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Toluidine blue cytometry test for sperm DNA conformation: comparison with the flow cytometric sperm chromatin structure and TUNEL assays

J.Erenpreiss[1,2,4], K.Jepson[1], A.Giwercman[1], I.Tsarev[2], Je.Erenpreisa[2] and M.Spano[3]

[1]Fertility Centre, Malmö University Hospital, SE 205 02 Malmö, Sweden, [2]Latvian University Biomedicine Centre, Ratsupites 1, LV 1067 Riga, Latvia and [3]Section of Toxicology and Biomedical Sciences, BIOTEC-MED, ENEA CR Casaccia, Via Anguillarese 301, 00060 Rome, Italy

[4]To whom correspondence should be addressed. E-mail: Juris.Erenpreiss@kir.mas.lu.se

BACKGROUND: Sperm DNA integrity (SDI) is an important factor in the prognosis of male fertility. Here we compare the toluidine blue (TB) image cytometry test, recently proposed by us for SDI assessment, with two other tests—the sperm chromatin structure assay (SCSA) and the terminal nick-end labelling (TUNEL) assay. METHODS: Sperm samples from 35 men were evaluated for standard sperm parameters and subjected to the TB test and SCSA. Eighteen of the 35 samples were also subjected to the TUNEL assay. RESULTS: The proportion of sperm cells with abnormal DNA integrity assayed by the TB test correlated strongly with the proportion of abnormal cells detected by the SCSA and TUNEL assay ($\rho = -0.84$ and $\rho = 0.80$, $P < 0.001$, respectively). Furthermore, the fractions of abnormal cells by the TB test corresponded closely to the sum of two SCSA parameters, the DNA fragmentation index (DFI) and the fraction of highly DNA-stainable cells (HDS) (medians 33.0 versus 32.0%, $P = 0.6$). CONCLUSIONS: Abnormal cells in a TB test correspond to the sum of DFI and HDS fractions in the SCSA. TB-positive cells may represent sperm with fragmented DNA and/or abnormal chromatin structure. Because the TB test is an easy and inexpensive method, its potential use as a routine test for sperm DNA integrity, complementary to standard semen parameters, should be investigated further.

Key words: flow cytometry/infertility/sperm chromatin/sperm DNA integrity/toluidine blue test

Introduction

Several methods are used to assess sperm chromatin/DNA status. These methods may yield diagnostic and prognostic information complementary to but distinct from that obtained from standard sperm parameters (concentration, motility and morphology) (reviewed in Evenson *et al.*, 2002; Agarwal and Said, 2003; Perreault *et al*., 2003). Sperm DNA integrity is an important, independent parameter of sperm quality that has been associated with male fertility potential both *in vivo* and *in vitro* (Lopes *et al*., 1998; Evenson *et al.*, 1999; Spano *et al.*, 2000; Larson-Cook *et al.*, 2003). A widely used test for chromatin/DNA integrity is the sperm chromatin structure assay (SCSA) (Evenson *et al*., 1980). In this test, sperm are exposed to acid and stained with the metachromatic DNA dye acridine orange (AO). DNA that denatures in the acid (fragmented, i.e. abnormal) fluoresces red, while intact, double-stranded DNA fluoresces green. The proportion of red versus green fluorescence in each sperm is monitored by flow cytomery (FCM). Cells are assumed to be abnormal when the amount of red exceeds a certain threshold. The proportion of those cells is regarded as a so-called DNA fragmentation index (DFI) (Evenson *et al*., 2002). The SCSA predicts infertility when the DFI exceeds 30% in natural conception (Evenson *et al*., 1999; Spano *et al*., 2000) and 27% in *in vitro* conception (Larson *et al*., 2000; Larson-Cook *et al*., 2003; Saleh *et al*., 2003), justifying its clinical application and wide use in ongoing studies. However, the SCSA requires the use of FCM, thereby making it unaffordable for some infertility clinics and thus limiting its application in routine infertility work-up.

In our previous studies (Erenpreiss *et al*., 2001; Erenpreisa *et al.*, 2003), we found that sperm cell nuclei with red fluorescence in an AO *in situ* denaturation test [performed on slides according to our protocol (Erenpreiss *et al.*, 2001) based on the recommendations of Rigler (1966)] corresponded to those that stained dark purple with the thiazine dye toluidine blue (TB) in a TB test on slides, whereas those with green fluorescence corresponded to those that stained pale blue with TB. Based on this AO test, the conditions for the TB staining were set and a threshold of optical density (OD) in CCD green image ≥ 0.16 was determined to distinguish dark purple sperm cells with presumably damaged DNA. The proportion of TB dark cells also correlated with the fraction of sperm cells containing DNA strand breaks

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measured with a direct method for detecting DNA fragmentation, i.e. the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay, which was also performed on slides. Thus, we have elaborated the image cytometry method to detect sperm cells with abnormal DNA integrity (Erenpreisa *et al.*, 2003) as a reliable but less expensive potential alternative to FCM-based tests.

However, it should be taken into account that the AO test relies on a visual interpretation of spermatozoa. Although this method provides a general picture of the status of sperm DNA denaturation susceptibility, sperm classification is limited to only a few categories (green, red and yellowish). Problems with interpretation are aggravated by rapidly fading fluorescence and heterogeneous slide staining, making it difficult to score cells that contain both red and green fluorescence in an accurate and consistent manner. These limitations have impaired a wider use of this technique in clinical andrology laboratories (Evenson *et al.*, 2002; Perreault *et al.*, 2003).

The primary factor for the AO metachromatic shift from green to red fluorescence is the critical equilibrium staining of AO which is fully accomplished in the SCSA. Compared with the AO test, the FCM SCSA offers unbeatable performances in terms of automation, sensitivity, precision, accuracy, repeatability and speed of measurements, producing results with a high statistical power based on several thousands of cells. By the simultaneous measurement of green and red fluorescence intensity emitted by every sperm in the sample, SCSA data allow visualization and quantification of two subpopulations of abnormal sperm, namely the fraction of sperm with fragmented DNA and the fraction of immature sperm with a high level of DNA stainability.

It is generally acknowledged that the performance of the TUNEL assay also improves when carried out as an FCM technique (Gorczyca *et al.*, 1993), as compared with slide-based protocols. Similarly to the SCSA, the FCM TUNEL assay allows rapid analysis of a large number of sperm in a short period of time and provides a highly sensitive means of defining sperm with fragmented DNA in a more objective and precise manner (Perreault *et al.*, 2003).

In the present study, we compared the TB test with the two robust FCM methods, i.e. the SCSA and the TUNEL assay, to document further the applicability of the former.

Materials and methods

Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St Louis, MO).

Semen collection

Thirty-five semen samples were obtained at the Fertility Centre of Malmö University Hospital from patients undergoing routine semen analysis for infertility. Samples were obtained by masturbation after the recommended time of sexual abstinence. Standard semen parameters (volume, concentration, motility and morphology) were measured according to the World Health Organization guidelines (World Health Organization, 1999). All 35 samples were analysed with both TB and SCSA. Eighteen samples with a sufficient volume were also analysed by TUNEL. Samples were processed randomly, and the evaluator was blinded to sample identity. The study protocol was approved by the ethical review board of Lund University.

Toluidine blue image cytometry test

The TB test was performed as described earlier (Erenpreisa *et al.*, 2003). Briefly, the ejaculate was allowed to liquefy at 37°C for 30 min. The liquefied sample was pelleted at 250 *g* for 10 min and resuspended in its own supernatant to an approximate concentration of 2×10^8 cells/ml. Thin smears were prepared on pre-cleaned defatted slides and then air dried for 30–60 min. Dried smears were fixed with freshly made 96% ethanol:acetone (1:1) at 4°C for 30 min–12 h and air dried. Hydrolysis was performed with 0.1 mol/l HCl at 4°C for 5 min followed by three changes of distilled water, 2 min each. TB (0.05% in 50% McIlvain's citrate phosphate buffer at pH3.5, Gurr-BDH Chemicals Ltd, Poole, UK) was applied for 5 min. Slides were rinsed briefly in distilled water, lightly blotted with filter paper, dehydrated in tertiary butanol at 37°C (2×3 min) and xylene at room temperature (2×3 min) (Histoclear RA Lamb Labs, USA), and mounted with DPX. The TB working solution was prepared monthly from a stock solution of 1% TB in distilled water and stored at 4°C. The stock solution can be stored at 4°C for up to 1 year.

The results of the TB test were estimated using oil immersion (10×100) on a Leica DM LB microscope supplied with a 3CCD colour video camera Sony DXC-390P and image analysis computer program (Image Pro Plus 4.1). Calibration of the video camera for measurement of true optical densities in three colour images was performed by using a set of neutral optical filters. The proportion of cells possessing an OD ≥ 0.16 in the green CCD image was calculated based on 200 sperm cells examined per sample. Stained objects with an area $< 3.5\ \mu m^2$ were automatically deleted from counting. Somatic cells and immature germ cells were removed manually. Only sperm cells possessing an elongated or a droplet-like form and a bipolar structure, with a clear light acrosome in one pole and a stained nucleus in the other were measured. Distinction between normal and abnormal sperm was not made.

The inter-assay variability ($< 8\%$) was verified by repeat assessments of control semen samples (results not shown).

Sperm DNA denaturation: SCSA

The SCSA was applied following the procedure described elsewhere (Spano *et al.*, 2000) with slight modifications. An aliquot of unprocessed semen ($\sim 13-70\ \mu l$) was diluted to a concentration of $1-2 \times 10^6$ sperm/ml with TNE buffer (0.01 mol/l Tris–HCl, 0.15 mol/l NaCl and 1 mmol/l EDTA, pH7.4). This cell suspension was treated with an acid detergent solution (pH1.2) containing 0.1% Triton X-100, 0.15 mol/l NaCl and 0.08 mol/l HCl for 30 s, and then stained with 6 mg/l purified AO (Polysciences Inc., Warrington, PA) in a phosphate-citrate buffer, pH6.0. Cells were analysed using a FACSort flow cytometer (Becton Dickinson, San Jose, CA), equipped with an air-cooled argon ion laser.

Evenson *et al.*, (2002) originally recommended starting the FCM analysis 3 min after staining. However, since in the TB test sperm have to dry on slides for 30–60 min, experiments were set up in the current study to investigate the stability of the AO staining time in the SCSA. Therefore, in a separate experiment, we performed SCSA on 28 samples and two reference samples at various times after staining: 3 min, 10 min, 30 min, 1 h and 4 h. The results indicated that the time after staining was not crucial for SCSA reliability (Table I), with a mean intra-donor coefficient of variation of only 5%. This stability was true for both good samples (DFI 6–15%) and poor samples (DFI 40–60%). Therefore, in the present study, all SCSA samples were analysed for SCSA 1 h after staining.

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Table I. Stability of the SCSA results (DFI) for the different AO staining times

Sample no.	AO staining time				
	3 min	10 min	30 min	1 h	4 h
1	10.89	11.86	12.41	11.34	11.65
2	18.40	19.00	18.74	18.29	17.19
3	9.91	10.42	10.66	10.78	9.81
4	23.68	23.95	22.46	23.78	23.39
5	23.11	24.16	13.91	23.15	24.01
6	13.05	12.01	13.34	12.72	12.75
7	18.52	15.96	16.52	18.03	18.40
8	20.13	22.33	20.65	20.49	20.70
9	29.15	29.94	29.85	27.79	29.60
10	19.58	22.00	19.39	19.03	19.57
11	16.45	15.80	16.38	16.90	17.12
12	23.97	23.74	22.74	21.62	22.87
13	61.03	62.96	61.16	62.77	60.88
14	70.37	70.91	69.92	68.49	68.10
15	39.74	39.13	39.82	41.06	38.56
16	57.05	57.92	57.63	58.41	56.43
17	6.02	6.54	6.44	6.71	7.23
18	14.52	13.74	15.12	12.76	12.99
19	21.26	19.09	17.76	21.05	17.99
20	26.03	25.34	26.10	26.74	25.11
21	16.22	17.03	15.91	16.40	16.18
22	44.99	47.24	47.28	46.27	46.55
23	14.28	14.90	14.39	14.80	13.55
24	14.75	14.18	14.65	14.57	14.54
25	27.20	30.10	26.41	27.16	25.62
26	9.49	7.75	8.85	9.33	7.74
27	9.24	9.17	9.85	9.16	10.08
28	32.36	33.16	34.29	35.60	33.91
29	6.28	6.68	5.87	7.11	6.16
30	11.50	11.29	11.47	11.92	12.28
Mean ± SD	23.6 ± 16.2	23.9 ± 16.6	23.3 ± 16.4	23.9 ± 16.9	23.4 ± 16.0

Green fluorescence was measured using a 530 ± 30 nm band-pass filter and red fluorescence using an LP620 long-pass filter. The two colours were separated by a 560 nm dichroic filter. Data were stored in list mode at 10-bit resolution. A total of 5000 events were accumulated for each measurement at a flow rate 200–300 cells/s. Under these experimental conditions, when excited with a 488 nm light source, AO that is intercalated in double-stranded DNA emits green fluorescence, while AO associated with single-stranded DNA emits red fluorescence. Thus, sperm chromatin damage can be quantified by FCM measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red (fragmented DNA) versus green (DNA stainability) fluorescence intensity cytogram patterns. Adopting guidelines published by Evenson *et al.*, (2002), the extent of DNA denaturation was expressed in terms of the DFI, which is the ratio of red to total (red plus green) fluorescence intensity, by using the ListView software (Phoenix Flow Systems, San Diego, CA). This conversion was necessary to calculate correctly the percentage of abnormal spermatozoa. The DFI value was derived for each sperm cell in a sample, and the detectable DFI values were calculated on the resulting DFI frequency histogram. The fraction of highly DNA-stainable (HDS) cells (previously termed HGRN) (Evenson *et al.*, 2002) was also assessed. The percentage of HDS cells was calculated by setting an appropriate gate on the scattergram (abscissa, green DNA fluorescence; ordinate, red fluorescence, denaturated DNA) and considering those events which exhibit a green fluorescence intensity higher than the upper border of the main cluster as representing the sperm population with non-detectable DFI. For the flow cytometer set-up and calibration, aliquots were used from a normal human ejaculate sample retrieved from the laboratory repository. Inter-assay variability (<5%) was verified by repeat assessments of control semen samples (results not shown). Fresh and frozen–thawed samples yielded similar results (<7% variability) (results not shown).

Sperm DNA fragmentation: TUNEL assay

The TUNEL assay was carried out according to the manufacturer's (Roche Diagnostics GmbH, Manheim, Germany) protocol with slight modifications. It was shown earlier that the results of the TUNEL assay remain unchanged after freezing–thawing (Duru *et al.*, 2001); therefore, for convenience, the assay was processed on frozen samples without cryoprotectant. Samples were quickly thawed in a 37°C water bath, centrifuged (500 *g* for 6 min) and washed twice in phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) (pH7.4). The pellet was resuspended in PBS/1% BSA to a final concentration of 1.5×10^6 cells/100 µl of sample. A 100 µl aliquot of 1% paraformaldehyde was added on a shaker for 1 h at 15–25°C. After fixation, the cells were washed once with 1 ml of PBS/1% BSA, and subsequently the fixative was removed. For permeabilization, the cells were re-suspended in 100 µl of permeabilization solution containing 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. After washing once with PBS/1% BSA, the labelling reaction was performed by incubation with 50 µl of labelling solution (supplied with the *in situ* Cell Death Detection Kit, Fluorescin, Roche Diagnostics GmbH, Mannheim, Germany) containing terminal deoxynucleotidyl transferase (TdT) for 1 h at 37°C in the dark. For each sample, a negative control was prepared by omitting TdT from the reaction mixture. After labelling, two subsequent washes in PBS/1% BSA were performed and the sample was resuspended in PBS/0.1% BSA to a volume of 500–700 µl

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and counterstained with propidium iodide (0.5 μl/ml). Positive controls for each sample were prepared as described above but with an additional treatment with 2 IU of DNase (Roche Diagnostics GmbH) for 15 min at 37°C before the labelling reaction.

Green fluorescence (TUNEL-positive cells) was measured using a 530 ± 30 nm band-pass filter, and red fluorescence (propidium iodide-labelled cells) using a 620 nm long-pass filter. The two colours were separated by a 560 nm dichroic filter. A total of 10 000 events were accumulated for each measurement at a flow rate of 200–300 cells/s. Data were processed off-line by CELLQUEST analysis sowftware (Becton Dickinson) after gating out debris and aggregates.

The inter-assay variability (<7%) was verified by repeat assessments of control semen samples (results not shown).

Data analysis

Results are expressed as medians (ranges). Bivariate correlations between the traditional sperm parameters and proportions of sperm cells with abnormal DNA integrity as detected by the TB test, the SCSA and the TUNEL assay were calculated with Spearman's ρ. Furthemore, the Wilcoxon test for paired data was used to compare the results obtained by the different sperm DNA tests.

Since the TUNEL assay is the only applied method that directly assesses the amount of DNA strand breaks, the predictive values of TB as well as of SCSA DFI for the fraction of TUNEL-positive cells were evaluated in a linear multiple regression model. For this analysis, TUNEL-positive cells were computed as the dependent variable. The proportion of TB-positive sperm or the percentage DFI was used as an independent variable, with sperm concentration, percentage motile and percentage morphologically normal cells as co-variables.

All testing was two-sided with a *P*-value of <0.05 regarded as significant. Analyses were conducted with SPSS 11.0 for Windows (SPSS Inc., Chicago, IL).

Results

The proportion of sperm cells with abnormal DNA conformation, detected by the TB test (dark cells with OD green ≥ 0.16) correlated significantly with the proportion of spermatozoa containing denaturable DNA detected as SCSA percentage DFI ($\rho = 0.84$, $P < 0.001$, Figure 1a) and with the fraction of spermatozoa with fragmented DNA in the FCM TUNEL test ($\rho = 0.80$, $P < 0.001$, Figure 1b). Median values of cells with abnormal DNA integrity in 18 samples in the TB test were 28.5% (10–64), 16.0% (6–37) in the SCSA, and 10.5% (4–27) in the TUNEL test ($P < 0.01$). The median values were also higher in the TB test [33.0% (6–64)] compared with the SCSA test [19.5% (6–64), $P < 0.001$] for all 35 samples analysed with these two methods.

The results of the SCSA and the TUNEL assay also exhibited a statistically significant although somewhat weaker correlation ($\rho = 0.63$, $P = 0.005$, Figure 1c). Moreover, in a linear multiple regression analysis with sperm concentration, percentage motile and morphologically normal cells as covariates, the TB test was a significant predictor ($P = 0.04$, standardized β coefficient 0.83) of TUNEL assays results, but no for DFI in the SCSA ($P = 0.19$, standardized β coefficient 0.57). Sperm concentration, motility and morphology

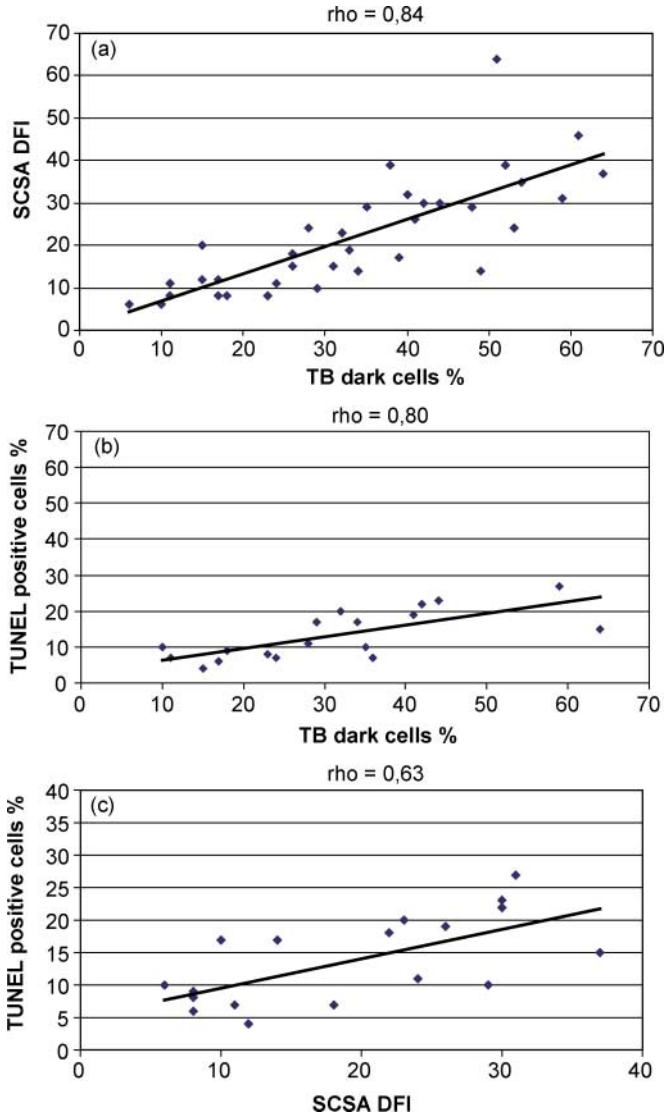

Figure 1. Relationships between the proportion of cells with abnormal DNA integrity as measured by the TB test, SCSA (DFI) and FCM TUNEL assay. All three parameters were strongly correlated with each other.

data did not have a significant predictive power for results of any of sperm DNA integrity assays ($P > 0.08$).

As shown in Table II, all three assays showed negative correlations with sperm concentration, motility and normal morphology.

An additional group of pathological sperm is represented by the HDS group in the SCSA test. These cells possess an abnormally high green fluorescence intensity. The sum of HDS + DFI [median 32.0% (13–69)] correlated significantly ($P < 0.001$) with the proportion of TB dark cells [median 33.0% (6–64)] (Figure 2), and the absolute values in 35 samples analysed were very close and did not differ statistically from each other ($P = 0.6$).

Discussion

TB is a classic nuclear (cationic) dye used for external metachromatic and orthochromatic staining of chromatin, which

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Table II. Correlations between the proportion of cells with abnormal DNA integrity as detected by the TB test (dark cells), SCSA (DFI) and FCM TUNEL assay, and sperm concentration, motility (fast plus slow progressive motility) and normal morphology

	TB dark cells %	SCSA DFI	TUNEL-positive cells
Sperm concentration (ml/ml)	-0.70, $P < 0.001$	-0.64, $P < 0.001$	-0.57, $P = 0.014$
Sperm motility (%)	-0.74, $P < 0.001$	-0.77, $P < 0.001$	-0.71, $P = 0.001$
Normal morphology (%)	-0.72, $P < 0.001$	-0.71, $P < 0.001$	-0.71, $P = 0.001$

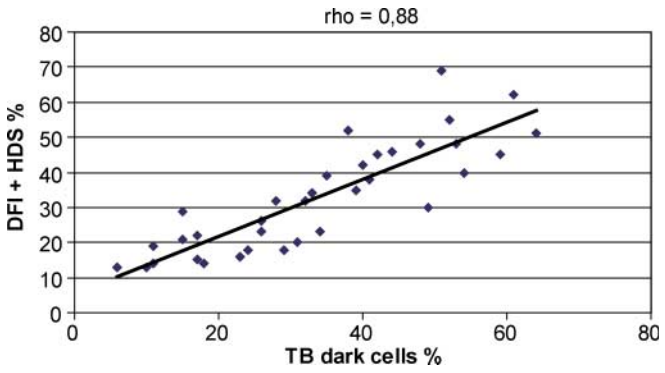

Figure 2. Relationships between abnormal sperm integrity as assessed by TB dark cells and the sum of DFI and HDS by SCSA.

overall is negatively charged (Michaelis, 1947; Sylven, 1954; Sculthorpe, 1978; Erenpreisa *et al.*, 1992). Thus, orthochromatic (blue) staining is the result of monomeric dye forms characteristic for either low dye concentrations or low accessibility of sites on the chromatin. Metachromatic (purple) staining, on the other hand, is the result of polymeric dye forms characteristic of cooperative binding to the DNA phosphate residues. The first situation corresponds to highly packaged chromatin of mature sperm cells with their very low stainability by external dyes (Darzynkiewicz, 1990). The second situation arises when chromatin proteins are more loosely electrostatically bound to the DNA and can dissociated from it easily, for example by short acid pre-treatment as used in our AO test (Erenpreiss *et al.*, 2001). In turn, DNA–protein interactions depend on the integrity and superhelicity of the DNA (Darzynkiewicz, 1990). In agreement with this, nicked DNA was shown to be more weakly electrostatically bound to chromatin proteins in somatic cells, therefore favouring binding and polymerization of external thiazine dyes (Erenpreisa *et al.*, 1988, 1997).

The present study confirms and extends our previous findings for the TB test (Erenpreisa *et al.*, 2003) and the AO test that the proportion of metachromatically and hyperchromically stained (red by AO and dark purple by TB) sperm cell nuclei correlates with the proportion of cells possessing DNA strand breaks, as revealed in the TUNEL reaction. The present results are also in line with previous published data, where the fraction of sperm showing DNA fragmentation by TUNEL assay was correlated with the SCSA-derived DFI fraction (Gorczyca *et al.*, 1993; Aravindan *et al.*, 1997; Zini *et al.*, 2001). However, although the three sperm nuclear tests analysed here are closely correlated to each other, they are not identical. The absolute values of TB-positive cells were significantly higher than those for DFI in the SCSA, and both were higher than those for the TUNEL test. It is noteworthy that it was also found in this study that the TB test is capable of detecting the total abnormal chromatin conformation considering the aggregate of HDS and DFI fractions revealed by the SCSA. HDS is thought to be indicative of immature sperm cells in which the chromatin might be less compact and more accessible to nuclear dyes (Evenson *et al.*, 2002). Higher levels of HDS also appear to predict decreased fertility potential both *in vivo* and *in vitro* (Evenson *et al.*, 1999; Larson *et al.*, 2000).

The formula 'TB dark cells = DFI + HDS' suggests that the TB probe in the chosen conditions is sensitive for both DNA strand breaks and chromatin packaging changes. This was also suggested previously by an increase of the TB dark cells by both DNase and dithiothreitol provocation, the latter mimicking immature sperm lacking protamines (Andreetta *et al.*, 1995; Erenpreisa *et al.*, 2003). The difference between both structural probes (TB test and SCSA) is explainable by different staining mechanisms. TB is only an external dye. External staining by TB is sensitive to the degree of DNA–protein binding, packaging regularity and also, in a cooperative manner, to DNA integrity (Erenpreisa *et al.*, 1992, 1997). AO, according to DNA substrate, can intercalate or bind externally. The thermodynamic conditions, which favour intercalary interaction of staining ligands with twisted DNA, are energetically favourable (Darzynkiewicz and Kapuscinski, 1990) and compete with the external staining (Kruglova and Zinenko, 1993). Therefore, unless DNA is not nicked, external staining in AO-based tests is limited. Thus, theoretically, the SCSA should be less sensitive to the changes in DNA–protein interactions, and more specific for DNA double strand breaks (DSBs).

Therefore, each of the two DNA structural probes (TB and SCSA) has its own advantages. At present, we do not know the real contribution of the whole spectrum of the sperm chromatin pathology (including rare DSBs, single strand breaks and incomplete maturation) on fertilization outcome. The TB structural probe, with its absorbance spectrum sensitive to different DNA conformations, may provide potential for discrimination of these factors. These applications of the TB test are the subject of current research by our group, in both cytochemical and clinical trials, to test their infertility prognosis power.

Our data are in agreement with those of other investigators (using a variety of assays) showing significantly negative correlations between sperm DNA integrity and semen quality (Sun *et al.*, 1997; Irvine *et al.*, 2000, Zini *et al.*, 2002; Benchaib *et al.*, 2003; Saleh *et al.*, 2003). However, neither

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sperm concentration, motility nor morphology were statistically significant predictors of the proportions of sperm cells with DNA strand breaks as assessed by means of TUNEL analysis, confirming sperm DNA integrity as an independent parameter for sperm quality.

In conclusion, we have demonstrated that the TB results correlate with results of other tests for sperm DNA and chromatin structure damage, suggesting that it may be considered as an alternative to these tests, especially in the absence of an FCM facility. Several assays are now available to detect sperm chromatin abnormalities. Due to the heterogeneous nature of human semen samples and to our incomplete knowledge of the complex process of sperm chromatin packaging, their complementary and overlapping levels are currently under active scrutiny in order to evaluate a possible clinical relevance for each of them.

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