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Flow cytometry application in the assessment of sperm DNA integrity of men with asthenozoospermia

Małgorzata Piasecka[1], Dariusz Gączarzewicz[2], Maria Laszczyńska[1], Andrzej Starczewski[3], Agnieszka Brodowska[3]

[1]Laboratory of Embryology and Developmental Biology, [3]Reproduction and Gynecology Department, Pomeranian Medical University, Szczecin, Poland
[2]Department of Animal Reproduction, University of Agriculture, Szczecin, Poland

Abstract: Sperm genomic integrity and ultrastructural features of ejaculated spermatozoa contributing to the assessment of gamete fertility potential in patients with asthenozoospermia are discussed. The proportion of TUNEL-positive cells was significantly higher in the semen of patients with low sperm motility (n=40; $p<0.01$) as compared to men with normal sperm motility (n=54). Sperm DNA fragmentation negatively correlated (n=94) with sperm motility, concentration, and integrity of the sperm plasma membrane (HOS-test). Two categories of patients were distinguished: (1) patients (23 out of 94 subjects) with ≤4% of TUNEL-positive cells and (2) patients (71 subjects) with >4% of TUNEL-positive cells. A significant difference was noted in the sperm motility and HOS-test results between patients from both groups. Large numbers of immature spermatozoa with extensive cytoplasmic retention, ultrastructural chromatin and midpiece abnormalities, and conglomerates containing sperm fragments were present more frequently in the semen of asthenozoospermic subjects with >4% of TUNEL-positive sperm cells. Low sperm motility seems to be accompanied by serious defects of gamete chromatin expressed as diminished sperm genomic integrity and abnormal DNA condensation as well as by defects of sperm midpiece. These abnormalities may reflect developmental failure during the spermatogenic remodeling process. The DNA fragmentation test may be considered as an additional assay for the evaluation of spermatozoa beside standard analysis and taken together with an electron microscopy may help to determine the actual number of "healthy" spermatozoa thereby playing an important role during diagnosis and treatment of male infertility.

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

Introduction

Sperm nuclear DNA integrity is a novel marker of male fertility potential [1-5]. DNA fragmentation frequently affecting abnormal spermatozoa present in large numbers in the semen of subjects with low sperm motility [6-13] may prevent or hinder fertilization and embryo development, and increase the risk of genetic defects [2,4-6,8,10,14-18]. It should be emphasized that detection of DNA damage is often beyond the scope of routine seminological analysis which in many cases is decisive for qualification to assisted fertilization.

Rapid development of molecular diagnostics often based on the binding of fluorescent dyes to sperm chromatin has created the possibility of identifying sperm DNA abnormalities. In turn, precise qualitative and quantitative evaluation of spermatogenic defects visualized by fluorochromes is usually performed with the flow cytometer. Cytofluorometric studies of semen allow to analyze several thousand cells per sample (*e.g.* 10 or 20 thousand) in a short time. Therefore, flow cytometry as a screening test for sperm alternations is extensively used and is of considerable predictive value regarding sperm fertilizing ability [10-12, 19-24].

The purpose of this work was to determine the number of ejaculated spermatozoa with nuclear DNA strand breaks and to find specific sperm ultrastructural features in asthenozoospermia. Furthermore, we decided to establish the relationship between sperm genomic integrity and routine sperm characteristics.

Correspondence: .M. Piasecka, Laboratory of Histology and Reproductive Biology, Pomeranian Medical University, Żolnierska Str. 48, 71-210 Szczecin, Poland;
tel.: (+4891) 4800907, fax.: (+4891) 4800905,
e-mail: mpiasecka@ipartner.com.pl

Materials and methods

Subjects and semen preparation. Semen obtained from 94 patients of the ART (assisted reproductive techniques) Laboratory at the Department of Reproduction and Gynecology of the

Pomeranian Medical University was analyzed according to standard criteria recommended by WHO [25]. The men were partners in couples treated for infertility. This study was approved by the Ethics Committee of the University. The cut-off value for normal sperm morphology was set at ≥ 15% and for normal sperm cellular integrity at >60% (HOS-test positive spermatozoa – hypo-osmotic swelling test). Two categories of men were distinguished: (1) men with normal sperm motility (54 out of 94 subjects; ≥ 50% spermatozoa with rapid + slow progressive motility or ≥ 25% with rapid progressive motility) and (2) men with low sperm motility (asthenozoospermia; 40 out of 94 subjects). Twenty-six patients with normal sperm motility exhibited normal sperm concentration ($\geq 20 \times 10^6$/mL) and morphology (N - normozoospermia), 23 subjects presented with abnormal morphology (T - teratozoospermia) and 5 with abnormal concentration and morphology (OT - oligoteratozoospermia). Isolated asthenozoospermia (A) was found in 3 men only, asthenoteratozoospermia (AT) in 28 subjects and oligoasthenoteratozoospermia (OAT) in 8 men (Table 1).

The liquefied semen was centrifuged for 15 min at 400 x g at room temperature. Seminal plasma was removed and the sperm suspension was washed twice in PBS (phosphate buffered saline, Sigma-Aldrich Chemie GmbH, Germany). The sperm pellet was resuspended in 1 mL PBS and was used for cytofluorometric (n = 94), fluorescence and electron microscopic (n = 58) studies.

DNA fragmentation analysis. The incidence of spermatozoa with nuclear DNA strand breaks was identified by TUNEL method (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling assay) using in situ Cell Death Detection Kit (APO-BrdU a complete Kit for measuring apoptosis by dual color flow cytometry, BioSource International, Inc. - APO-BrdU test) according to the manufacturer's instructions. The negative control was obtained omitting TdT from the reaction mixture. All spermatozoa were treated with propidium iodide/RNase solution. The TUNEL-positive reaction in FITC-antiBrdU mAb labeled cells was checked in fluorescence microscope (Axioskop, Carl Zeiss, Jena GmbH, Germany).

Flow cytometry measurements. Sperm DNA fragmentation was measured 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 (480-530 nm) and red (580-630 nm) fluorescence 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. Emission date were collected and analysed by means of Cell Quest software (Becton-Dickinson) [26].

Transmission electron microscopy (TEM). Semen samples for electron microscopy were prepared using a conventional method described by Piasecka and Kawiak [27]. Ultrathin sections of samples were examined in JEM-1200 EX (JEOL Ltd, Tokyo, Japan) transmission electron microscope at 80 kV. Pathomorphological evaluation of semen was performed according to Bartoov *et al.* [28]

Statistical analysis. All results were expressed as mean ± standard deviation (mean ± SD) and median. The conformity of variables with the normal distribution was examined using Shapiro-Wilk W-test. Mann-Whitney U-test was employed to assess statistical differences between groups. Spearman rank correlation (r_s) test was used to determine the relationships between sperm nuclear DNA fragmentation and sperm motility, concentration, morphology, and integrity of sperm cellular membrane. Statistical significance was taken at $p<0.05$. All computations were performed using the Statistica ver. 6.0 software.

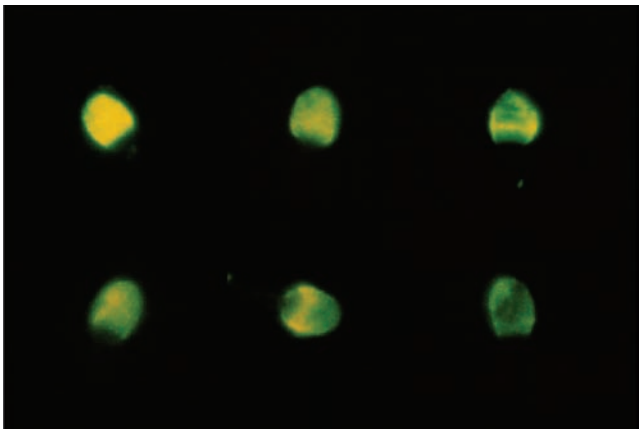

Fig. 1. TUNEL-positive spermatozoa labeled FITC-antiBrdU mAb are detected in fluorescence microscope. Note the different distribution of bright green-yellow fluorescence in the sperm heads; × 5500.

Results

Fluorescence microscopy and flow cytometry of spermatozoa with APO-BrdU test

Spermatozoa with normal integrity of DNA (TUNEL-negative) displayed background fluorescence only, while those with fragmented DNA revealed bright green-yellow fluorescence of the heads (Fig. 1).

The percentage of spermatozoa with nuclear DNA strand breaks was calculated on the basis of results obtained from histogram analysis (Fig. 2) described by Piasecka *et al.* [26]. The proportion of TUNEL-positive sperm was significantly higher ($p<0.01$) in the semen of asthenozoospermic patients (15.10 ± 10.96%; median 12.00%; Q_1-Q_3 = 6.00-23.00%; n=40; $p<0.01$) as compared to men with normal sperm motility (11.69 ± 15.74%; median 6.50%; Q_1-Q_3=4.00-12.00%; n=54) (Table 1).

According to data of Host *et al.* [29,30], Benchaib *et al.* [6] and Huang *et al.* [8], we distinguished two categories of patients using DNA fragmentation threshold value of 4%: (1) patients (23 out of 94 total subjects) with ≤ 4% of TUNEL-positive cells and (2) patients (71 out of 94 subjects) with 4% of TUNEL-positive cells. The former group included 5 asthenozoospermic men (AT=4; OAT=1), while the latter one included as many as 35 asthenozoospermic men (A=3; AT=24; OA=1; OAT=7) out of 40 total studied men with asthenozoospermia. Our studies revealed 32 subjects with >4-10% (N=10; T=7; A=3; AT=9; OT= 1; OA=1; OAT=1), 21 subjects with >10-20% (N=6; T=4; AT=8; OT=1; OAT=2) and 18 subjects with >20% (N=2; T=2; AT=7; OT=3; OAT=4) of sperm with fragmented DNA. A significant difference was found only in the integrity of sperm cellular membrane and sperm motility between patients from both groups.

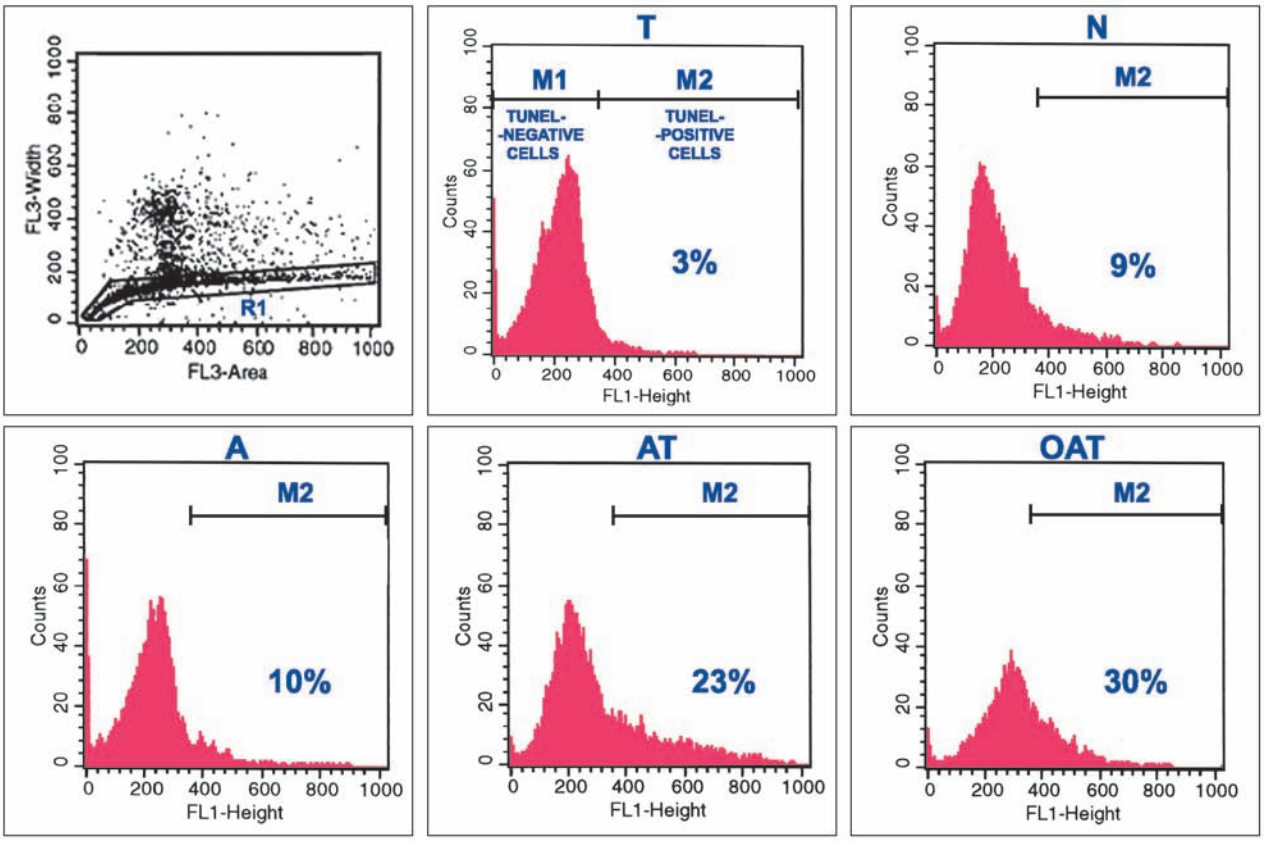

Fig. 2. Flow cytometry analysis of semen samples with APO-BrdU test. Density plot (FL3A versus FL3W) obtained after propidium iodide staining of spermatozoa. Cells gated in R1 region were analyzed, while debrids and aggregates were excluded from the analysis. FL3 channel - the intensity of fluorescence in the red spectrum. Representative histograms of FITC-antiBrdU mAb labeled spermatozoa (FL1-H) in subjects with teratozoospermia (T), normozoospermia (N), asthenozoospermia (A), asthenoteratozoospermia (AT) and with oligoasthenoteratozoospermia (OAT). The percentages of TUNEL-positive spermatozoa with nuclear DNA strand breaks (emitting green fluorescence, see Fig. 1.) read from the M2 marker, but the cells without fragmented DNA (TUNEL-negative) from the M1 marker. Position of M1 marker was adjusted according to the result of negative control (without TdT) which allows to set a region including all TUNEL-negative cells [26]. The x-axis: FL1 channel - the intensity of fluorescence in the green spectrum, the y-axis: depicts the frequency in terms of the number of cells; the fluorescence intensity scale is expressed as "channel number" (0-1000).

Subjects with 4% of TUNEL-positive gametes displayed significantly lower percentages of HOS-positive sperm and of spermatozoa with progressive motility ($p<0.01$) than subjects with ≤ 4% of TUNEL-positive cells (Table 2).

There were significant correlations (n=94) between the percentage of sperm with DNA fragmentation and percentages of sperm with progressive motility (r_s=-0.38), without movement (r_s=0.35), as well as sperm concentration (r_s=-0.33) and percentage of HOS-test positive sperm (r_s=-0.43; n =75). No significant correlation was found between percentage of TUNEL-positive cells and percentages of sperm with non-progressive motility or with normal sperm morphology.

TEM

Electron microscopy revealed a large number of immature spermatozoa usually with excessive cytoplasmic remnants, chromatin and midpiece defects as well as sperm conglomerates, more frequently in the semen of asthenozoospermic subjects with TUNEL-positive spermatozoa exceeding 4%. Granular substructure of sperm chromatin, pale vacuoles and myelin-like, lamellar, granular and electron-dense inclusions in the nucleus of spermatozoa were found (Fig. 4A, B, C). Thickened and diversely deformed sperm midpieces with irregular arrangement of mitochondria and with additional (supernumerary, redundant) mitochondria located usually in the residual cytoplasm were observed (Fig. 4C-E). Moreover, immature gametes with cytoplasmic "sack" instead normal midpiece and flagellum were also noted (Fig. 4B). Cytoplasmic conglomerates contained sperm structures (head, midpiece, principal piece) and their fragments (chromatin, acrosome, mitochondria, outer dense fibers, axoneme, fibrous sheath). In some cases the sperm structures within the conglomerates were degraded (Fig. 5).

Table 1. Results of flow cytometry and conventional analysis of semen. Comparison of sperm DNA fragmentation rates between patients with normal sperm motility and patients with low sperm motility.

Patients	Parameter	TUNEL-positive sperm (%)	Routine sperm characteristics							
			Concentration ($x10^6$/ml)	Morphology (% of normal sperm forms)	Sperm motility (% of spermatozoa)					Hos-test positive sperm (%)
					Rapid + slow progressive (A + B)	Rapid (A)	Slow (B)	Non-progressive	Immotile sperm	
Total (n = 94) N = 26; T = 23; A = 3; AT = 28; OA = 1; OT = 5; OAT = 8	Mean ± SD	13.14 ± 13.95	60.56 ± 40.73	10.12 ± 8.04	50.74 ±16.67	6.16 ± 7.89	44.59 ± 13.98	11.87 ± 8.66	37.38 ± 15.37	64.52 ± 10.70 n = 75
	Median	8.50	60.00	8.00	52.00	3.00	45.00	10.00	36.00	62.00
	Range	1.00–76.00	2.00–180.00	0.00–30.00	15.00–78.00	0.00–36.00	10.00–75.00	1.00–45.00	12.00–77.00	38.00–90.00
	Q_1–Q_3	5.00–16.00	26.00–90.00	2.00–16.00	40.00–66.00	0.00–9.00	36.00–51.00	6.00–14.00	26.00–48.00	58.00–72.00
With astheno-zoospermia (n = 40 out of 94) A = 3; AT = 28; OA = 1; OAT = 8	Mean ± SD	**15.10 ± 10.96** [a]	45.05 ± 35.86	6.93 ± 6.49	34.73 ± 10.58	1.28 ± 1.99	33.45 ± 10.17	15.00 ± 10.38	50.28 ± 12.34	58.94 ± 9.33 n = 31
	Median	12.00	32.50	6.50	35.50	0.00	35.00	11.50	48.50	60.00
	Range	2.00–42.00	4.50–150.00	0.00–24.00	15.00–49.00	0.00–9.00	10.00–49.00	4.00–45.00	24.00–77.00	38.00–80.00
	Q_1–Q_3	6.00–23.00	19.00–60.00	0.00–11.50	25.00–43.50	0.00–2.00	24.50–40.50	7.50–19.50	42.00–59.00	54.00–64.00
With normal sperm motility (n = 54 out of 94) N = 26; T = 23; OT = 5	Mean ± SD	**11.69 ± 15.74** [b]	72.06 ± 40.59	12.48 ± 8.31	62.61 ± 8.24	9.78 ± 8.65	52.83 ± 10.18	9.56 ± 6.28	27.83 ± 9.16	68.45 ±9.90 n = 44
	Median	6.50	70.00	13.00	63.50	7.50	50.00	9.50	28.00	66.00
	Range	1.00–76.00	2.00–180.00	0.00–30.00	50.00–78.00	0.00–36.00	30.00–75.00	1.00–36.00	12.00–46.00	54.00–90.00
	Q_1–Q_3	4.00–12.00	50.00–100.00	5.00–18.00	56.00–69.00	3.00–14.00	46.00–60.00	5.00–12.00	20.00–36.00	60.00–79.00

Q_1-Q_3 - lower quartile-upper quartile; SD - standard deviation; ab - means with different letters are significantly different at $p < 0.01$.

Discussion

In this study, we confirmed that asthenozoospermic men had significantly higher proportion of spermatozoa with nuclear DNA strand breaks (15.10 ± 10.96%; median 12.00%) than men with normal sperm motility ($p<0.01$). Some authors [6,7] obtained similar results. Gandini *et al.* [7] discovered 11 ± 4.3% and Benchaib *et al.* [6] found > 12% TUNEL-positive sperm in the semen of men with abnormal sperm motility. Huang *et al.* [8] presented lower percentage of TUNEL-positive sperm (6.1 ± 6.1% or 9.7 ± 7.2%), but other authors [9-13] noted larger numbers of sperm with fragmented DNA in subjects with low sperm motility. Liu *et al.* [9] discovered 18.8%, Varun *et al.* [11] found 19.7 ± 8.7% and 21.9 ± 9.5%, Zini *et al.* [13] reported 27 ± 2.5%, Marchetti *et al.* [10] observed 30 ± 2% and Vicari *et al.* [12] noted 37 ± 1.7% sperm with DNA damage. Men with abnormal sperm parameters (especially with asthenozoospermia), who were frequently infertile, usually exhibited higher percentage of spermatozoa with diminished DNA integrity in the semen as compared to men with normozoospermia, normal sperm motility or fertile [6-13,22,24]. Moreover, our research reveled as many as 35 asthenozoospermic men with >4% TUNEL-positive spermatozoa out of 40 total studied men with asthenozoospermia. It should be emphasized that a selected fraction of semen with low sperm motility displays more frequent abnormalities of sperm chromatin than a fraction with high sperm motility [31,32]. Our findings taken together with the reported results [8,13,14,31-38] revealed a relationship between DNA fragmentation and sperm motility.

Table 2. Comparison of conventional sperm parameters between patients with ≤ 4% TUNEL-positive sperm and patients with > 4% TUNEL-positive sperm.

Subjects with various levels of sperm DNA fragmentation rate (%)	Parameter	TUNEL-positive sperm (%)	Routine sperm characteristics					
			Concentration ($x10^6$/ml)	Morphology (% of normal sperm forms)	Sperm motility (% of spermatozoa)			Hos-test positive sperm (%)
					Rapid + slow progressive (Aa + B)	Non-progressive	Immotile sperm	
≤ 4% of tunel-positive sperm (n = 23 out of 94) N = 8; T = 10; AT = 4; OAT = 1	Mean ± SD	3.00 ± 1.00	68.28 ± 34.55	12.17 ± 9.46	**58.35 ± 16.00 ^{A}**	10.61 ± 6.47	**31.04 ± 13.74 ^{a}**	**71.06 ± 10.73 ^{A}** n = 17
	Median	3.00	60.00	12.00	64.00	10.00	28.00	72.00
	Range	1.00–4.00	4.50–130.00	0.00–30.00	17.00–76.00	2.00–25.00	16.00–62.00	52.00–86.00
	Q_1–Q_3	2.00–4.00	50.00–90.00	4.00–20.00	50.00–69.00	4.00–14.00	20.00–42.00	60.00–80.00
> 4% of TUNEL-positive sperm (n = 71 out of 94) N = 18; T = 13; A = 3; AT = 24; OA = 1; OT = 5; OAT = 7	Mean ± SD	16.42 ± 14.61	58.06 ± 42.46	9.45 ± 7.48	**48.28 ± 16.24 ^{B}**	12.28 ± 9.26	**39.44 ± 15.39 ^{b}**	**62.60 ± 10.00 ^{B}** n = 58
	Median	12.00	50.00	8.00	50.00	10.00	38.00	61.00
	Range	5.00–76.00	2.00–180.00	0.00–28.00	15.00–78.00	1.00–45.00	12.00–77.00	38.00–90.00
	Q_1–Q_3	7.00–21.00	25.00–90.00	2.00–15.00	34.00–59.00	6.00–14.00	29.00–48.00	58.00–68.00

Q_1-Q_3 - lower quartile-upper quartile; SD - standard deviation; ABab - means with different letters are significantly different (AB at $p < 0.01$; ab at $p < 0.05$).

A significant negative correlation between DNA damage and sperm progressive motility ($p<0.001$; n=94) and a significantly lower percentage of sperm with progressive motility ($p<0.01$) in men displaying >4% TUNEL-positive gametes suggested that abnormal sperm genomic integrity could be associated with poor sperm movement. This suggestion is consistent with conclusion of Ramos *et al.* [37]. They considered the sperm motility as a relevant physiological marker for intact DNA. Similar interpretation have been drown by Zini *et al.* [13] and Muratori *et al.* [36].

Sperm DNA breakage negatively correlated not only with sperm motility but also with HOS-test results ($p<0.001$), and sperm concentration ($p<0.01$). DNA fragmentation did not significantly correlate with normal sperm morphology ($p=0.06$). The hypo-osmotic swelling test reflects the viability of spermatozoa expressed as percentage of cells with functional/integral cellular membrane. Our data are agreement with other reports correlating the percentages of sperm with fragmented DNA with percentage of HOS-test positive sperm [35,36]. Regarding the correlation between DNA integrity and sperm concentration, our results are consistent with those of other investigators [14,15,23,35], but are in contrast with study of Muratori *et al.* [36]. Thus, it can be concluded that semen sample with a low percentage of sperm with normal motility and with low percentage of sperm with functional cellular membrane may contain a high percentage of TUNEL-positive sperm. Furthermore, decreased sperm concentration may be associated with increased percentage of spermatozoa with nuclear DNA fragmentation. It should be stressed, that patients with poor standard seminological parameters and low predictive value concerning the sperm fertilizing ability are usually enrolled in ART [1,4-6,14,15,29,30]. Moreover, men with low sperm progressive motility may display an increased rate of sperm aneuploidy [39-41]. The intracytoplasmic sperm injection (ICSI) bypasses a natural selection mechanisms preventing the interaction of both gametes. Current reports reveal that DNA fragmentation has an impact on fertilization, embryo development, pregnancy and may eventually contribute to spontaneous miscarriages, genetic anomalies and childhood cancer in the offspring. Therefore, the extent of nuclear DNA damage in spermatozoa has important clinical significance in natural and assisted procreation. From this reason, sperm DNA fragmentation must not be ignored [2,4-6,8,10,14-18,42].

So far, the threshold value for the TUNEL assay to discriminate between fertile and infertile men has not been established. Some investigators proposed 4%

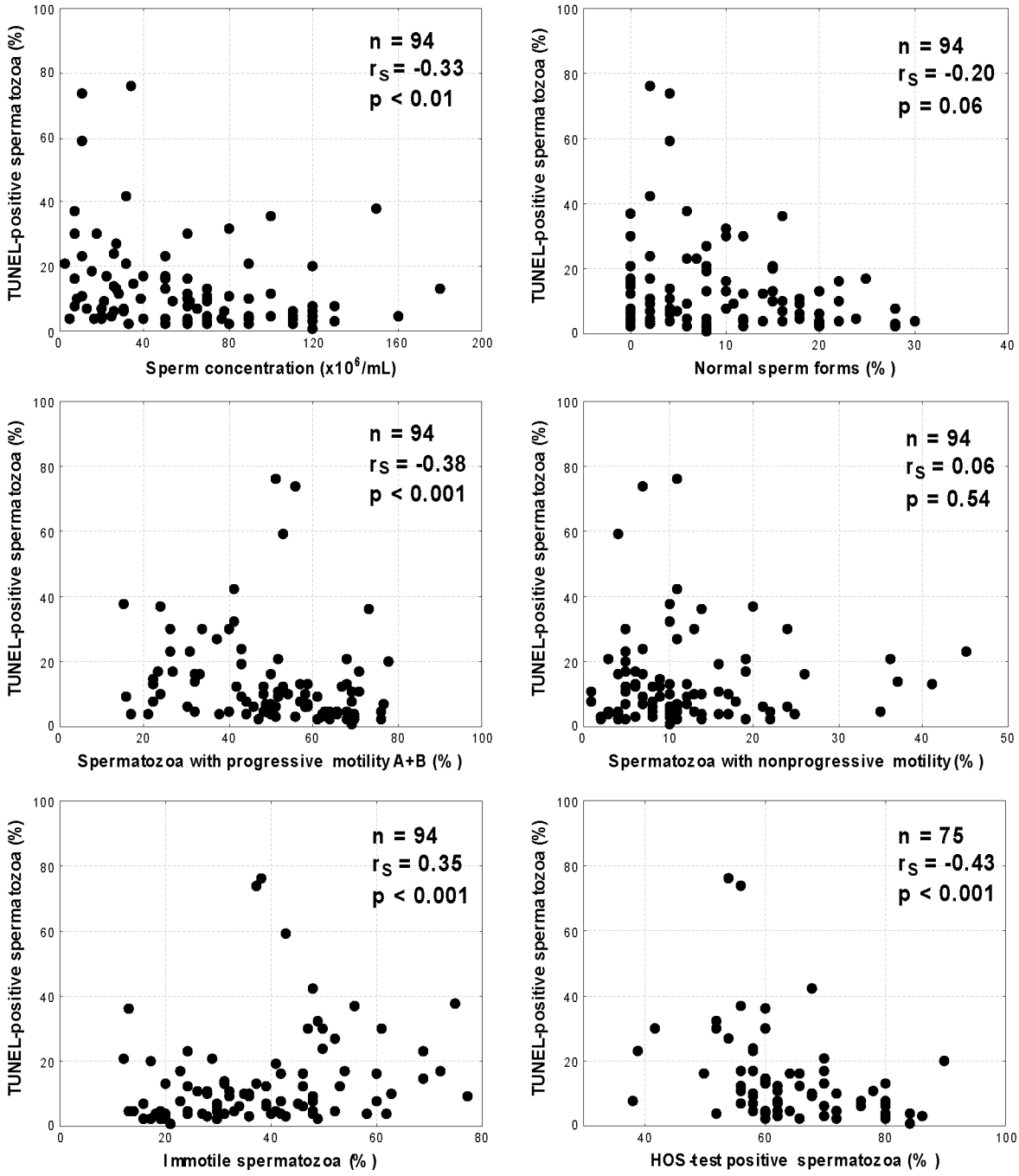

Fig. 3. Spearman rank correlation coefficients (r_s) for the percentage of TUNEL-positive spermatozoa and routine sperm characteristics.

[6,8], 10% [6,14], others 12% [34], 20% [14,17,24], or as much as 36.5% [29] TUNEL-positive sperm cells as a cut-off value for predicting TUNEL positivity of sperm samples, fertilization, or pregnancy. According to several reports [6,8,29,30] and using a DNA fragmentation threshold value of 4%, we distinguished two categories of patients: with ≤4% or with >4% TUNEL-positive cells. Host *et al.* [29,30] and Huang *et al.* [8] observed a significantly higher fertilization rate when <4% or ≤4% spermatozoa with fragmented DNA were present in semen. In the present research, only 23 men with ≤4% were disclosed among 94 subjects.

Huang *et al.* [8] found that the fertilization rate was affected when sperm DNA fragmentation was >10%. Similar results were reported by Benchaib *et al.* [6] who noted that the fertilization rate was significantly higher for DNA fragmentation below 10% and no pregnancy was obtained when DNA fragmentation

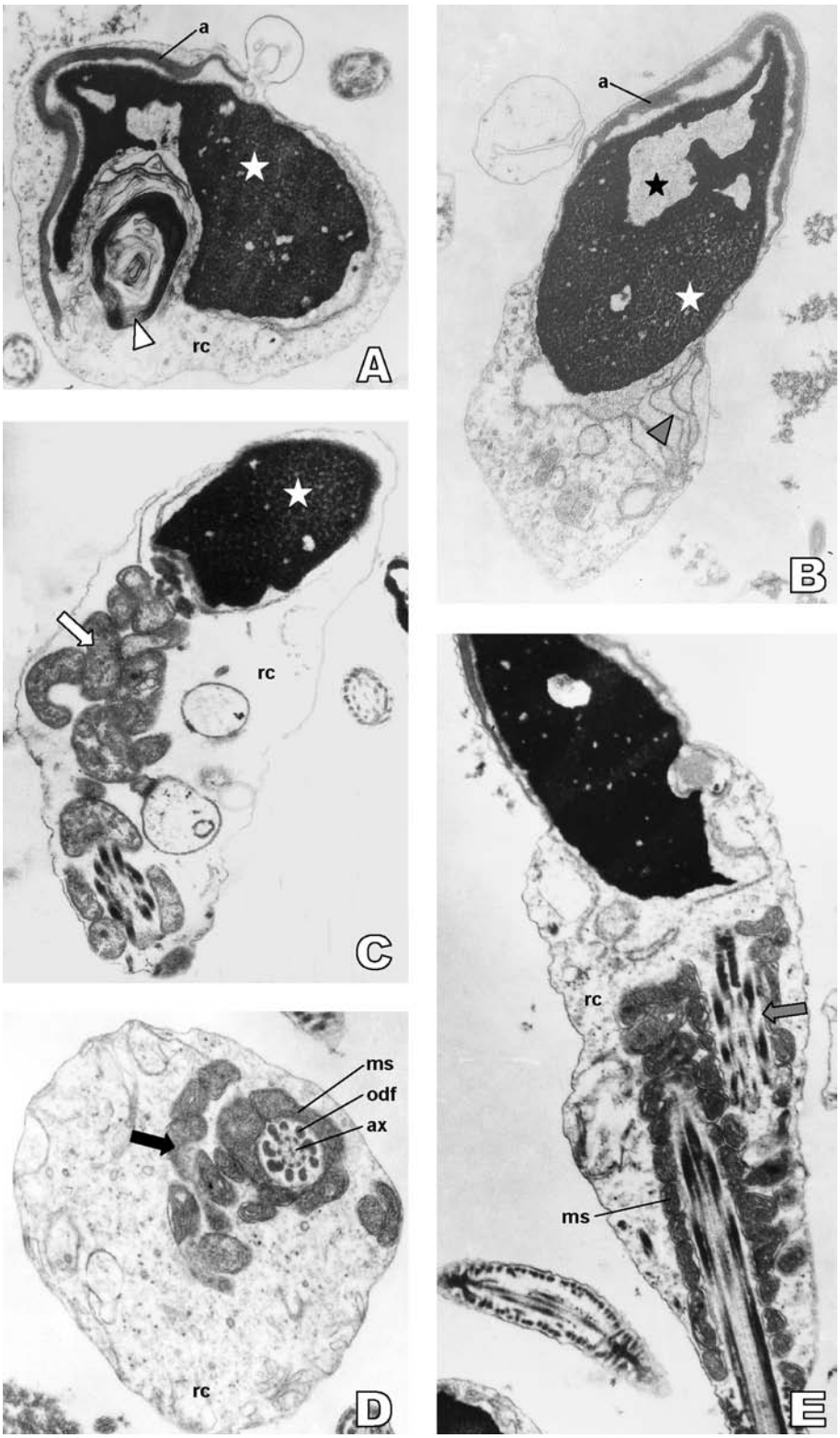

Fig. 4. Representative ultrastructural features of immaturity of ejaculated spermatozoa reflecting developmental failure in the spermatogenic remodeling process in patients with low sperm motility and TUNEL-positive spermatozoa exceeding 4%. Abnormal packaging of sperm chromatin: immature chromatin with granular substructure (white asterisk, A, B, C), vacuole (black asterisk, B) and with myelin-like structure (white arrowhead, A); abnormal morphogenesis of the midpiece: spermatozoon containing cytoplasmic "sack" with granular, membranous material and with redundant membranes of nuclear envelope (gray arrowhead, B). Midpiece with irregular arrangement of mitochondria (some of which have abnormal shape and size, white arrow, C), with supernumerary, redundant mitochondria shed outside the mitochondrial sheath into the residual cytoplasm (black arrow, D). Spermatozoon with additional section through the midpiece (gray arrow, E); note excessive cytoplasm in the head (A, C, E), the neck (C, E) and in the midpiece (C, D, E); Baccetti *et al.* [45] have identified the spermatozoon shown in Fig. B as apoptotic; a - acrosome; ms - mitochondrial sheath; odf - outer (peripheral) dense fibers; ax - axoneme; rc - residual cytoplasm; A, B, C: × 19 000; D: × 18 750; E: × 21 740.

exceeded 20%. Borini *et al.* [14] found significantly a higher percentage of clinical pregnancy and significantly lower percentage of pregnancy loss for men with <10% TUNEL-positive spermatozoa than for >10%. Duran *et al.* [34] reported that no semen sample having >12% sperm with fragmented DNA resulted in pregnancy. In turn, Seli *et al.* [17] obtained 50% more blastocysts for subjects with <20% TUNEL-positive male gametes as compared to ≥ 20%. Some authors observed a significant negative correlation between

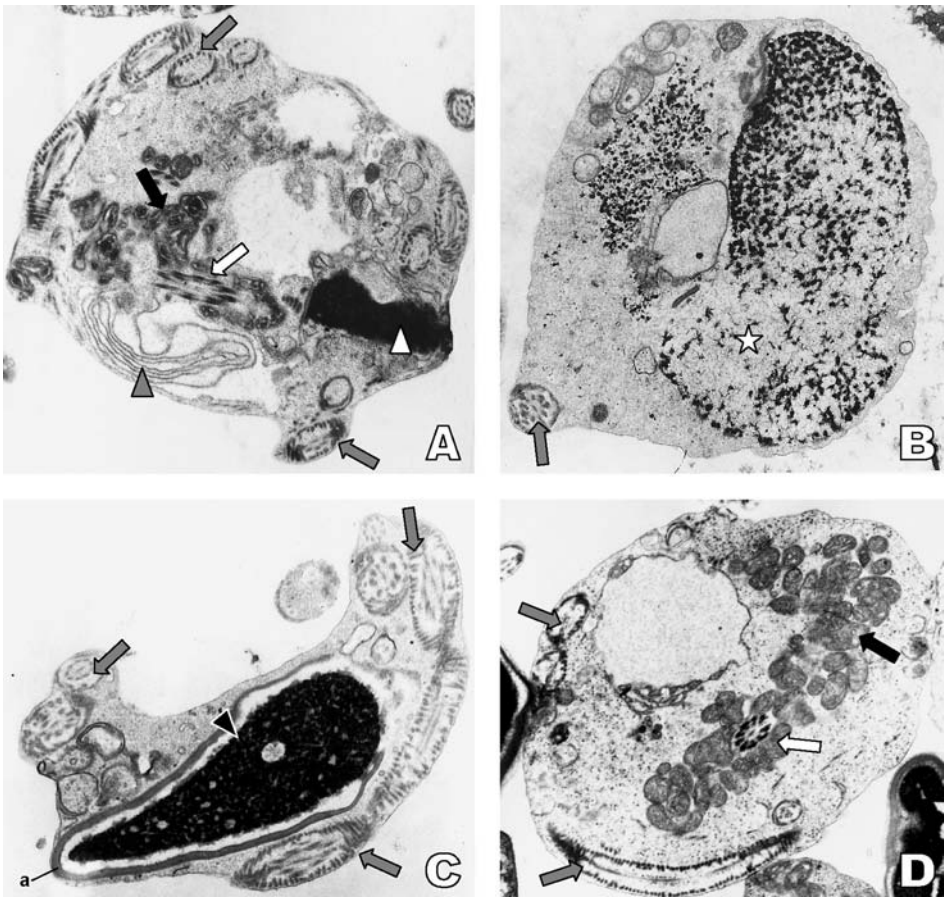

Fig. 5. Semen conglomerates containing sperm structures reflecting abnormal spermiogenesis in men with low sperm motility and TUNEL-positive spermatozoa exceeding 4%. The cytoplasm reveals granular and membranous material, condensed chromatin (white arrowhead, A), degraded chromatin (white asterisk, B), sperm head with acrosome (black arrowhead, C), redundant membranes of nuclear envelope (gray arrowhead, A), midpiece (white arrow, A, D), redundant mitochondria (black arrow, A, D) and sections through the tail which can be rolled-up (gray arrow, A-D); structures resemble degeneration of abnormal, elongating, highly differentiated spermatids with fully developed midpiece (A) and tail (A, B) and can resemble immature spermatozoa with excessive cytoplasm in the head (C) and at the midpiece (D); Baccetti *et al.* [45] have identified the ultrastructure in Fig. B as an apoptotic body; a - acrosome; A: × 9000; B: × 15 000; C: × 20 500; D: × 14 300.

DNA damage and fertilization rate [10,14,29,30], blastocyst development [17], or clinical pregnancy [15]. Our study revealed 32 subjects with >4-10%, 21 subjects with >10-20%, and 18 subjects with >20% sperm with fragmented DNA. Taking into account the findings of other researchers [6,8,14,34], the chance for fertilization will be lower for patients with >10-20% TUNEL-positive sperm. Likewise, the pregnancy rate may be diminished for patients with >20% spermatozoa having nuclear strand breaks.

We tried to find a relationship between the molecular disorders of sperm chromatin and sperm ultrastructural characteristics. The electron microscopy of semen was a probe attempting to specify eventual reasons for decreased sperm genomic integrity. According to our results, it seems that sperm DNA fragmentation in asthenozoospermic subjects was a result of defective chromatin condensation/packaging. The granular substructure of sperm chromatin, diverse lamellar inclusions and pale vacuoles in the sperm nucleus reflected immaturity of the chromatin [36,43-45]. Incompletely packed DNA (denaturated DNA) contains persistent endogenous nicks. Strand breaks in nuclear sperm DNA can be an effect of the failed replacement of histones by protamines - impaired protamination (protamine deficiency or abnormal activity of endogenous nuclease and topoisomerase II - enzymes involved in the creation and ligation of DNA nicks during protamination). Therefore, the observed ultrastructural features of sperm chromatin may indicate the molecular disorders in nuclear remodeling process during the differentiation of round to elongated spermatids, while TUNEL-positive spermatozoa with endogenous nicks in ejaculate may be the product of incomplete sperm chromatin compaction [2,4,5,13,36,46]. On the other hand, some spermatozoa may be identified as apoptotic cells [7,43,45]. Hence, TUNEL-positive spermatozoa can be considered as cells entering programmed death [7,22,23,45]. The used test is able to detect single and double stranded DNA breaks and may suggest a final move to programmed and also non programmed cell death [7,10,13,22]. However, the damaged DNA is not always indicative of apoptosis. It should be emphasized that interpretation of DNA-fragmented spermatozoa is still equivocal as well as mechanisms involved in generating sperm DNA lesions are not fully elucidated yet. Abnormal sperm chromatin condensation, sperm apoptosis and oxidative stress at reduced antioxidant protection are currently considered as causes of DNA strand breaks [4,5,7,16,22,36,46,47].

In the present research, abnormalities of sperm midpiece frequently accompanied by excessive residual cytoplasm and spermatozoa with cytoplasmic "sack" instead flagellum were found in the semen of asthenozoospermic men, especially with >4% TUNEL-positive sperm cells. In considering these ultrastructural results can be related to sperm maturity within the midpiece. One may assume that abnormal morphogenesis of mitochondrial sheath and failed cytoplasmic retention appeared during spermiogenesis in studied men with low sperm motility beside impaired chromatin condensation. Sperm defects demonstrated in our study were observed by other authors also in the semen of human subjects with low sperm motility [7,36,40,41], with Hodgkin's disease [7], testicular cancer [7,45], vericocele [43,45] and with endocrine [45] or genetic [40,43,44] disorders. Sperm ultrastructural characteristics may suggest an apoptosis or immaturity [7,36,40,44,45]. It is believed that immature spermatozoa with persistent cytoplasm generate reactive oxygen species (ROS) which contribute to the loss of sperm DNA integrity [2,5,15,16, 31,37,47-50]. Our previous studies showed (1) normal oxidoreductive capability (NADH-dependent NBT assay) of spermatozoa with residual cytoplasm involved in generating of ROS and (2) functional supernumerary mitochondria (JC-1 test) localized in sperm cytoplasmic remnants in some cases of asthenozoospermia [27]. Mitochondria are known to produce ROS [16,49,51]. Therefore, the abnormal spermatozoa noted in the semen of men with low sperm motility may be a source of ROS. High DNA fragmentation in spermatozoa of asthenozoospermic subjects seems to be understandable.

We may speculate that the immature spermatozoa with molecular and morphological defects can assemble and form sperm conglomerates discovered in semen of asthenozoospermic men. These conglomerates contained abundant cytoplasmic material, sperm fragments, and other structures. Our findings suggest that abnormal cells are degraded within the conglomerates. However, interpretation of these electron microscopic findings is difficult. Without doubt, sperm conglomerates reflect spermatogenic failure. Moreover, formation of the conglomerates may be a adequate way to eliminate abnormal cells which can develop during differentiation of spermatids. In this case the sperm conglomerates are not destroyed in the testis or epididymis, but they are liberated from seminiferous epithelium and finally appear in the ejaculate. Some of the conglomerates can be related to apoptotic sperm, spermatids or bodies [7,36,45].

In conclusion, our study suggest that asthenozoospermia is accompanied by (1) intense defects of gamete chromatin expressed as diminished sperm genomic integrity and abnormal DNA condensation and (2) intense defects of sperm midpiece. These abnormalities may reflect the developmental failure during the spermatogenic remodeling process. In such cases, intense elimination of abnormal, immature cells possibly associated with their death and with the increase of sperm conglomerates in the semen may take place. The DNA fragmentation test may be considered as an additional assay in evaluation of spermatozoa beside a standard analysis and taken together with the ultrastructural study may help to determine the actual number of "healthy" spermatozoa, thus playing an important role during proper diagnosis and treatment of male infertility.

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