Article

Double probing of human spermatozoa for persistent histones, surplus cytoplasm, apoptosis and DNA fragmentation

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Abstract

Individual spermatozoa were assessed with pairs of probes for persistent histones and cytoplasmic retention, persistent histones and DNA fragmentation, and persistent histones and apoptotic markers. The individual spermatozoa were treated sequentially with combinations of probes for these cytoplasmic and nuclear biochemical markers. Sperm fields were recorded with computer-assisted imaging, and staining patterns with the two probes in the same spermatozoa were examined and scored as light, intermediate or dark (mature to arrested-maturity spermatozoa). The effects of arrested sperm maturation were similar with respect to the cytoplasmic and nuclear characteristics of spermatozoa in 84% of cells, indicating that cytoplasmic and nuclear attributes of arrested sperm maturation are related. However, there were moderate (intermediate–dark or intermediate–light patterns, 14.5% of cells) or major (light–dark patterns, 1.6% of cells) discrepancies in the intensity of the double staining patterns. Thus, testing with single maturity markers may not be fully reliable. These findings are important with respect to: (i) arrested sperm maturation; (ii) potential efficacy of antioxidant and similar therapeutic strategies in subfertile men, as spermatozoa with infrastructure defects due to mismaturation or maturation arrest are unlikely to respond to interventions; and (iii) detection of adverse male environmental exposures.

Keywords: *anti-oxidant therapy, apoptosis, DNA fragmentation, multiple biochemical probes, persistent histones, sperm maturity*

Introduction

DNA integrity is an attribute of paramount importance in spermatozoa, because fragmented DNA in spermatozoa adversely affects the paternal contribution to fertilization and conception, and to zygote development (Aitken *et al*., 2003, 2004; Seli *et al*., 2005; Borini *et al*., 2006; Tarozzi *et al*., 2007). DNA chain fragmentation is thought to be related to the various nuclear attributes of arrested/diminished sperm maturity, as shown by excessive persistent histones, which indicate a lower efficiency of histone–protamine replacement (Dadoune *et al*., 1988; Foresta *et al*., 1992; Hammadeh *et al*., 1996; Morel *et al*., 1998). Lower concentrations of the protamine–DNA complex render the DNA chains more vulnerable to damage due to inappropriate DNA folding and packaging (Spano *et al*., 2000; Steger *et al*., 2003; Ozmen *et al*., 2007).

Further, studies on sperm maturity and expression levels of the heat shock protein (HspA2) chaperone protein indicated that the low expression of HspA2 in diminished maturity spermatozoa contribute to a reduced DNA repair capacity (Eddy, 1999; Huszar *et al*., 2000). Indeed, the destruction of the gene for the homologous chaperone protein, HSP70–2, in mice caused diminished sperm production, apoptosis

and male infertility (Dix *et al.*, 1996). Conversely, in human spermatozoa with arrested maturity and low HspA2 concentrations, increased levels of the apoptotic process have been found (Cayli *et al.*, 2004).

Another relevant aspect of DNA damage is the relationship between production of sperm reactive oxygen species (ROS) and arrested sperm maturation. This relationship was established based on proportionally increased ROS production and cytoplasmic retention in spermatozoa, as detected by higher concentrations of sperm creatine kinase (CK) and other cytoplasmic proteins (Aitken *et al.*, 1994; Huszar and Vigue, 1994). A further relationship was demonstrated between sperm cytoplasmic content and the rate of lipid peroxidation measured by malonyl dialdehyde production (a direct end product of lipid peroxidation). Regarding spermatozoa with arrested maturation, in combined fractions of mature spermatozoa and spermatozoa with arrested maturity subjected to repeated co-centrifugation and co-coincubation at 37°C that provided extensive sperm-to-sperm contact, there was no increase in ROS production in the mature sperm fraction. The conclusion was drawn that increased sperm ROS production is primarily an 'inborn' error of arrested spermatogenetic maturation, rather than a process that may be 'acquired' by mature spermatozoa (Huszar and Vigue, 1994). The CK probe of cytoplasmic retention also indicated that in semen samples, there is a polymorphism, as well as a day-to-day and a subject-to-subject variation regarding the proportion of mature and arrested maturity spermatozoa, whether in normozoospermic and oligozoospermic men (Huszar *et al.*, 1988, 2007).

With the various sperm biochemical markers aimed at the cytoplasmic and nuclear attributes of spermatozoa, one can detect features of diminished sperm maturity, including cytoplasmic retention with CK (Huszar and Vigue, 1993), persistent histones by aniline blue staining (Dadoune *et al.*, 1988; Foresta *et al.*, 1992; Morel *et al.*, 1998), and the presence of active apoptotic process with caspase-3 staining (Cayli *et al.*, 2004). The assessment of DNA degradation is also possible by in-situ DNA nick translation (Irvine *et al.*, 2000).

Most recently, methods were developed the authors' laboratory that allow the application of multiple biochemical probes to the same spermatozoa, in order to explore the hypothesis that there is a relationship between the various nuclear and cytoplasmic attributes in arrested sperm maturation. In the present experiments, this relationship was studied with probe combinations for persistent histones and cytoplasmic retention, persistent histones and DNA degradation, and persistent histones and apoptotic markers.

Materials and methods

Overview of experimental design and methods

The double probing studies in the same spermatozoa were carried out as outlined in **Figure 1**. The steps were as follows. The spermatozoa, smeared onto glass slides, were fixed with

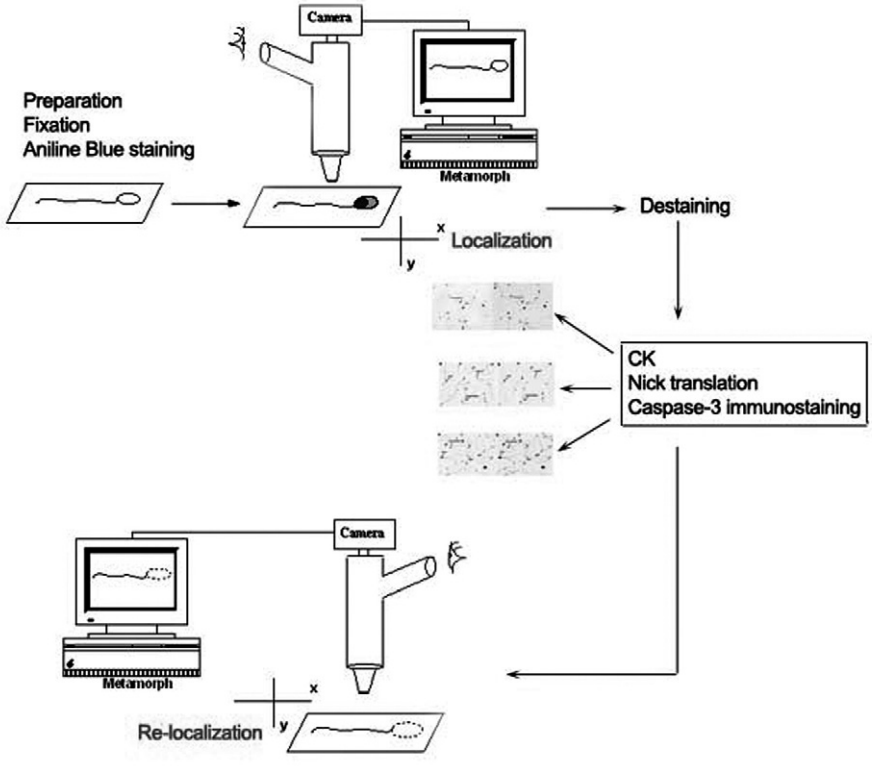

Figure 1. Flow chart of the experimental design. CK = creatine kinase.

methanol–acetic acid, and the sperm cells were stained with aniline blue, a stain that detects persistent histones. Images of sperm fields and individual spermatozoa were then captured using the Metamorph™ imaging program (Universal Imaging Co. Downingtown, PA, USA) (Celik-Ozenci *et al.*, 2003). Subsequently, with the slides still on the microscope platform, the X–Y co-ordinates of the fields were determined, in order to facilitate the re-localization of the same sperm fields. Following this step, the sperm slides were de-stained from aniline blue by overnight incubation in the fixative solutions that were appropriate for the second biochemical markers (methanol for DNA nick translation, and paraformaldehyde for the immunocytochemistry studies). In further steps, the de-stained spermatozoa were treated with one of the second biochemical probes: (i) CK immunocytochemistry to demonstrate cytoplasmic retention in diminished-maturity spermatozoa (Huszar *et al.*, 1993); (ii) DNA nick translation for detection of DNA chain breaks (Irvine *et al.*, 2000); and (iii) caspase-3 immuno-staining to detect the apoptotic process in spermatozoa (Cayli *et al.*, 2004).

Following the staining steps with the second probes, the previously noted X–Y co-ordinates were used to re-locate the same fields that were captured by the Metamorph program following the aniline blue staining. Next, the images of the same spermatozoa treated with the second probe were captured, in order to compare side-by-side the same spermatozoa stained with aniline blue and the second probe applied (**Figures 2**, **3** and **4**). The sperm cells, based on the staining intensity, were classified as light (mature), intermediate or dark (arrested-maturity spermatozoa), as described by Huszar *et al.* (2003). In this latter study and in a subsequent study (Huszar *et al.*, 2007), the spermatozoa were probed with the various single biochemical markers, and the sperm staining patterns of parts (**a**) or (**b**) of **Figures 2**, **3** and **4** in this work were in all aspects comparable with the earlier images.

The men studied presented for semen analysis in the Sperm Physiology Laboratory, Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine. All studies were approved by the Yale Human Investigation Committee.

Sperm preparation

Aliquots of liquefied semen were assayed for sperm concentration and motility, and the semen samples were diluted with physiological saline containing 0.3% bovine serum albumin and 30 mmol/l imidazole pH 7.2 (SAIM) up to a final volume of 10 ml. The semen samples were then centrifuged at 500 *g* for 18 min at room temperature. After the supernatant was discarded, each sperm pellet was re-suspended in the SAIM solution to a concentration of 10–25 × 10^6 sperm/ml. Sperm slides were prepared by smearing 5–10 μl of sperm suspension onto clean glass slides and allowing them to air-dry. The sperm cells were treated with the various biochemical markers as described below. For the aniline blue/DNA nick translation studies only, the semen samples were first purified by centrifugation through 1.5 ml of a 40% single-phase isolate gradient (Irvin Scientific, Santa Ana, CA) at 500 *g* for 10 min at room temperature, and the re-suspended sperm pellet fraction was used for the studies.

Aniline blue staining of sperm chromatin

Sperm smears were dried on glass slides and stained with a 5% aniline blue solution (Sigma Co., St Louis, MO, USA) acidified to approximately pH 3.5 with acetic acid. The slides were washed, air-dried and a cover slip was applied before evaluation. Mature spermatozoa, having completed histone–protamine replacement, stained very lightly with aniline blue (light = mature spermatozoa), the slightly immature spermatozoa were stained more extensively (intermediate = diminished maturity), and immature spermatozoa with substantial degrees of persistent histones were darkly stained (dark = immature spermatozoa Huszar *et al.*, 2003). It is of note that the first fixation–aniline blue staining treatment prior to application of the second probe was identical in each of the three double staining studies (**Figure 1**).

For the second probe treatments, the methods used were varied, as the conditions required for the immunological and the DNA nick translation studies were different.

Immunostaining of spermatozoa for CK

After recording the sperm fields, the aniline blue-stained sperm cells were de-stained with 0.5% paraformaldehyde incubation in phosphate buffer/sucrose (PB–suc) overnight at room temperature. All de-staining procedures were carried out on a shaking platform. The formaldehyde was removed by three washing steps with PB–suc, the slides were allowed to air dry, and the spermatozoa were exposed to a 3% bovine serum albumin (BSA) blocking solution in PB–suc at room temperature. After further washing, the spermatozoa were overlaid with a 1:1000 dilution of polyclonal anti–CK-B antiserum (Chemicon Co, Temecula, CA, USA). Following further PB–suc washes, the slides were treated with a biotinylated second antibody (Sigma-Aldrich, Milwaukee, WI) at a 1:1000 dilution and were exposed to a Vector horseradish peroxidase/ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. The avidin–biotin complex (ABC)-treated slides were further processed with diaminobenzidine and hydrogen peroxide (Sigma, St Louis, MO, USA). The developed brown colour highlighted spermatozoa with various degrees of cytoplasmic retention (**Figure 2**). The specificity of the CK staining was established by using pre-immune serum (Sigma-Aldrich, Milwaukee, WI, USA) in place of the first antibody, or by applying the second antibody only. In these experiments, 1284 spermatozoa were studied (four men, concentration: 10.9 ± 2.2 × 10^6/ml, motility: 32.5 ± 4.8%, 321 sperm/man, range: 209–610).

Immunostaining of spermatozoa for caspase-3

The de-staining and other procedures for caspase-3 immunocytochemistry were carried out similarly to those described for CK immunostaining (Cayli *et al.*, 2004). However, after the spermatozoa were exposed to the 3% BSA blocking solution, they were treated with a 1:300 dilution of active caspase-3 (PharMingen, San Diego, CA, USA) antibody overnight at 4°C. Further, the slides were processed with 1:1000 dilution of a biotinylated anti-rabbit second antibody (Vector

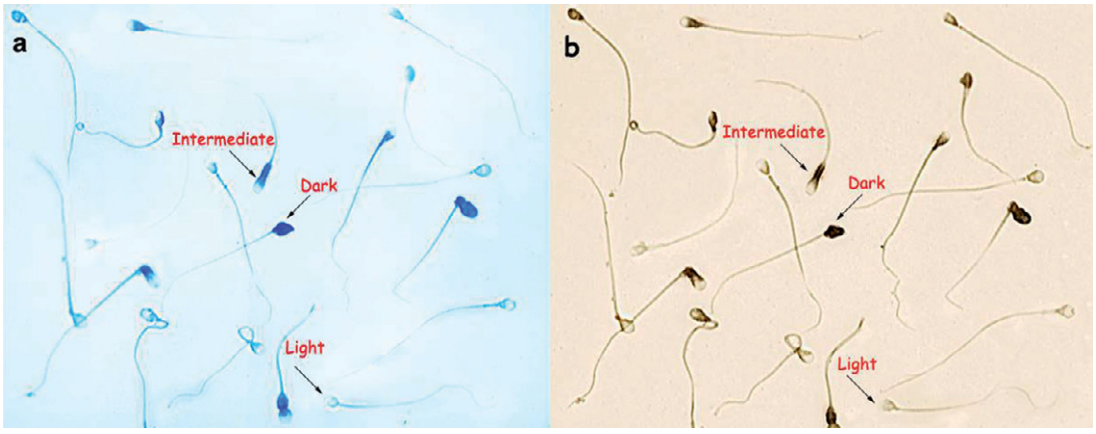

Figure 2. (**a**) Aniline blue staining and (**b**) creatine kinase (CK)-immunostaining of the same spermatozoa. Note the substantial degree of similarity in the light-, intermediate- and dark-staining patterns with aniline blue and CK.

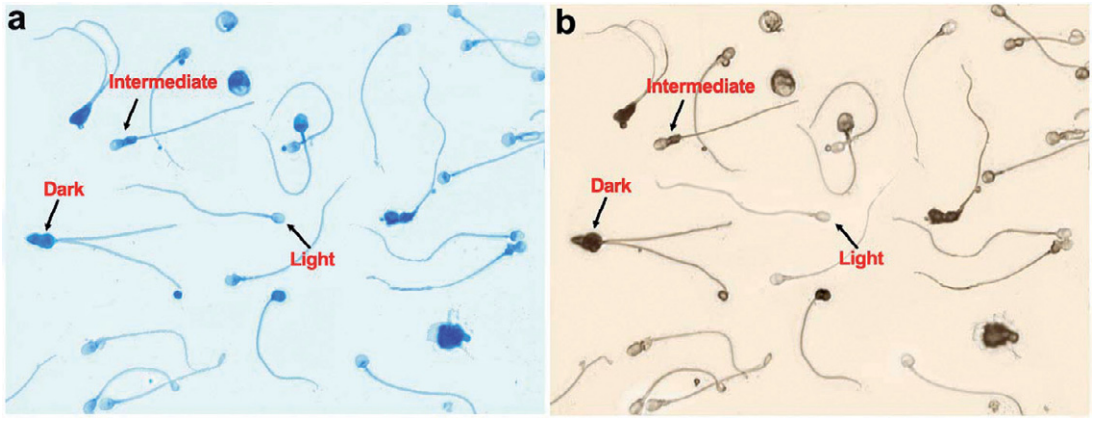

Figure 3. (**a**) Aniline blue staining and (**b**) caspase-3 immunostaining of the same spermatozoa. Note the similarity in the light-, intermediate- and dark-staining patterns of the aniline blue and caspase-3 panels. Also, caspase-3 immunostaining is present in the mid-piece of intermediate-type spermatozoa, whereas in dark spermatozoa with more extensive maturity arrest both the head and the mid-piece are stained (**a** and **b**).

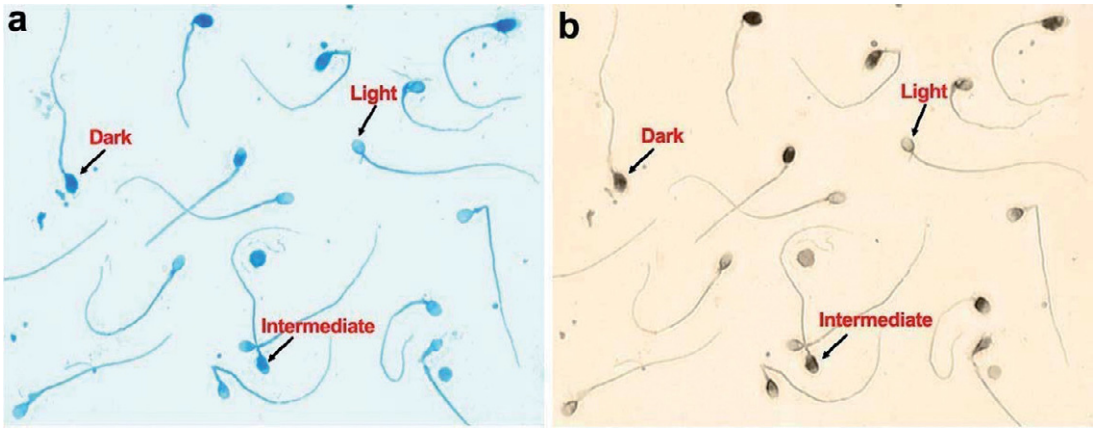

Figure 4. (**a**) and (**b**) DNA nick translation of the same spermatozoa. Note the substantial degree of similarity in the light-, intermediate- and dark-staining patterns with the two methods.

Laboratories). The brown colour representing the caspase-3 content of spermatozoa was developed by the ABC method (Vector and Sigma; **Figure 3**). The specificity of staining was established by applying the secondary antibody only. These studies were carried out in 2101 spermatozoa (4 men, sperm concentration: 11.9 ± 1.4 × 10^6 ml, motility: 34.0 ± 6.6%).

Assessment of DNA integrity by nick translation

The basic concept of the assay is as follows. The enzyme DNA polymerase repairs DNA strand breaks by incorporation of nucleotides. However, one of the repair elements is biotin-labelled dUTP, which serves as the anchor for avidin-conjugated horseradish peroxidase. Thus, after the assay, the horseradish peroxidase-generated colour in a spermatozoon is proportional to the extent of DNA breaks and repair (Irvine *et al.*, 2000).

All steps were carried out at room temperature. After recording of the fields, the aniline blue-stained sperm cells were de-stained in 30% methanol overnight. Further, the slides were covered with 20 mmol/l of imidazole buffer pH: 7.0 for 1 h, and 30% methanol applied for 15 min, followed by air drying. The slides were further treated with methanol–glacial acetic acid (3:1) for 15 min. After exposure to a dehydrating ethanol series (70, 85, 100%), the slides were air dried and treated with 10 mmol/l dithiothreitol (DTT) (in 100 mmol/l Tris, pH: 7.2) for 30 min to initiate DNA decondensation. After a washing step with 100 mmol/l Tris, pH 7.2, the slides were exposed to 10 mmol/l lithium diiodosalicylic acid in 100 mmol/l Tris, pH 7.2 for 3 h. Following further washing with PBS, the slides were blocked with biotin (1:10 dilution of a 0.01% stock) for 20 min, washed again, and then blocked with avidin (1:10 dilution of a 1 mg/ml solution) for 20 min. Further, the slides were exposed to a solution containing 1 mmol/l DTT, 10 mmol/l $MgSO_4$, 50 mmol/l Tris–HCl, pH 7.2, 0.01 mmol/l biotin-16-dUTP, and a 0.01 mmol/l mix of dGTP, dCTP, dATP, and DNA polymerase I (0.025 IU/ml) for 30 min. The repair with biotin-labelled nucleotides was detected by avidin–biotin horseradish peroxidase using the Vector ABC method (Vector Laboratories; all other chemicals were from Sigma). After further washing, the slides were developed in 3,3-diaminobenzidine solution for 10–15 min. The slides were counterstained with Coomassie Blue (0.04% in 25% isopropanol, 10% glacial acetic acid) for 30 s, in order to optimise visualization of the sperm contours. The slides were then washed, and mounted with Permount (Sigma-Aldrich, Milwaukee, WI) (**Figure 4**). In these experiments, 2446 spermatozoa were studied (5 men, sperm concentrations: 17.6 ± 1.1 × 106 sperm/ml, motility: 38.4 ± 5.9%, 489 sperm/man; range: 276–602).

Evaluation of sperm morphology

Sperm smears were dried on glass slides and stained with Diff-Quik (Dade-Behring, Newark, USA) according to the manufacturer's instructions. The slides were scored by two investigators in a blinded manner according to the Tygerberg criteria (Kruger *et al.*, 1986, 1988; Menkveld *et al.*, 1990; WHO, 1999). The results were averaged for each slide.

Statistical analysis

Data analysis was carried out using Sigma-Stat 2.0 (Jandel Corporation, San Rafael, CA, USA). Differences among groups, and the various morphometric parameters, were compared using one-way analysis of variance (ANOVA) on normally distributed data, and one-way ANOVA on ranks test on data that were not normally distributed. Following ANOVA, Dunn's *post hoc* test was performed. Data analysis was carried out using R statistical software (R Core Development Team, Vienna, Austria) and Sigma-Stat 2.0 (Jandel Corporation). All data are presented as mean ± SEM.

In order to compare the level of agreement between probe pairs in the same spermatozoa, weighted kappa analysis with quadratic weights was used. A rating scale for kappa, suggested by Altman (1991), indicates that a kappa value >0.8 represents an excellent level of agreement.

Results

Experimental design

In this series of studies, two biochemical probes were applied on the same sperm cell following the experimental scheme of **Figure 1**. For these experiments, oligozoospermic or low normal sperm concentration samples were selected in order to achieve an adequate representation of arrested/diminished maturity spermatozoa. Approximately 5600 double stained spermatozoa were studied, originating from semen samples from 13 men (mean sperm concentration: 25.0 ± 6.2 × 10^6/ml, motility: 36.2 ± 6.5%.) It is of note that the double-stained sperm cells in **Figures 2**, **3**, and **4** are from individual semen samples.

Double probing of spermatozoa with CK-immunocytochemistry and aniline blue staining

In the first study, the spermatozoa were double stained with nuclear and cytoplasmic markers of arrested sperm maturation. Initially, aniline blue was applied to highlight the presence of persistent histones, and this step was followed by CK immunocytochemistry. As **Figure 2a**,**b** indicates, the spermatozoa stained light, intermediate or dark, represent spermatozoa that are mature, intermediate maturity or diminished maturity respectively. There was good agreement between the two staining patterns with the aniline blue and CK probes. The dark spermatozoa showed high degrees of persistent histones as well as retained cytoplasm. A quantitative analysis of the 1284 cells evaluated is presented in **Table 1**. The actual number of spermatozoa with light–light, intermediate–intermediate and dark–dark staining, and the proportion of spermatozoa expressed as a percentage of the total, is presented.

The majority of the spermatozoa follow a light–light (approximately 40%), intermediate–intermediate (approximately 25%) and dark–dark (approximately 18%) pattern. The representation of intermediate–light or intermediate–dark is approximately 18%, whereas spermatozoa with the discordant

staining pattern of light–dark and dark–light occur only in <1% of the cells. Thus, 82% of the spermatozoa showed conforming staining patterns with the biochemical markers of nuclear (persistent histones) and cytoplasmic (cytoplasmic retention) probes of sperm maturity/arrested maturity.

Double probing of the same spermatozoa with aniline blue and for DNA chain fragmentation with DNA nick translation

In this study, similar to the previous experiment, the sperm fields were stained first with aniline blue, and in-situ DNA nick translation was subsequently performed on the same cells. **Figure 4a**,**b** demonstrates a substantial agreement between the staining patterns with the two nuclear markers. In **Table 2**, the distribution of the 2446 spermatozoa studied with both probes is presented. The overwhelming majority of spermatozoa showed staining patterns that were conforming, as light–light (n = 1261), intermediate–intermediate (n = 498), and dark–dark (n = 291). The data are also expressed as a proportion of all spermatozoa, as light–light (approximately 52%), intermediate–intermediate (approximately 20%) and dark–dark (approximately 12%), 84% in all. Sperm cells with heterogeneous staining of light–intermediate, dark–intermediate, intermediate–light or intermediate–dark, were approximately 15% of the population, whereas the discordant light–dark or dark–light spermatozoa represented approximately 1.2%. As with the aniline blue–CK experiment, 84% of the spermatozoa showed an identical staining pattern between aniline blue and DNA fragmentation. Thus, spermatozoa with arrested maturity and persistent histones also exhibited a high level of DNA degradation (about 12% of the population), and about 52% of the sperm population tested normally developed mature cells, devoid of both persistent histones and DNA fragmentation.

Table 1. Double probing of spermatozoa with aniline blue and creatine kinase (CK) immunocytochemistry.

Aniline blue staining	*CK staining* *Light*	*Intermediate*	*Dark*
Light	511 (39.8)	117 (9.1)	3 (0.2)
Intermediate	34 (2.6)	313 (24.4)	47 (3.7)
Dark	1 (0.1)	34 (2.6)	224 (17.4)

Total of 1284 spermatozoa studied from four men (sperm concentration: 10.9 ± 2.2 × 10^6/ml, motility: 32.5 ± 4.8%).
Values are actual numbers of spermatozoa (% of total).

Table 2. Double probing of spermatozoa with aniline blue and DNA nick translation.

Aniline blue staining	*DNA nick translation* *Light*	*Intermediate*	*Dark*
Light	1261 (51.6)	162 (6.6)	20 (0.8)
Intermediate	80 (3.3)	498 (20.4)	71 (2.9)
Dark	8 (0.3)	55 (2.2)	291 (11.9)

Total of 2446 spermatozoa studied from five men (sperm concentration: 17.6 ± 1.1 × 10^6/ml, motility: 38.4 ± 5.9%).
Values are actual numbers of spermatozoa (% of total).

Double probing of spermatozoa with aniline blue and caspase-3

In **Figure 3a**,**b**, fields of spermatozoa double stained with aniline blue and caspase-3 immunostaining can be seen. As **Table 3** indicates, 2101 spermatozoa were evaluated for the staining patterns. There was light–light staining in 1047 spermatozoa, intermediate–intermediate pattern in 483 spermatozoa and dark–dark staining in 261 cells. Light–light pattern occurred in approximately 50% of the spermatozoa, intermediate–intermediate pattern in 23% and dark–dark diminished maturity pattern in about 12% of spermatozoa. Thus, as with the other cytoplasmic and nuclear markers (**Tables 1** and **2**), approximately 85% of the double stained spermatozoa showed conforming staining patterns with aniline blue and the apoptotic marker. Similarly to the other markers, intermediate–light or intermediate–dark sperm staining occurred in about 14% of the cells, and the discordant pattern of dark–light and light–dark staining was <1.0%.

Sperm shape and sperm maturity

Sperm morphology evaluated according to the Tygerberg strict criteria seems to be related to the sperm biochemical maturity because the abnormal head and mid-piece shapes, and abaxial insertion of the tail occur as a consequence of cytoplasmic retention in spermatozoa with arrested spermatogenetic maturation. The shorter sperm tail, characteristic for spermatozoa with arrested maturity, is also a component of the Kruger strict morphology evaluation.

Table 3. Double probing of spermatozoa with aniline blue and caspase-3.

Aniline blue staining	*Caspase-3 staining* *Light*	*Intermediate*	*Dark*
Light	1047 (49.8)	123 (5.9)	3 (0.1)
Intermediate	77 (3.7)	483 (23.0)	46 (2.2)
Dark	0 (0.0)	61 (2.9)	261 (12.4)

Total of 2101 spermatozoa studied from four men (sperm concentration: 11.9 ± 1.4 × 10^6/ml, motility = 34.0 ± 6.6%).
Values are actual numbers of spermatozoa (% of total).

The relationship between Tygerberg normal morphology and aniline blue staining intensity was studied in five samples by counting approximately 400 spermatozoa for each patient (n = 3882 in all, evaluated by two investigators independently). Within the groups of spermatozoa that stained light (normal spermatozoa), intermediate (intermediate maturity) and dark (diminished maturity), the proportion of Tygerberg normal spermatozoa were 9.5 ± 1.3, 2.3 ± 0.9 and 0.5 ± 0.3% respectively. There were significant differences in the light versus intermediate ($P < 0.05$), and intermediate versus dark groups ($P < 0.01$). Thus, there is a relationship between the proportion of spermatozoa with various degrees of arrested maturity and abnormal Tygerberg morphology.

Inter-subject variation in staining patterns with the various probes

In order to better evaluate the validity of the two probe staining pattern data, variations among the men studied with each probe combination are presented.

Within the group of four men whose spermatozoa were studied with the aniline blue–CK immuno-probe combination, the proportion of light–light spermatozoa was 36.0 ± 4.4% (mean ± SEM), and in the individual men 34.4, 30.3, 30.4 and 48.9%, respectively, the mean incidence of intermediate–intermediate staining was 24.9 ± 4.1% (25.8, 15.8, 35.4 and 22.8%), whereas spermatozoa with dark–dark pattern were present in 21.1 ± 5.9% (28.7, 33.3, 12.7 and 9.5%).

In the group of five men whose spermatozoa were studied with aniline blue-DNA nick translation, the mean light–light pattern was 52.0 ± 6.7% (54.8, 66.4, 32.9, 55.2 and 50.8%), the mean intermediate–intermediate pattern was 19.7 ± 1.9% (20.6, 16.6, 25.9, 20.5 and 15.0%). The dark–dark pattern was 12.2 ± 1.7% (12.5, 7.2, 16.1, 9.8 and 15.4%).

Finally, within the group of four men whose spermatozoa were double stained with aniline blue–caspase 3, the proportion of light–light spermatozoa was 49.4 ± 6.7% (44.5, 56.7, 33.2 and 63.3%) and the intermediate–intermediate stained spermatozoa were 23.3 ± 3.5 (30.3, 26.4, 20.9 and 14.4%). Further, the dark–dark spermatozoa were 12.7 ± 5.5% (11.3, 3.5, 28.3 and 7.6%).

Interpretation of the staining patterns: a close relationship among probes

The consistently high agreement (83–85% overlap) of probe signal within the mature, intermediate and arrested/diminished maturity spermatozoa, indicates that cytoplasmic retention, DNA chain fragmentation and the presence of the apoptotic process are all related to persistent histones.

This relationship among the various nuclear and cytoplasmic probes was quantified with regression analysis based on the weighted kappa method. There was a very close correlation (kappa = 0.8) among the attributes of arrested maturation within the same sperm cell. Thus, double probing of the same spermatozoa is an extremely sensitive demonstration of the proportion of mature and immature spermatozoa in semen.

Discussion

In these experiments, individual spermatozoa have been studied with probes for persistent histones combined with other probes for cytoplasmic retention, for apoptotic processes, and for DNA fragmentation with in-situ nick translation. All these probes provide light, intermediate and dark staining patterns representing mature, intermediate maturity and arrested/diminished maturity spermatozoa respectively (Huszar *et al.*, 2003). In line with the present hypothesis, approximately 84% of the spermatozoa studied showed maturity levels with aniline blue that were consistent with the staining patterns for cytoplasmic retention, DNA chain fragmentation, and the presence of the apoptotic process. These attributes are also related to persistent histones, and presumably also to lower expression of transition proteins and protamines (Steger *et al.*, 2003; Aoki *et al.*, 2006).

Thus, there is substantial agreement between the cytoplasmic and nuclear probes of maturity in individual spermatozoa.

Confirming that the cytoplasmic and nuclear attributes of arrested sperm maturity are related, there was >80% agreement in the presence or absence of the cytoplasmic and nuclear biochemical marker combinations within individual spermatozoa. Thus, the majority of sperm cells with DNA fragmentation also showed diminished sperm maturity, as detected by cytoplasmic retention, aniline blue staining and apoptotic processes. In **Figure 3a**,**b**, one can observe fields of spermatozoa double stained with aniline blue and caspase-3 immunostaining. Caspase-3 is an important marker because it is a probe of active apoptotic processes in conjunction with cytoplasmic retention, and in some spermatozoa with the expression of the anti-apoptotic protein, Bclx2 (Carrell *et al.*, 2003; Cayli *et al.*, 2004; Seli *et al.*, 2005).

In addition to the relationship between arrested sperm maturity and DNA integrity, the data also contribute to the compensatory concepts of sperm DNA fragmentation, and to the limitations of antioxidant therapy and similar strategies. It is likely that a substantial proportion of spermatozoa with arrested maturation and related defects of spermatogenesis, as well as the consequential infrastructure deficiencies, would not and could not respond to such therapeutic interventions (Gil-Guzman *et al.*, 2001; Aitken *et al.*, 2003; Steger *et al.*, 2003; Suzuki *et al.*, 2003; Agarwal and Said, 2005; Comhaire *et al.*, 2005; Greco *et al.*, 2005; Lewis and Aitken, 2005; Aitken and Baker, 2006; Ménézo *et al.*, 2007).

Considering the nuclear probes, the aniline blue–DNA fragmentation and the aniline blue–caspase combinations indicated that approximately 12% of spermatozoa showed arrested maturity, whereas with the aniline blue–cytoplasmic retention probes there was a slightly higher, approximately 17%, incidence of spermatozoa with dark and intermediate staining. It is unclear whether this difference arises from the probe specific attributes alone, or because spermatozoa that show nuclear impact of immaturity survive at a lower rate.

Approximately 15% of spermatozoa tested with any probe combination showed an intermediate–light or intermediate–dark maturity pattern. In these spermatozoa one can see signs of

arrested maturity with one probe that is not detected to the same extent with another probe. This is an important finding for two reasons. First, this heterogeneity in staining intensity or in the regional distribution of markers in the head and mid-piece is in line with the polymorphic attributes of spermatozoa. Thus, even in arrested maturity the sperm cell may follow a variety of pathways (Huszar *et al.*, 2003, 2007). Second, due to this polymorphism, it has become apparent that one cannot determine sperm maturity reliably with a single probe. However, the quick aniline blue test is very useful in detecting the proportion of spermatozoa that are suspected to be of arrested maturation in semen (Dadoune *et al.*, 1988; Foresta *et al.*, 1992; Liu *et al.*, 1992; Morel *et al.*, 1998).

The association between aniline blue staining and caspase-3 in the same spermatozoa suggests that DNA degradation is also related to arrested maturity. An earlier study investigated why these spermatozoa with increased levels of cytoplasmic retention, caspase-3 content and DNA fragmentation survive to be ejaculated rather than, as one would expect, being eliminated by apoptosis within the adluminal area or in the epididymis (Cayli *et al.*, 2004). The probe studies demonstrated the simultaneous presence of caspase-3 and the anti-apoptotic Bclx2 protein in these surviving arrested maturity cells. Further, caspase-3 and BclX2 were located almost exclusively together in the mid-piece. These data suggest that these spermatozoa overcome apoptotic decay due to the protective Bclx2.

Additional factors that may lead to DNA strand breaks in arrested maturity spermatozoa include an impaired capacity for DNA repair due to the low levels of HspA2 chaperone, which would normally deliver the enzymes and other DNA repair components. Low HspA2 chaperone levels cause meiotic errors and chromosomal aneuploidies, diminished zona pellucida binding, low fertilization and oocyte activation rates, and increased miscarriage rates due to defects of paternal contribution of spermatozoa (Aitken and Baker, 2006, Huszar *et al.*, 2007). Whatever cause may prevail, in the present study it has been established that DNA chain breaks are related to arrested sperm maturity, whether shown by one or more of the nuclear and cytoplasmic probes. Additional adverse effects related to persistent histones may reflect the upstream events of spermatogenesis, as indicated by aberrant protamine-1/protamine-2 ratios at both the mRNA and protein levels in spermatozoa with arrested maturity (Steger *et al.*, 2003; Aitken *et al.*, 2004; Agarwal *et al.*, 2005; Seli and Sakkas 2005).

Another relevant aspect is that spermatozoa with intermediate or dark staining with probes of arrested maturity may also exhibit shape properties that reflect the spermatogenetic defects with respect to cytoplasmic retention and tail sprouting, such as lower proportion of spermatozoa with normal head shape, abaxial insertion of the tail, shorter tail length, or lower tail length/head long axis ratio (Gergely *et al.*, 1999; Celik-Ozenci *et al.*, 2003; Chemes and Rawe, 2003). The shorter sperm tail, characteristic for spermatozoa with arrested maturity, is also a component of the Tygerberg strict morphology evaluation. (Menkveld *et al.*, 1990; Gergely *et al.*, 1999; WHO, 1999; Carrell *et al.*, 2003; Celik-Ozenci *et al.*, 2004). Mature spermatozoa present in sperm–hemizona complexes are clear headed. This supports the idea that sperm shape may also be considered as a biochemical parameter (Huszar *et al.*, 2007).

The study of individual spermatozoa with the various probes provides enhanced levels of statistical power in the analysis of mature and immature spermatozoa, and with this approach, a close correlation was demonstrated between the maturity attributes (kappa = 0.8). It is suggested that this type of analysis, and the use of biomarkers, will be useful in men exposed to environmental reproductive toxicity. It is further suggested that this may be particularly true in the early stages of exposure, when the toxic agents interfere only with sperm maturation, but have not yet placed stress on daily sperm production to cause a decline in sperm concentrations.

The presence of spermatozoa with various degrees of maturity in semen samples further supports the day-to-day and man-to-man variations in sperm maturity, independent of sperm concentrations (Huszar *et al.*, 1992, 2003, 2007). The pregnancy success in couples treated with intrauterine insemination or IVF was inversely related to the proportion of spermatozoa with arrested maturation, as indicated by sperm cytoplasmic retention and HspA2 concentrations (Huszar *et al.*, 2007). Further, IVF fertilization rates are inversely related to DNA damage (Sakkas *et al.*, 1999; Greco *et al.*, 2005), although intracytoplasmic sperm injection fertilization rates seem to be normal with spermatozoa with damaged DNA. This discrepancy may be due to the fact the paternal genome is only expressed at around the 4- to 8-cell stage of human embryos; thus, the DNA breaks may not have an effect in fertilization or in very early development, but the negative effect becomes apparent in the later embryonic stages. Indeed, the proportion of spermatozoa with DNA fragmentation was related to the time needed to achieve pregnancy, and DNA fragmentation was more extensive in men whose wives suffered recurrent pregnancy loss compared with sperm donors in the general population. Also, the incidence of miscarriage is higher with the less mature testicular spermatozoa, compared with ejaculated spermatozoa (Evenson *et al.*, 1999; Spano *et al.*, 2000; Virro *et al.*, 2004).

The present data are also relevant to therapies directed to improve male subfertility, including several lines of research exploring new strategies or therapeutic interventions, i.e. antioxidant therapy, for the prevention or repair of sperm DNA fragmentation, which causes a decline in fertility and the paternal contribution of spermatozoa to the zygote (Aitken *et al.*, 2003; Suzuki *et al.*, 2003; Agarwal and Said, 2005; Comhaire *et al.*, 2005; Greco *et al.*, 2005; Lewis and Aitken, 2005; Tesarik *et al.*, 2006). However, intermediate and diminished maturity spermatozoa, approximately 40% of spermatozoa in this study, in addition to DNA chain fragmentation, show cytoplasmic retention, persistent histones, apoptotic processes, and structural defects of spermatogenetic and spermatogenetic origin. Those cells with a defective infrastructure are not likely to be amenable to repair. Thus, the study of sperm maturity is relevant to assessment of the potential sperm pool that would be available for improvement by therapeutic interventions. Otherwise, the proportion of spermatozoa that are subject to rescue might be overestimated (Ménézo *et al.*, 2007).

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