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Toluidine Blue Test for Sperm DNA Integrity and Elaboration of Image Cytometry Algorithm

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Background: Sperm DNA integrity is of paramount importance in the prognosis of fertility. We applied image cytometry to a toluidine blue (TB) test we recently proposed.

Methods: Sperm samples from 33 men were assayed for standard sperm parameters and classified as normal or abnormal. Sperm smears were subjected to the TB test, DNA denaturation testing with acridine orange (AO), and terminal deoxyuridine triphosphate biotin nick end labeling (TUNEL). In CCD image analysis, TB-stained sperm cell heads were microscopically assigned to one of four color groups (dark, blue, light violet, and light blue). The optical densities of 6,600 cells in green and red CCD images were used to elaborate an algorithm for discrimination of these groups.

Results: The proportions of sperm in TB color groups, as estimated with the developed image cytometry algorithm, correlated with microscopic features. The number of TB dark cells correlated with the number of AO-red and TUNEL$^+$ cells. The proportion of TB dark cells in normal samples did not exceed 35%. Light-blue sperm cell heads prevailed in normal samples, whereas dark and blue sperm cell heads dominated in abnormal samples.

Conclusions: The TB test was suitable for the assessment of sperm cell DNA integrity. The elaborated image cytometry algorithm can be used for this purpose and for finer determination of sperm nucleus status. Cytometry Part A 52A:19–27, 2003. © 2003 Wiley-Liss, Inc.

Key terms: infertility; sperm DNA integrity; toluidine blue test; image cytometry

Sperm DNA integrity is one parameter of sperm quality that has paramount importance in the prognosis of fertility and the outcome of assisted reproductive procedures (1–7). In andrologic practice, mostly indirect methods for estimation of the DNA integrity are used. These methods are based on the ability of some stains to test the conformation of sperm chromatin, which in turn depends on DNA strand breaks and DNA interaction with proteins (8,9). The idea to test sperm chromatin with acridine orange (AO) after DNA denaturation occurred in the 1960s (10–12). In conjunction with flow cytometry, this method is known as sperm chromatin structure assay (SCSA) (13). The ability of cell nuclei to produce metachromatic (red) AO fluorescence when they contain single-stranded DNA as opposed to othochromatic (green) fluorescence in intact nuclei is the basic principle of the method. After applying the SCSA test in a dual (red/green) parameter system, a broadly heterogeneous pattern was found indicative of reduced or absent fertility (14). By SCSA, the infertility threshold has been established by different investigators (4,5,7) as being 27–40%. This is the percentage of cells containing damaged or abnormal DNA; over this threshold, the sperm are judged to be unable to fertilize an egg. However, the SCSA method requires the use of flow cytometry, which is affordable only in large centers. The other, relatively widely used microscopic method of Tejada et al. (15) with the use of AO needs practically immediate scoring of samples due to their quick fading. Another AO protocol developed at this laboratory allows scoring of fluorescence within 48 h (9), but it is rather time consuming.

We also published a description of a microscopic method of staining of sperm smears with the thiazine dye, toluidine blue (TB), showing its comparability with the currently used microscopic methods of affinity staining (9). Our work originated from previous studies demon-

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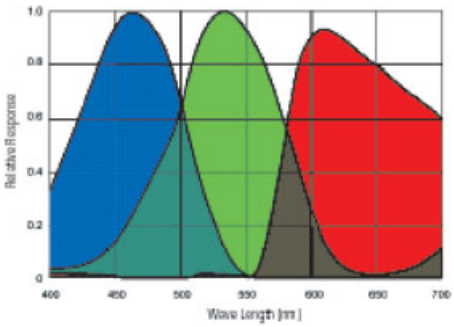

FIG. 1. CCD spectral curves (without optical and protective filters). A relatively high response (0.6–1.0) covers wavelengths from 500 to 575 nm in the green filter and from 580 to 700 nm in the red filter. Thus, the green image is sensitive to the metachromatic and the main toluidine blue (TB) absorption peaks (520–570 nm). The maximum response in the green image (530 nm) corresponds to the metachromatic area of the TB absorption. The red image includes the TB orthochromatic absorption peak (630–660nm). The blue image has low sensitivity to the TB absorption spectral area. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

strating the similarity between AO and TB staining after short acid treatment on somatic apoptotic cells displaying metachromasia in the nuclei with fragmented DNA (16). As reported by several investigators, the spermatogenetic program involves apoptosis (17,18) and introduction of functional DNA breaks (19). In common with somatic apoptotic cells, sperm cells containing damaged DNA, as measured by the AO test, are positive for DNA double-strand breaks (DSBs) (20,21) in the terminal deoxyuridine triphosphate biotin nick end labeling (TUNEL) reaction (22).

In our previous study (9), we found that sperm cell nuclei with red fluorescence by AO test are stained darkly (purple) by the TB procedure as opposed to light TB staining of the nuclei that fluoresce green in the AO test. A similar method applying TB (but with acid in Carnoy's fixative) has been proposed by Andreeta et al. (23). The TB method is simple and economic, with no time constraints on scoring. However, the association of positive TB test results with DNA damage remained to be directly proved, and the infertility threshold referring to quantified sperm parameters (not just to fertility anamnesis) (9) needed to be established. In addition, we considered the possibility of using image cytometry to make the scoring more objective.

The classic investigations of Michaelis and others (24,25) and our own research (16,26) described three peaks in TB absorption spectra that characterize various conformations of the DNA. There is an orthochromatic peak (blue monomeric staining) at 630–660 nm and a metachromatic peak (red oligopolymeric staining) with absorption maximum at 520–550 nm. The broad peak, which is always present (as determined by Michaelis et al. as dimeric staining), with a maximum at 568 nm, can shift toward orthochromatic or metachromatic wings if these are correspondingly exaggerated. As seen in Figure 1, where the spectral sensitivity of the CCD camera is presented, this sensitivity fits mostly into the TB spectral range known for somatic cells.

In the present work, we also paid attention to intermediate forms of sperm heads always observed with TB staining. The state of the chromatin, the integrity of DNA in the sperm cell heads of intermediate colors, and their relation to clinical pathology have not been studied. We also wondered whether these forms could be distinguished by image cytometry.

The present research had four objectives: (a) to make a preliminary determination of the infertility threshold for the TB test (in our protocol) by using standard sperm quality parameters and the AO test as a reference method; (2) to characterize cytochemically the differently TB-stained sperm heads into main color groups: dark (D), light violet (LV), blue (B), and light blue (LB); (3) to clarify the relation of the color staining groups to sperm standard quality; and (4) to elaborate an algorithm for the application of this test in color image cytometry and obtain objective data for individual spectrospermatograms.

MATERIAL AND METHODS

The Institutional Review Board approved the study protocol. Thirty-three sperm samples were obtained from men of infertile couples and healthy volunteers.

Fresh sperm was used to determine standard sperm parameters (sperm concentration, motility, and normal morphology) according to the recommendations of the World Health Organization (WHO) (27). In comparative assessments of standard sperm quality, we used the sum of normalized sperm parameters according to the formula we previously developed (28):

$$\text{sperm quality} = \text{Conc} \times 10/\text{Conc}_{\max} + \text{Motil} \times 10/\text{Motil}_{\max} + \text{Nmorph} \times 10/\text{Nmorph}_{\max}$$

where Conc is the sperm concentration in millions per milliliter, Motil is group A fast progressive motility in percentage, and Nmorph is the percentage of cells with normal morphology. Maximum values for this set of samples, arbitrarily equal to 10 U each, were 300 for $\text{Conc}_{\max}$, 34 for $\text{Motil}_{\max}$, and 36 for $\text{Nmorph}_{\max}$. In addition, sperm samples were classified as abnormal if one or more parameter was under the following critical WHO (27) criteria: 20 million/ml for sperm concentration, 25% for group A motility, and 20% for normal morphology.

Our previous work with a modified AO test on approximately 200 sperm samples (28) showed the same infertility threshold as found with the SCSA test (4), which was approximately 30% of sperm cells with abnormal DNA in unsorted samples. Therefore, in this investigation, our AO test was used as a reference method to compare and estimate the TB results. The same sperm samples were stained on parallel slides by both methods.

Staining Protocols

The following protocols were applied after semen liquefaction at 37°C for 30 min.

Sperm was pelleted at 1,000 rpm for 10 min and resuspended in its own supernatant up to an approximate concentration of 2×10^8 cells/ml and then air dried for 30–60 min.

AO staining. Dried smears were fixed with freshly made 96% ethanol:acetone (1:1) at 4°C for 30 min to a maximum of 24 h. The slides were not allowed to air dry after fixation. Rehydration consisted of bathing slides in 96% ethanol for 5 min, 70% ethanol for 5 min, and 30% ethanol for 3 min at room temperature (RT). Treatment in phosphate buffer saline (PBS) for 5 min was followed by treatment with 1 N HCl for 1 min at 60°C. Slides were rinsed three times in distilled water for 2 min. Slides were then dipped into McIlvain's citric phosphate buffer (pH 4) for 5 min, stained with 0.038 mg/ml (10^{-4} M) of AO for 15 min (AO was prepared daily from a stock solution consisting of 7.6 mg of AO [Sigma Chemical Co., St. Louis, MO] in 1 ml of distilled water and stored in the dark at 4°C for 4 months), and rinsed three times for 5 min with 10^{-6} M of AO in the same buffer. Coverslips were placed over this solution and sealed with nail polish. Samples were kept in dark at 4°C and scored within 24 h. Sperm cell heads of good DNA integrity showed green (green-yellow) fluorescence, and impaired cell heads showed orange-red fluorescence.

TB staining. Dried smears were fixed with freshly made 96% ethanol:acetone (1:1) at 4°C for 30 min and air dried (and can be preserved at room temperature for about 1 month). Hydrolysis was done with 0.1 N HCl at 4°C for 5 min and rinsing three times in distilled water for 2 min per rinse. Staining with 0.05% TB (Gurr-BDH Chemicals Ltd., Poole, UK) was applied for 5 min. The staining buffer consisted of 50% McIlvain's citrate phosphate buffer (pH 3.5). Slides were rinsed briefly in distilled water and lightly blotted with filter paper. For permanent preparations, dehydration was done 2×3 min with tertiary butanol at 37°C and 2×3 min with xylene (Histoclear RA Lamb Labs, USA), followed by embedding in DPX. The TB working solution in buffer was prepared monthly and stored at 4°C. A stock solution of 1% TB in distilled water was preserved at 4°C for no longer than 1 year.

Light sperm cell heads (LB, LV, and B) were scored as possessing DNA of normal integrity, and those with dark (purple or DB) were scored as having damaged DNA.

Optical Density (OD) Measurements

In the AO test, the percentage of sperm cells with abnormal DNA integrity (ADI) as demonstrated by red-orange fluorescence was scored for every 300 cells by one observer with the use of a Nikon TE 300 fluorescence microscope (Nikon GmbH, Dusseldorf, Germany) and a mercury lamp (excitation band-pass filter, 420–490 nm; emission was observed through a suppression filter with a 515-nm limit; magnification, 10×40). For TB-stained specimens, ADI was estimated independently for 200 cells by a second observer using oil immersion (10×100) on a Leica DM LB microscope supplied with a CCD TV COHU 2200 and image analysis program (Image Pro Plus 4.1). Calibration of the COHU 2200 camera for measurement of ODs in three color images by using the reference slide PRESS-PRO21 displayed ideal curves for each image, with correlation coefficients close to 1 (not shown).

The average OD in three color images (blue, green, and red) was determined over the entire sperm head (including the acrosomal area). Immature sperm cells, leukocytes, and detritis were toggled off. Simultaneously, each sperm cell head was visually classified on a color display into four color categories: D, LV, B, and LB. Subsequently, image ODs of each cell, grouped into these visually classified categories, were collected and averaged.

To verify the CCD discrimination of the samples with different ADIs and the manual classification of the four color groups, spectra were obtained from a proportion of the samples with the use of a cytospectrophotometer (Lietz MPV-3) and a plug area of 1 μm (settled over the denser part of the chromatin) and a monochromator step of 5 nm. OD was measured in 200 random cells representing the entire sample and in 50 cell heads for each visually discriminated color group.

Cytochemical Experiments

To test and model the TB staining of sperm head color groups, several reagents were applied to the sperm of donors with excellent standard parameters. Cell smears were prepared and air dried for 30 min. Afterward they were fixed in ethanol:acetone at 4° C for 30 min or methanol:acetone at −20°C for 20 min, air dried for 30 min, and treated as follows:

DNAse I (Sigma Chemical Co.) was used for introducing multiple DSBs: 4 μm/ml of DNAse I in a buffer at RT for 60 min; the DNaseI buffer consisted of 30 mM of Tris/HCl buffer (pH 7.2) in water treated with diethylpyrocarbonate (DEPC), 1% bovine serum albumin, 140 mM of sodium cacodylate, 4 mM of $MgCl_2$, 1 mM of $CoCl_2$, 0.1 mM of ditiothreitol (DTT; Sigma Chemical Co.); smears were washed three times in DEPC water

DNAse I buffer was used without DTT and bivalent salts at RT for 60 min

0.1 mM of DTT in 30 mM of Tris/HCl buffer in DEPC water (pH 7.2) at RT for 60 min; controls were treated with DTT alone or with buffer alone

BSp681 restrictase (size of DNA fragments, 58 kb) was purchased from Fermentas (Vilnius, Lithuania) and applied to sperm smears at a concentration of 0.4 μm/μl at 37° C for 30 min in 50 mM of Tris/HCl buffer (pH 7.5), 10 mM of $MgCl_2$, 100 mM of NaCl, and 0.1 mg/ml of bovine serum albumin, and three washes in PBS; buffer alone was applied as a control

In addition, fresh sperm was washed in PBS and treated with 0.06% H_2O_2 in PBS for 10 min at RT, washed in PBS, resuspended in spermal supernatant, prepared as slide smears, and fixed as indicated above

The nontreated and treated (with different reagents) sperm cells were assessed by the TB test. The results were similar for both types of fixation. To identify whether sperm heads in the different color groups had DNA strand breaks, the TUNEL reaction in a fluorescent variant (FISEL)

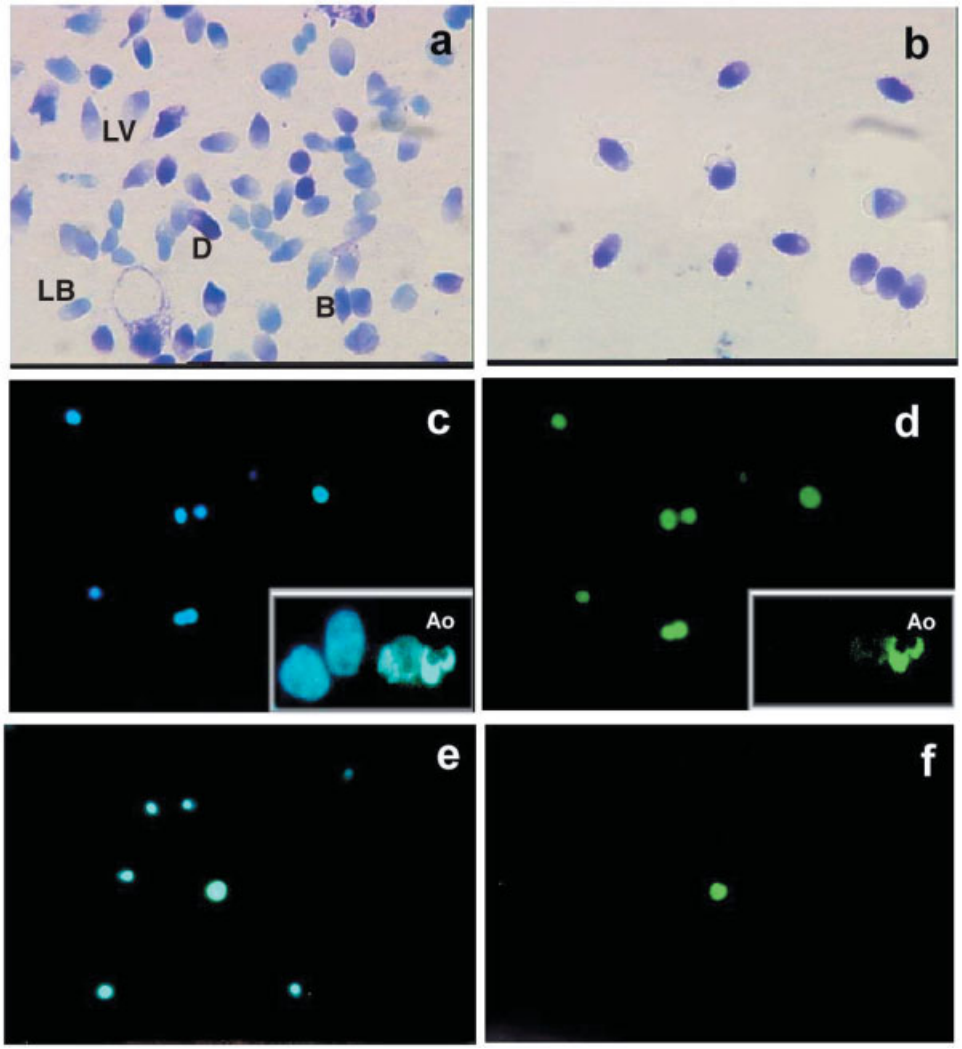

FIG. 2. Typical results of the toluidine blue (TB) and TUNEL tests on sperm smears. **a:** TB staining of sperm from infertile men. Dark (D), light-blue (LB), and intermediate color (LV, light violet; B, blue) cells can be seen. **b:** TB test on healthy donor sperm treated by DNAse I as a positive control for the TUNEL reaction. All sperm heads are dark. **c,d:** Characterization of the sensitivity of the applied fluorescent TUNEL reaction: the TUNEL reaction (cell nuclei positive for fluorescein isothiocyanate; FITC) after introduction of double-strand breaks by DnaseI; DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI in c; FITC in d). All sperm heads were DAPI and TUNEL$^+$. Insets show the TUNEL reaction applied to irradiated lymphoma cells: three cell nuclei are stained by DAPI and of these only the disintegrating apoptotic cell (Ao) with characteristic nuclear crescents is TUNEL$^+$. **e,f:** Representative TUNEL reaction in an intact sperm sample (DAPI in e, FITC in f). Only one sperm cell head among five stained by DAPI is TUNEL$^+$. Original magnification, 400×.

was carried out in preparations fixed by methanol:acetone (−20°C) as recommended by Ansari et al. (29) according to the protocol of Smith and Haaf (19), with some modifications as follows.

For only the positive controls, DNaseI (Sigma Chemical Co.) was applied, as indicated above. For DSBs, the reaction mix was applied to slides under cover glass at 37°C for 1 h, and the reaction was arrested in a stopping solution (605 mg of Tris buffer, 372 mg of ethylene-diaminetetraacetic acid, and 100 ml water; pH was adjusted to 7.4) for 5 min; slides were rinsed in 30 mM of Tris/HCl buffer (pH 7.2) for 3 min, washed 2 × 5 min in PBS, washed in 1 M HEPES (MHBS) for 5 min, incubated in avidin conjugated to fluorescein isothiocyanate (FITC; 1:200 in MHBS; Vector Laboratories, Burlingame, CA) at RT for 45 min, washed 3 × 5 min in MHBS, stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.; 1 μg/ml in MHBS) for 5 min, embedded in photoprotecting medium, and sealed with nail polish. The TUNEL reaction mix (30 μl/sample) consisted of 0.5 μl (10 U) of terminal deoxynucleotidyl transferase (Fermentas), 6 μl of 5× cacodylate buffer (supplied with enzymes), 0.15 μl of 1 mM of biotin-11-deoxyuridine triphosphate (Sigma Chemica Co.), 0.45 μl of 1 mM 2′-deoxythymidine 5′-triphosphate (dTTP) (Fermentas), and 22.9 ml of DEPC-treated water.

Slides treated with the fluorescent TUNEL reaction were scored under a Leica fluorescent microscope using two separate filters: (a) an excitation filter (ultravioloet + violet; band pass, 355–425 nm) and a suppression filter (long pass, 470 nm) for DAPI-stained nuclei and (b) an excitation filter (blue; band pass, 450–490 nm, and a suppression filter (long pass, 515 nm) for FITC$^+$ cells containing DSBs.

Statistical analysis was performed with power analysis and two-tailed Student's *t*-test with the use of Microsoft Excel 97.

RESULTS

According to WHO standard quality criteria (27), 12 sperm samples were classified as normal and 21 as abnor-

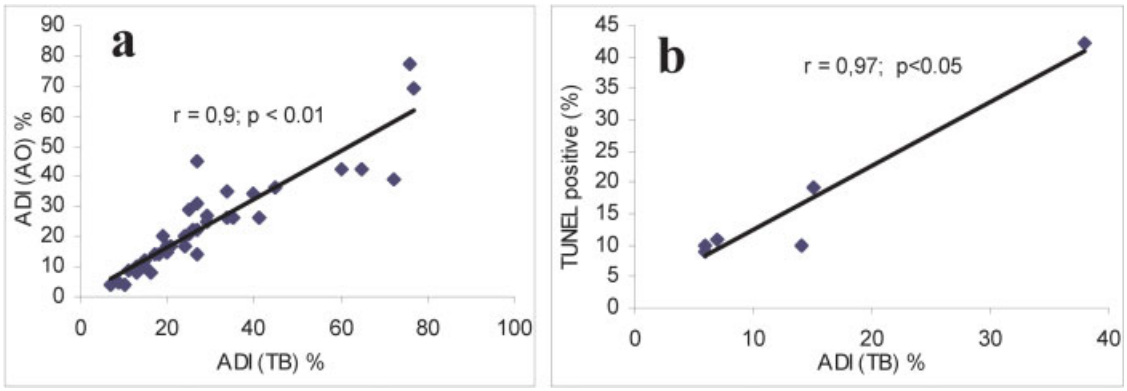

FIG. 3. Verification of the toluidine blue (TB) test as an indicator of DNA abnormality. **a:** There is a strong correlation between metachromatic red-orange sperm cell heads in the Acridine Orange (AO) test and dark heads in the TB test on the same samples. **b:** There is a strong correlation between TB-dark and TUNEL$^+$ cells on the same samples. The results of each test were scored by independent observers. ADI, abnormal DNA integrity.[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mal. A typical abnormal sperm smear stained with the TB test is shown in Figure 2a. The presence of LB and D cells can be seen, as can some cells with intermediate colors (LV and B). Across all samples, the index for abnormal DNA (ADI of the TB test when counted as a percentage of D sperm cell heads) correlated linearly with the ADI of the AO test when counted as a percentage of red-orange cell heads (Fig. 3a). Figure 3b shows good correlation between ADI in the TB test and the proportion of TUNEL$^+$ cells. ADI values in the AO and TB tests plotted against sperm quality, assessed as a sum of normalized standard parameters for each specimen, showed similar patterns for both staining reactions (Fig. 4).

Spectral characteristics of the TB-stained sperm samples from three patients with very different degrees of DNA abnormality were compared by measuring 200 cells with the cytospectrophotometer and using three-color image cytometry. The following features of the monochromator spectra (Fig. 5a) were observed: (a) a larger ADI (more cells with damaged DNA) was associated with higher OD in the green part of the spectrum; and (b) a larger ADI was associated with a shift toward the short wavelength region (increasing red polymeric component of staining). Both deviations in the TB spectra caused by DNA damage were superimposed in the OD of the CCD green image (Fig. 5b), which allowed good discrimination between samples of different ADIs (TB). The CCD spectral sensitivity curves presented in Figure 1 show a broad green image band with a maximum at 530 nm that includes the area of the main TB absorption peak (about 570 nm) and, to a greater extent, that of DNA metachromasy (520–550 nm). The CCD blue image (maximum, 450 nm) had low sensitivity to TB staining because this wavelength is nearly out of the TB absorption spectrum, as seen by comparing the spectra in Figure 5a and 5b. However, the red image (maximum, 610 nm; with a wide right wing seen in Fig. 1) was well within the orthochromatic part of TB absorption in sperm cells.

We investigated whether we could discriminate the four TB color groups with the use of image cytometry. Visual discrimination of the four color groups was proved for several samples by cytospectrophotometry (Fig. 5c).

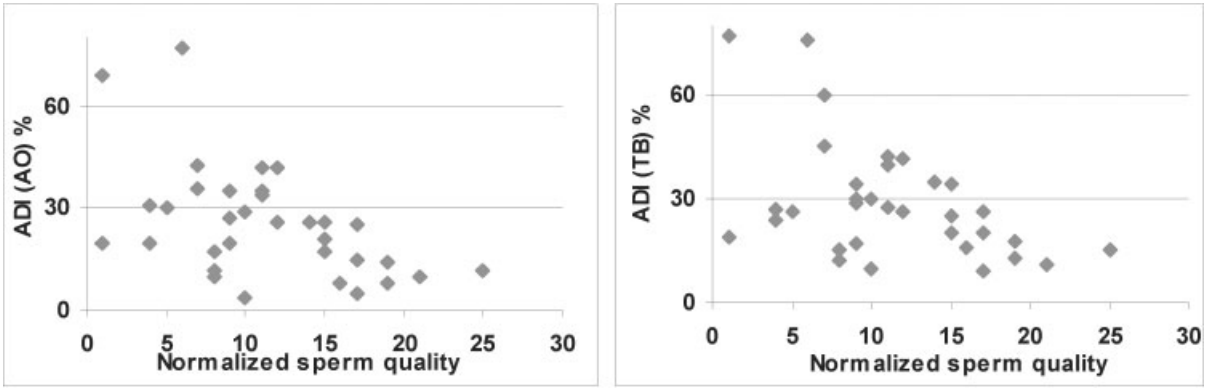

FIG. 4. Comparative distribution of abnormal DNA integrity (ADI) in Acridine Orange (AO) and toluidine blue (TB) tests in relation to normalized sperm quality in the same sperm samples. Samples of good quality have low ADIs (<30%).

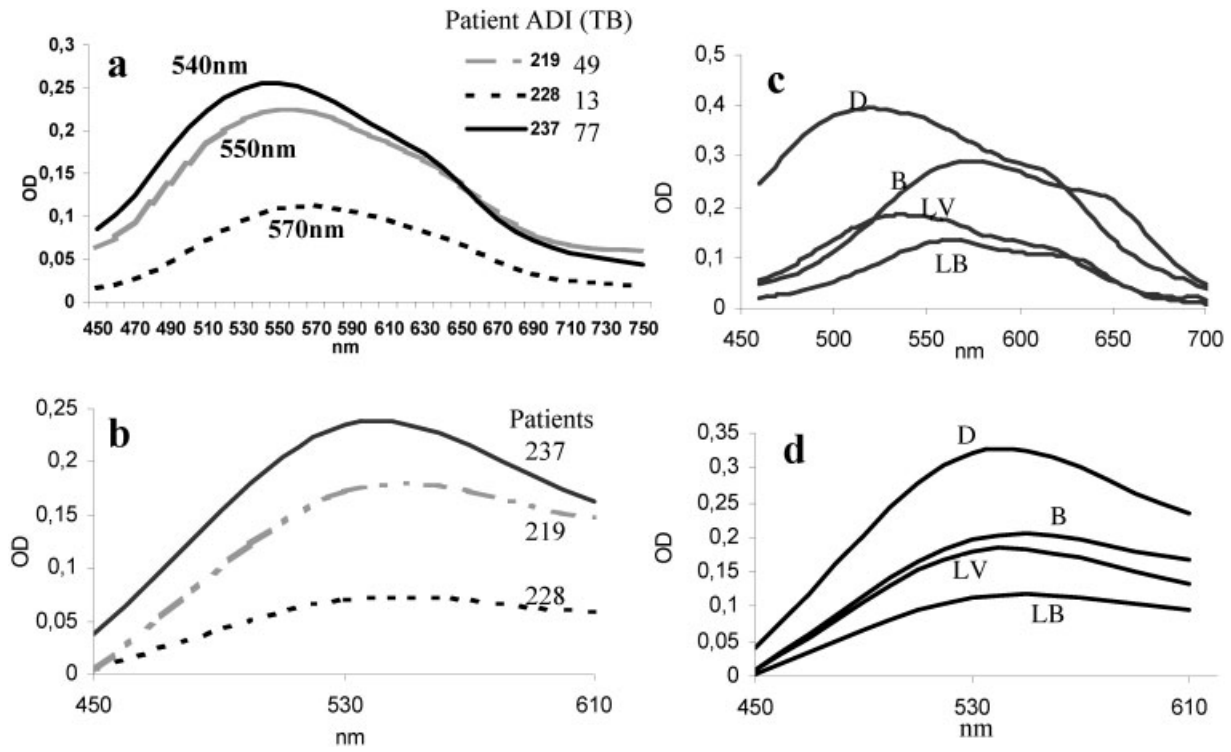

FIG. 5. Spectral characteristics of sperm staining in the toluidine blue (TB) test as recorded by a spectrophotometer with a small monochromator step (a,c) and by densitometry in three image colors, where the smoothed curves were obtained from only three measurement points (b,d). **a,b:** Comparison of spectra recorded in 200 random sperm cells for three patients (219, 228, and 237) with very different abnormal DNA indexes (ADIs). **c,d:** Comparison of spectra recorded in 50 cells from each of the four color groups in sperm of one patient. b: There is good discrimination between samples, with different ADIs in the green image. d: There is good discrimination between dark (D) and light-blue (LB) color groups and somewhat poorer for the light-violet (LV) and blue (B) groups. A higher absorption in the red image in relation to absorption in the green image seems to be the only way to distinguish blue cells of different staining intensities from dark and light-violet cells. OD, optical density.

Also, CCD curves differed for the visually discriminated four color categories of sperm heads in TB staining (Fig. 5d). We determined OD limits for each color group in green and red images by using 6,600 cells from 33 samples (Fig. 6a,b). Figure 6 shows a marginal overlap in the green image between the ODs of LB and LV cells and between the D and B cells. Higher OD red/OD green for blue cells allowed their differentiation from D and LV cells.

The results of the algorithm developed for this analysis in comparison with visual classification of the same cells is presented in Figure 6c–f. There is very good discrimination between D and LB cells and fairly good discrimination for the intermediate color groups, i.e., B and LV.

To determine whether the color groups had any relevance to sperm quality, the samples were categorised as normal or abnormal according to standard quality WHO criteria, as detailed in Materials and Methods. The proportions of the four color categories in normal and abnormal sperm samples are presented in Figure 7a. In sperm of normal standard quality of presumably fertile men, the average percentage of D cells was 22 ± 10% (one standard deviation did not exceed 32%) as opposed to 35 ± 16% in abnormal sperm of presumably infertile men ($P = 0.009$, statistically significant). Thus, the infertility threshold for the TB-test was set at about 35% ADI. Despite a rather large standard deviation for each color group in both sets, a statistically significant difference was found: there was a higher proportion of LB sperm heads in normal than in abnormal sperm ($P = 0.03$). Further, the B sperm heads were significantly more abundant in the poor quality sperm ($P = 0.015$), whereas the difference in the proportion of LV sperm heads in the both sets was insignificant ($P = 0.48$).

Cytochemical experiments were undertaken to determine which changes of DNA and proteins could be responsible for these sperm cell head color groups. The TUNEL reaction is sensitive to late apoptosis in somatic cells (Fig. 2c,d, insertions). In the positive control for TUNEL with DNAse I, all sperm cell heads were TB-D (Fig. 2b) and TUNEL$^+$ (Fig. 2c,d). When buffer alone was used, a more saturated color was obtained: LB heads became bright blue and LV heads became bright violet (not shown). After changing the components of the DNaseI buffer, we found that the color change was due to the presence of DTT. However, treatment with DTT in 30 mM of Tris/HCl buffer in DEPC water tested in three experiments in donor sperm increased neither the number of D (purple) cells nor the counts of TUNEL$^+$ cells (not shown). To discover which changes in TB sperm color could be introduced by small amounts of DNA DSBs, the

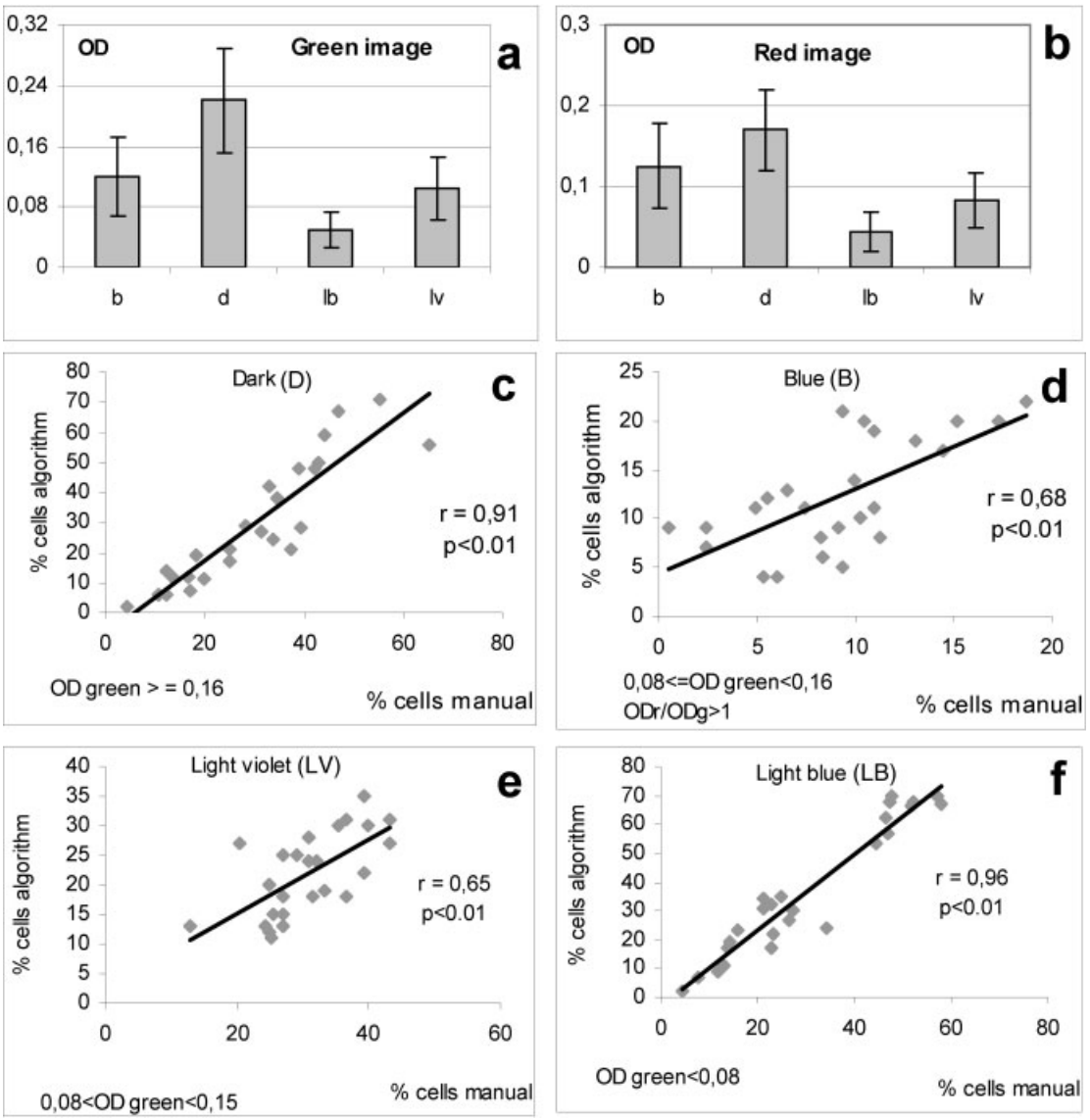

FIG. 6. Discrimination of sperm cell head toluidine blue (TB) color groups. **a,b:** Data for optical density (OD) in green and red images obtained with Image Pro Plus 4.1 from 6,600 sperm cells, which had been sorted into four TB colors. Bars represent averages, and lines represent standard deviations. **c–f:** Relations between the number of cells of each color category discriminated manually and with the aid of the algorithm. A good linear correlation is observed, particularly for dark (D) and light-blue (LB) cells (B, blue; LV, light violet). The formula for each color group is given.

Bsp681 restrictase was applied to an excellent donor sperm smear. This treatment increased the number of LV cells from 10% (in restrictase buffer alone) to 53%. The proportion of D cells was at the same low level as in the buffer control (12%). Treatment of fresh sperm samples with hydrogen peroxide increased the number of AO-red, TB-D, and $TUNEL^+$ cells with altered head morphology, i.e., very large, round heads with uneven contours (not shown).

An example demonstrating the utility of the spectrospermatogram using the algorithm and monitoring treatment in a male with secondary infertility is presented in Figure 7b. A significant change in the spectrospermatogram within the first months of therapy was observed: ADI decreased from 77% to 22%, under the infertility threshold. The number of B cells also decreased. The motility and concentration of sperm cells were greatly improved. In contrast, the proportion of LV cells increased to over 40% and the morphology remained under the WHO limit. Despite normalized ADI, at that time the patient was unable to fertilize naturally or by an intracytoplasmic sperm injection.

DISCUSSION

TB is a classic nuclear dye used for metachromatic and orthochromatic staining of chromatin. These features have been shown to be a sensitive structural probe for DNA secondary structure and packaging in situ (16,26).

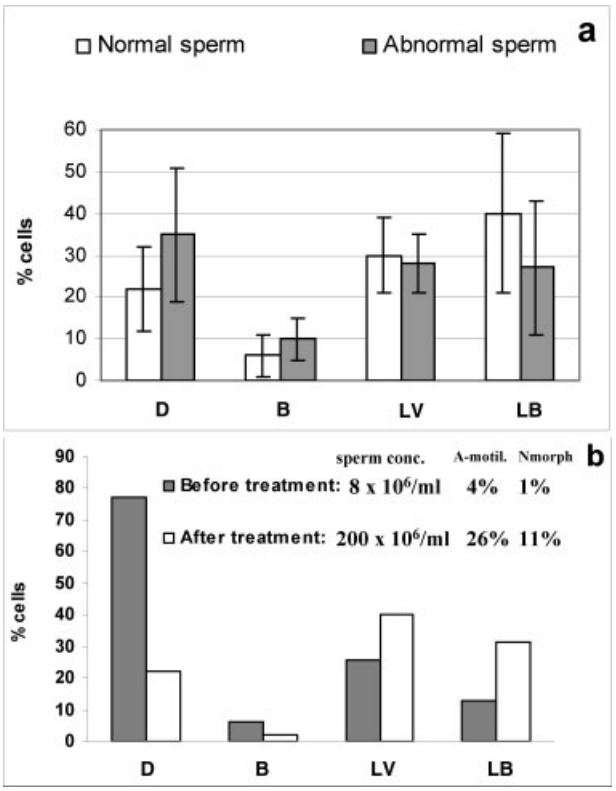

FIG. 7. Proportions of four color sperm cell head groups obtained by the toluidine blue (TB) test and image cytometry. **a:** In normal (n = 11) and abnormal (n = 22) sperm samples according to criteria of the World Health Organization, a statistically significant difference was found for dark (d), blue (b), and light-blue (lb) cells (lv, light violet). **b:** TB test and spectrospermatograms of a patient with secondary infertility before and after treatment with antioxidants.

We found that TB-stained D sperm cells are TUNEL$^+$ and could be mimicked by DNaseI treatment after introducing a large number of DNA DSBs. Experimentally, TB-D, AO-orange, and TUNEL$^+$ sperm cells could be induced in fresh sperm by oxidative damage due to hydrogen peroxide. The infertility threshold for the TB test was estimated at about 35%, a few percentages higher than that obtained for our AO reference test in the present study and for a large number of samples in a previous study (28). The 35% threshold is within the range reported by others: from 27–30% (4,7) to 40% (5).

The present results showed that the calibrated CCD camera and software, which permitted precise measurement of OD of the green and red images, can be applied to an objective assessment. This type of equipment is readily available for small family centers and can improve their work by allowing them to develop reference values.

The results obtained for the TB-stained sperm heads, intermediate between poor (D) and very good (LB), are interesting. The cytochemical study showed that DTT pretreatment can saturate (i.e., increase) the staining of DNA phosphate groups in the LB cells and convert them to B cells without shifting the color spectrum. Thus, the secondary structure of the DNA is evidently not changed by DTT pretreatment.

With this treatment, LB cells became blue, similar to natural B sperm cell heads. A comparison of the TUNEL reaction with the AO results in our samples suggested that the natural blue sperm cell heads do not contain DSBs. The cytochemical mechanism of DTT likely was due to its ability to reduce covalent disulfide bonds (presumably of sperm protamines). This action would relieve exposure of DNA phosphates for staining by cationic dyes. Our clinical data associated B cells with poorer sperm quality. DTT treatment mimics natural B sperm cell heads, so those heads may represent immature spermatozoa with histones not sufficiently replaced by protamines, apparently without primary DSBs.

LV cells, which are always present in large numbers in relatively good sperm samples, possess a weak red metachromatic component and an increased OD in the green image band as compared with LB heads. Although LB cells statistically prevail in normal quality samples and, from our experience, are abundant in the best samples, this is not the case for LV cells. In our previous studies, we associated the metachromatic shift in TB staining with DNA strand breaks already appearing in early somatic apoptosis. Therefore, we assume that natural LV sperm cell heads also contain some DSBs, albeit in amounts below the sensitivity of the applied TUNEL reaction. The model experiment with Bsp681 restrictase, which introduced rare DNA DSBs (roughly one per DNA domain) and which increased the proportion of LV cells fivefold, did not affect the counts of TUNEL$^+$ cells, indirectly supporting this hypothesis. Rare DSBs may remain from incomplete spermatogenesis in testes. Conversely, this and a previous study (28) suggested that multiple DNA DSBs result mainly from oxidative stress, which is considered a key factor in the etiology of male infertility irrespective of its origin (30).

Many cases of sperm possessing low ADIs but with the men being infertile have been reported in the literature (4). Our previous work and the data shown in Figure 4 also showed that the poor quality sperm can display paradoxically low ADIs. However, ADI considers only cells with multiple DNA DSBs. Therefore, discrimination of intermediate TB color sperm head categories and clarification of their relation to the primary DNA damage, the ability of its repair, oxidative stress, and fertility need more research and may acquire practical significance in the future.

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