters [7-9, 16]. Host *et al.* [7-9] reported that the men with unexplained infertility suffered from the same malfunction as men with oligozoospermia. The mechanism responsible for sperm DNA fragmentation in patients with normal routine sperm parameters is still unclear and needs further study.

The objective of our study was to evaluate the incidence of spermatozoa with nuclear DNA strand breaks in normozoospermic patients who were partners in infertile couples. We propose to introduce electron microscopy of sperm chromatin to connect its molecular disturbances with ultrastructure defects. Our research is a probe attempting to specify reasons for decreased sperm genomic integrity which can be reflected in an elevated number of sperm with nuclear DNA strand breaks in idiopathic infertility.

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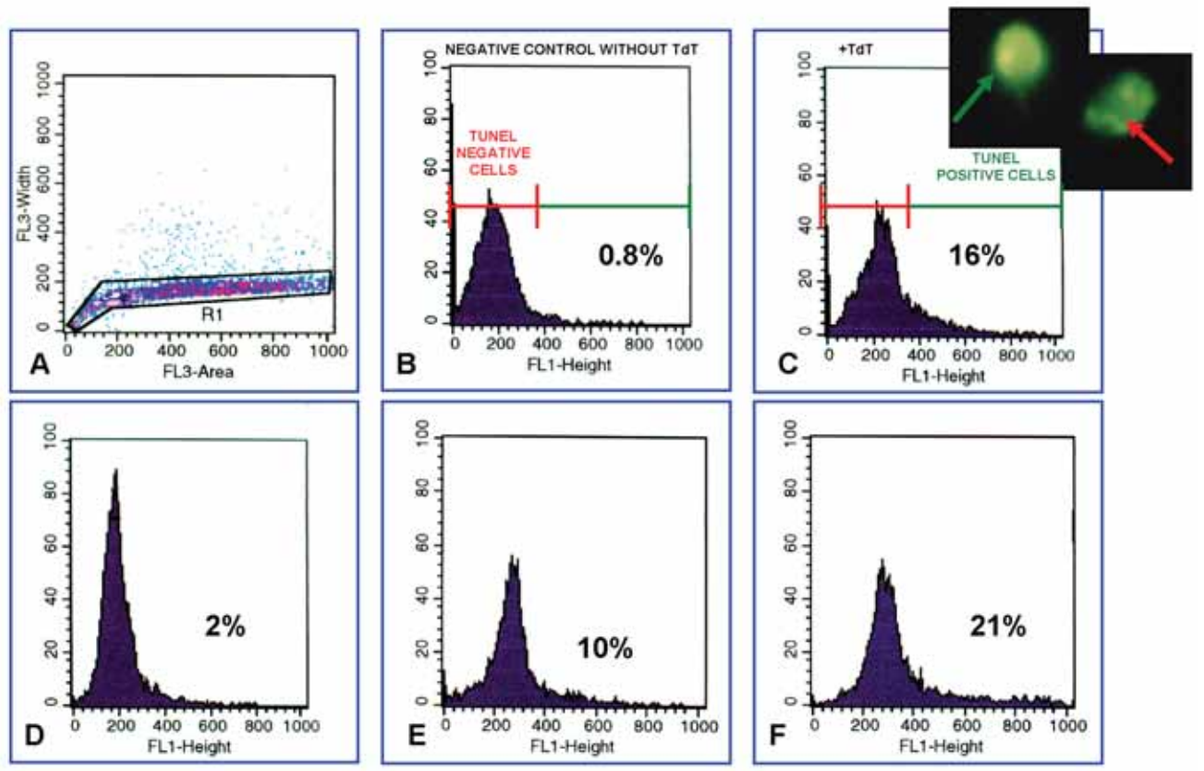

Fig. 1. Cytofluorometric analysis of TUNEL-positive spermatozoa obtained from normozoospermic patients. **A:** Density plot (FL3A versus FL3W) of PI stained spermatozoa. The cells gated in R1 region were analysed, debris and aggregates were excluded from the analysis, FL3 channel - the intensity of fluorescence in the red spectrum; **B:** frequency distribution histogram of TUNEL-negative spermatozoa; **C-E:** examples of frequency distribution histograms of TUNEL-positive spermatozoa, percentage of spermatozoa with fragmented DNA was estimated on the basis of spectrum for negative control which allows to set a region including all TUNEL-negative cells; Inserts: TUNEL-positive spermatozoa detected in fluorescence microscopy, spermatozoa with DNA fragmentation reveal intense bright green nuclear fluorescence in morphologically normal (green arrow) and abnormal (red arrow) head (in panel C), ×2800; X-axis: FL1 channel - the intensity of fluorescence in the green spectrum, Y-axis: depicts the frequency in terms of the number of cells; the percentage of TUNEL-positive cells are indicated; the fluorescence intensity scale is expressed as "channel number" (0-1000)

Materials and methods

Studies were performed on ejaculated sperm from normozoospermic patients (n=26) of ART Laboratory in the Reproduction and Gynecology Clinic of the Pomeranian Medical University, Szczecin, Poland. This study was approved by the Ethical Committee of the Pomeranian Medical University. The routine parameters of semen were determined by standard methods recommended by WHO . The cutoff for normal morphology was set at ≥15% [17] (Table 1).

The liquefied semen was centrifuged for 15 min at 400 × g at room temperature. The sperm pellet was washed twice in PBS (phosphate buffered saline, Sigma-Aldrich Chemie GmbH, Germany). Finally, the sperm pellet was resuspended in 1 mL PBS and was used for cytofluorometric and electron microscopic studies [14]. The sperm DNA strand breaks were identified with the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay. The TUNEL test was performed using *in situ* Cell Death Detection Kit (APO-BRDU a complete Kit for measuring apoptosis by dual color flow cytometry, BioSource International, Inc.) according to the manufacturer's recommended protocol (indirect method). The negative control was obtained omitting TdT from the reaction mixture. All cells were treated with propidium iodide/RNase solution. The TUNEL-positive cells (FITC-antiBrdU mAb labeled cells with DNA strand breaks) were evaluated in flow cytometer equipped with a 488 nm argon laser (FACSCalibur, Becton Dickinson, San Jose, CA, USA). Ten thousand events were measured for each sample at a flow rate of 100-200 events/s. Green fluorescence (480-530 nm) and red fluorescence (580-630 nm) were measured simultaneously in FL1 and FL3 channel respectively. The fluorescence data were obtained at fixed gain setting in logarithmic (FL1) and in linear (FL3) mode. Data were analyzed using Cell Quest software.TUNEL-positive cells were also detected using a fluorescence microscope (Axioskop, Carl Zeiss, Jena GmbH, Germany).

The preparation of spermatozoa for electron microscopy was performed according to a conventional method described by Piasecka and Kawiak [14]. Ultrathin sections of samples were examined in JEM-1200 EX (JEOL Ltd, Tokyo, Japan) transmission electron microscope at 80 kV.

All results were expressed as mean±SD and median. The conformity of variables with the normal distribution was examined using Shapiro-Wilk W-test. The Mann-Whitney U-test was employed to determine statistical differences between the groups. The Spearman rank correlation (r_s) test was used to evaluate the relationships between semen analysis parameters and cytofluorometric examination. Statistical significance was assumed at $p \leq 0.05$. All computations were performed using the Statistica v. 6.0 software.

Table 1. Results of conventional and cytofluorometric analysis of spermatozoa from normozoospermic patients

Patients	Parameter	Routine sperm characteristics					TUNEL-positive spermatozoa (%)
		Concentration (mln/mL)	Motility (% of cells)			Morphology (% of normal sperm forms)	
			Rapid + slow	Nonprogressive	Immotile		
Total (n=26)	Mean±SD	83.38±36.63	62.92±8.48	9.62±7.21	27.46±9.84	19.88±4.51	9.42±7.68
	Median	77.5	63	8	28	19	6.5
	Range	26-180	50-78	1-36	12-46	15-30	2-36
	Q_1-Q_3	60-120	57-69	5-12	18-36	16-22	4-13
<4% of TUNEL-positive cells (8 out of 26 subjects)	Mean±SD	82.13±32.89	64.38±8.25	8.63±4.81	27.00±8.60	**23.00±5.01**[a]	3.25±0.89
	Median	68.5	66	9	27.5	21	3.5
	Range	50-130	50-76	3-16	16-40	16-30	2-4
	Q_1-Q_3	55-115	59-69.5	4-12	19-33.5	20-28	2.5-4
>4% of TUNEL-positive cells (18 out of 26 subjects)	Mean±SD	83.94±39.08	62.28±8.73	10.06±8.14	27.67±10.57	**18.50±3.60**[b]	12.17±7.78
	Median	79	60	7.5	29	18	10
	Range	26-180	50-78	1-36	12-46	15-28	5-36
	Q_1-Q_3	60-120	57-69	5-12	17-36	16-20	6-16

Q_1-Q_3 - lower quartile - upper quartile; [ab] - means with different letters are significantly different ($p \leq 0.05$)

Results

The present study showed the presence of variable percentages of TUNEL-positive cells (9.42±7.68%; range: 2-36) in normozoospermic patients. Some of them exhibited a high proportion of sperm with nuclear DNA fragmentation (Fig. 1). Host *et al.* [8, 9] and Benchaib *et al.* [2] suggest that 4% of sperm with strand breaks can be considered as the threshold value of DNA fragmentation that allows to predict a possible conception or pregnancy. Therefore, two categories of patients according to a threshold value of 4% were obtained: (1) patients (8 out of 26 subjects) with ≤4% of TUNEL-positive cells and (2) patients (18 out of 26) with >4% of TUNEL-positive cells. The 18 selected subjects included 10 men with >4%-10%, 6 men with >10-20% and 2 men with >20% of TUNEL-positive cells. No significant differences were found in the concentration and motility of spermatozoa between patients from both groups. However, a significant difference between the groups was discovered in the sperm morphology (Table 1). A significant negative correlation was found between the proportion of sperm showing nuclear DNA strand breaks and proportion of normal sperm forms (Fig. 2).

Spermatozoa with normal integrity of DNA (TUNEL-negative) displayed only background fluorescence, while those with fragmented DNA revealed bright green nuclear fluorescence in the morphologically normal or abnormal heads (Fig. 1C). A large number of spermatozoa with several ultrastructural features indicating an immaturity of chromatin were present, more frequently in patients with >4% of TUNEL positive cells (11 out of 18 subjects) (Fig. 3).

Discussion

The etiology of sperm DNA damage has not been fully elucidated yet. TUNEL-positive spermatozoa can be related to apoptotic [5, 13] or immature cells [12, 15]. Moreover, numerous reports indicate that excessive amounts of seminal reactive oxygen species contribute to the loss of sperm DNA integrity at reduced antioxidant scavenging activities [1, 6, 11]. The used assay is known to detect single and double stranded DNA breaks and may reflect a final degradation phase of programmed and nonprogrammed cell death [5, 11, 18].

Our study shows the presence of variable numbers of TUNEL-positive spermatozoa in normozoospermic patients (9.42±7.68%; range 2-36%). Carrell *et al.* [3] and Zini *et al.* [18] obtained similar results (11.9±1.0; 13.3±2.5%), but other authors found lower (0.3±0.4%; 2.5±1.2%) [5, 7] or higher percentage of sperm with DNA fragmentation (23±2%) [11] in patients with normal semen parameters.

The high percentage of spermatozoa with DNA fragmentation can decrease fertilization and pregnancy rate in patients enrolled in an IVF (*in vitro* fertilization) program [2, 6, 8-11]. Host *et al.* [8, 9] reported a significantly better fertilization rate when sperm sample had ≤4% spermatozoa with DNA strand breaks. In the present study, only 8 out of 26 normozoospermic subjects with ≤4% of TUNEL-positive spermatozoa were found.

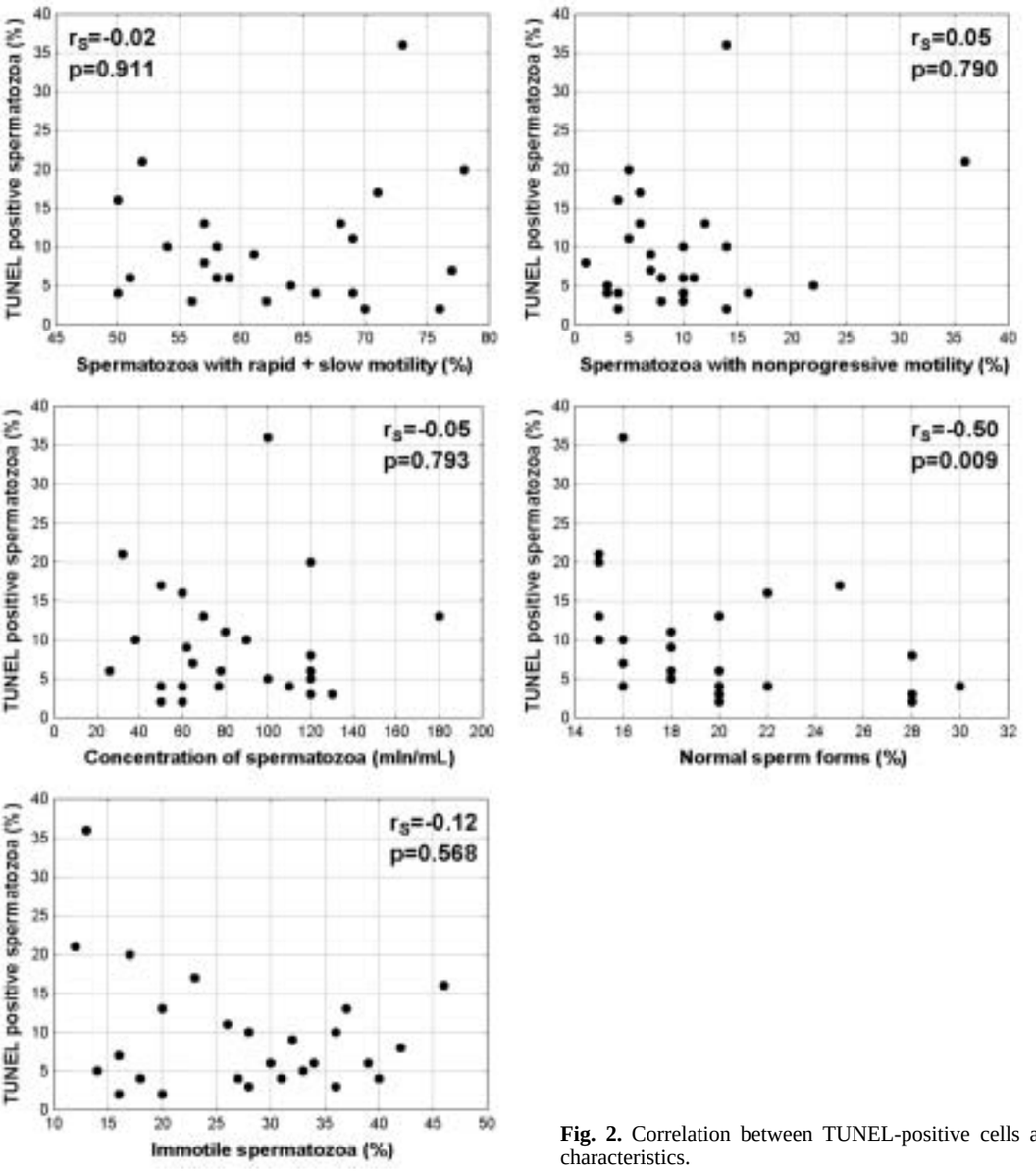

Fig. 2. Correlation between TUNEL-positive cells and routine sperm characteristics.

Similar proportion of patients with ≤4% of spermatozoa with fragmented DNA was noted in patients with abnormal semen parameters: 15 out of 68 subjects (unpublished data). Results suggest that a high proportion of patients (71 out of 94 total subjects - 26 normozoospermic subjects and 68 subjects with abnormal semen parameters) revealed >4% of spermatozoa with fragmented nuclear DNA.

Benchaib *et al.* [2] found that the fertilization rate was significantly higher for DNA fragmentation <10% and no pregnancy was obtained when DNA fragmentation was >20%. Similar findings were obtained by Huang *et al.* [10] who reported that the fertilization rate was affected when sperm DNA fragmentation was >10%. The value of 36.5% of TUNEL-positive sperm was considered as a threshold value for pregnancy rate by Henkel *et al.* [6]. Marchetti *et al.* [11] found a significant negative correlation between percentage of sperm cells with fragmented DNA and fertilization rate, similar observation was reported by other authors [8, 9]. Our studies revealed 10 men with >4-10%, 6 men with >10-20% and 2 men with >20% of TUNEL-positive sperm. In view of the results of other authors [2,10] the chance of fertilization *in vitro* may decrease for patients

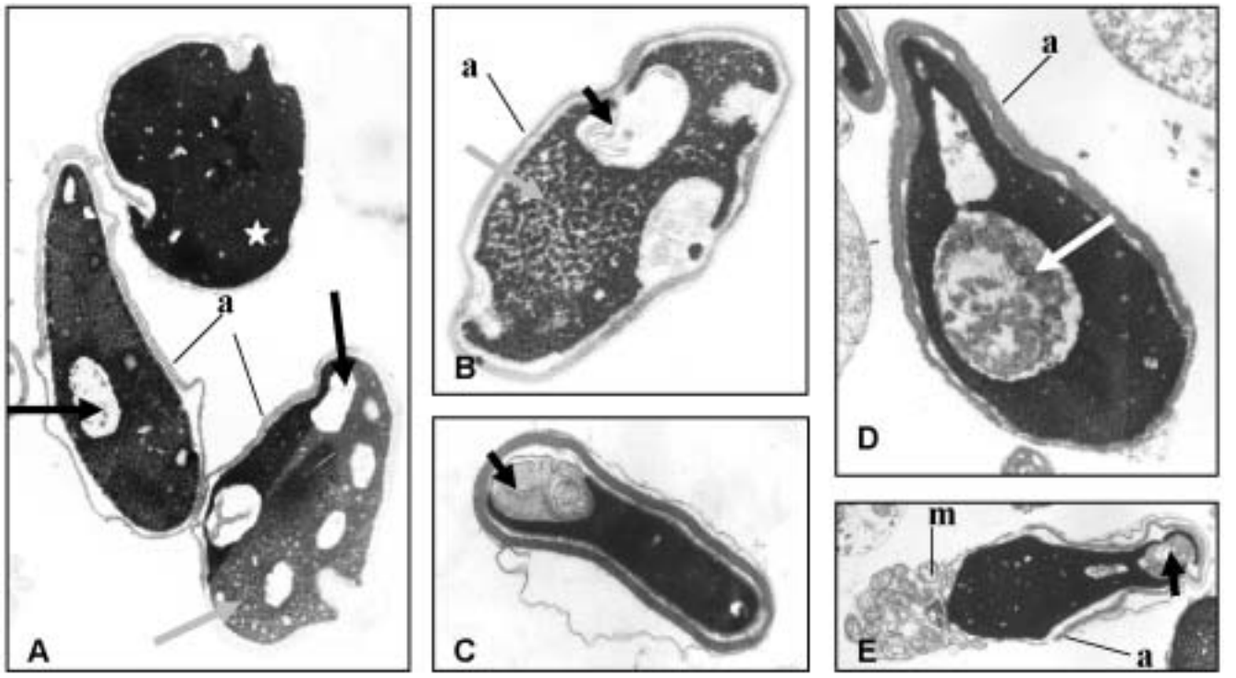

Fig. 3. Ultrastructural features indicating an abnormal packaging of sperm chromatin (immature chromatin) in normozoospermic patients. Vacuoles (large black arrow) with membranes (black arrow) or with granules (white arrow) in sperm chromatin; granular structure and low electron density chromatin (gray arrow); compact chromatin (asterisk); altered nuclear and acrosomal (a) shape (**A-E**) and disorganized mitochondria (m) in the cytoplasmic residue (**E**); A: × 13 000; B: × 26 500; C: × 22 500; D: × 20 000; E: × 9 000.

with >10-20% of TUNEL-positive sperm but pregnancy rate may diminish for patients with >20% of spermatozoa with fragmented nuclear DNA. It should be stressed that standard semen analysis not only fails to discriminate between fertile and infertile men but it also fails to predict successful *in vitro* fertilization. There seems to be a need for the discrimination criteria for spermatozoa with DNA strand breaks.

A significant negative correlation (r_s=-0.50) between DNA fragmentation and percentage of normal sperm forms and a significantly lower percentage of normal sperm forms in patients displaying >4% of TUNEL-positive cells suggest that DNA fragmentation may be associated with poor sperm morphology. The sample with a high percentage of TUNEL-positive sperm cells may exhibit low percentage of normal sperm forms. Our results are consistent with findings of other investigators [6, 12, 15, 18]. Thus it can be concluded that abnormal sperm forms may contain fragmented DNA. However, "normal" spermatozoon can also contain damaged DNA [2]. Other authors do not note any significant relationship between sperm DNA fragmentation and morphology [3, 5, 7]. Apparently the divergent findings may result from examination of different clinical groups of patients and from heterogeneity of spermatozoa. Our results may imply that the value of the percentage of normal sperm forms in normozoospermic patients, especially in unexplained infertility, may have a significant importance in *in vitro* fertilization because decreased percentage of normal sperm forms may be associated with increased percentage of spermatozoa with diminished genomic integrity which in turn may impact interaction of both gametes.

Our flow cytometric and electron microscopic studies suggest that a high percentage of TUNEL-positive spermatozoa in some normozoospermic patients may result from defective chromatin packaging. The compaction of sperm chromatin takes place during the transition from round to elongated spermatids at specific stages of spermiogenesis. Incompletely packed chromatin is an immature chromatin with persistent endogenous nicks which can be an effect of the impaired protamination [15, 18]. The observed ultrastructural defects of sperm chromatin (Fig. 3) seem to reflect molecular disturbances in nuclear remodeling process in patients with normal routine sperm parameters. Therefore, in some examined cases of normozoospermic men TUNEL-positive spermatozoa may be related to immature spermatozoa with reduced genomic integrity.

It should be emphasized that sperm DNA fragmentation not only can decrease fertilizing ability of spermatozoa and impair development of embryo or cause early embryonic death [2, 3, 6, 8, 9, 11], but also produce a risk of genetic disorders and eventually childhood

cancer in the offspring [1]. Therefore, the evaluation of sperm DNA integrity must not be neglected. The tests which display abnormal integrity of sperm nuclear DNA may have a diagnostic and prognostic value and may be considered as additional assays in evaluation of spermatozoa beside a standard semen analysis [4, 11, 16, 18]. TUNEL assay with flow cytometric assessment is an objective, quick and reliable screening method that may help to predict successful *in vitro* fertilization and eventually may help to exclude a patient from an IVF program.

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