

# Double probing individual human spermatozoa: aniline blue staining for persistent histones and fluorescence in situ hybridization for aneuploidies

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**Objective:** To study the potential relationship between two sperm nuclear attributes: persistence of histones and occurrence of chromosomal aneuploidies.

**Design:** The two variables were examined by double probing of the same spermatozoa.

**Setting:** Academic Andrology Laboratory.

**Patient(s):** Semen samples subjected for analyses were examined.

**Intervention(s):** We studied >58,000 spermatozoa, in seven men, first with aniline blue histone staining, graded as light (mature sperm), intermediate (moderately immature), and dark (severely arrested maturation). After recording the staining patterns and destaining, the same spermatozoa were subjected to fluorescence in situ hybridization (FISH), using centromeric X, Y, and 17 chromosome probes.

**Main Outcome Measure(s):** Proportions of sperm with light, intermediate, and dark staining were assessed, and ploidy of these sperm was evaluated.

**Result(s):** The aneuploidy frequencies in intermediate versus light (mature) spermatozoa were increased four- to sixfold. In addition, aneuploidy frequencies and proportions of intermediate sperm were related. There was no FISH signal detectable in the darkly stained, severely arrested mature sperm.

**Conclusion(s):** The data suggest that in sperm with arrested maturity and DNA fragmentation, the binding of FISH probes is diminished. DNA damage is further aggravated by the decondensation and denaturation steps of FISH. Thus, there is a strong likelihood that in oligozoospermic men, with a higher proportion of sperm with arrested maturation, the sperm disomy frequencies are historically underestimated. (Fertil Steril® 2010;93:2255–61. ©2010 by American Society for Reproductive Medicine.)

**Key Words:** Arrested sperm maturation, persistent histones, aneuploidy frequencies

During spermiogenesis the developing round spermatids undergo complex events in both the nuclear and cytoplasmic compartments, including meiosis, histone transition protein–protamine replacement, acrosome development, sprouting of the sperm tail, and cytoplasmic extrusion (1, 2). In addition, in late spermiogenesis, there is a remodeling of the sperm plasma membrane, a step that facilitates formation of the zona pellucida (ZP) and hyaluronic acid-binding sites (3–5).

There are various nuclear and cytoplasmic markers that reflect arrested sperm maturation. However, the major hallmark is the low expression of the chaperon protein HspA2, which

in normal sperm development is expressed in two waves (6). First, in primary spermatocytes, HspA2 is part of the synaptonemal complex and supports meiosis (1, 7). A second HspA2 expression occurs in elongating spermatids, which coincides with delivery of DNA repair and other enzymes, and the major protein movements of cytoplasmic extrusion, nucleoprotein transport, and plasma membrane remodeling. Regarding the nucleoprotein replacement cycle, a close relationship was found between HspA2 chaperon function and transition protein transport (4, 8, 9).

Low HspA2 expression is also associated with other attributes of arrested sperm cellular maturation, such as surplus cytoplasm, persistent histones, the apoptotic enzyme caspase 3, and DNA chain fragmentation (5, 10–13). Because HspA2 is a component of the synaptonemal complex, low expression of HspA2 is also associated with increased frequency of chromosomal aneuploidies (12, 14–19). In line with this concept, sperm with surplus cytoplasm showed a 2–3 times higher frequency of chromosomal aneuploidies compared with normally developed sperm. The frequency of sperm with surplus cytoplasm and aneuploidies were related in semen ( $r = 0.72$ ,  $P < .001$ ) (14). In addition, in sperm studies with morphology and morphometry, there was a relationship

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**TABLE 1****Sperm attributes of the seven semen samples studied.**

Men	Sperm Conc. 10 <sup>6</sup> /mL	Motility (%)	Cells scored (n)	AB Light (%)	AB Intermediate (%)	AB Dark (%)
1	21.0	38.0	8,174	84.6	11.5	3.9
2	18.0	38.0	9,155	75.5	20.9	3.6
3	10.0	49.0	8,193	73.9	21.8	4.2
4	10.0	25.0	8,322	69.5	23.9	6.7
5	16.0	47.0	8,372	69.6	26.2	4.2
6	16.0	49.0	8,721	75.0	19.0	6.0
7	13.0	29.0	7,856	63.8	29.0	6.5
Mean ± SEM	14.8 ± 1.5	39.2 ± 3.6	8,399 ± 159.6	73.1 ± 2.3	21.8 ± 2.1	5.0 ± 0.5

*Note:* AB = aniline blue staining.

Óvári. *Sperm aneuploidy and persistent histones. Fertil Steril* 2010.

between increased sperm head size (related to surplus cytoplasm), higher frequencies of chromosomal aneuploidies, as well as a deficiency in plasma membrane remodeling during terminal spermiogenesis causing diminished sperm–hyaluronic acid binding (12, 20, 21).

Regarding the nuclear compartment of sperm with arrested cellular maturity, earlier studies reported an association with diminished histone transition protein–protamine exchange detected by aniline blue staining of excess persistent histones (22–27). Morel et al. (23) found that in semen with increased proportion of aniline blue-stained sperm, there was also an increased frequency of chromosomal aberrations, based on a limited number of spermatozoa tested with fluorescence in situ hybridization (FISH).

In the present study we conducted a more detailed examination of the nuclear events related to persistent histones and aneuploidies. [1] The relationship between aniline blue staining and chromosomal aneuploidies were studied within the same double probed spermatozoa. [2] Approximately 8,000 sperm per man were examined using probes for the X, Y, and 17 chromosomes. [3] Finally, aniline blue staining intensity was quantified as light, intermediate, and dark, thus classifying the degree of arrest in histone transition protein–protamine replacement sequence, along with FISH within approximately 58,000 sperm.

## MATERIALS AND METHODS

### Experimental Design

Spermatozoa in semen of seven moderately oligozoospermic men were studied. The sperm were subjected to double probe treatment with aniline blue staining and FISH. After the aniline blue staining, the sperm fields were recorded by the computer-assisted Metamorph program (MDS Inc., Toronto, Canada), and the sperm were evaluated for staining intensity (Table 1). To facilitate the relocation of sperm fields, we introduced diamond-scratched lines on the slide, and recorded the X/Y microscope coordinates. The spermatozoa

were classified according to their staining intensity, which reflects sperm cellular maturity and persistence of histones.

Subsequently, the aniline blue was destained, and the sperm were subjected to FISH with three chromosome probes. The chromosome complements were evaluated under fluorescence microscopy (14, 28, 29). The order of the aniline blue staining and FISH procedures was important because after the decondensation and denaturation steps of FISH, the aniline blue staining pattern was not reliable. All studies were approved by the Yale School of Medicine HIC.

### Sperm Preparation

After determination of sperm concentration and motility, aliquots of the sperm was washed in 10 volumes of physiological saline containing 0.3% bovine serum albumin (BSA) and 30 mM of imidazole at pH 7.2 (SAIM solution) by centrifugation at  $500 \times g$  for 18 minutes at room temperature. Each sperm pellet then was resuspended in the SAIM solution to  $30\text{--}40 \times 10^6$  sperm/mL. The sperm slides were prepared by smearing 5–10  $\mu\text{L}$  of sperm onto clean precleaned, poly-L-lysine-coated glass slides and were allowed to air dry.

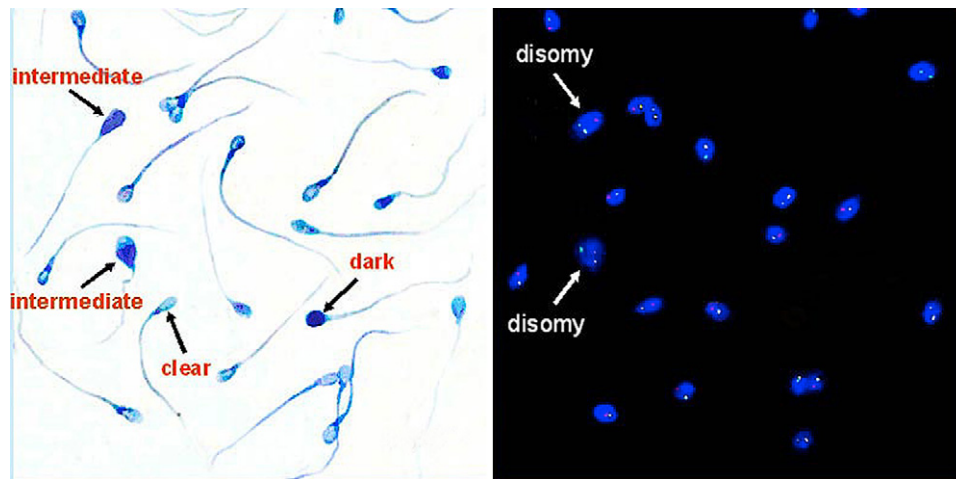
### Aniline Blue Staining of Sperm Chromatin

The slides were stained by 5% aniline blue solution (Sigma Co., St. Louis, MO) acidified to approximately pH 3.5 with acetic acid for 5 minutes, then washed with tap water and allowed to completely air dry (22). The sperm were evaluated at  $\times 600$  magnification, and images of random microscopic fields were recorded. At least 8,000–10,000 spermatozoa were captured per man (30–40 sperm in 250–300 fields). Destaining of the aniline blue, before FISH occurred in 5% aqueous acetic acid. In addition, the slides were dehydrated in an ethanol series, and were stored at room temperature.

Mature sperm, having completed histone transition protein–protamine replacement, stained very lightly (clear, mature sperm, Fig. 1), the slightly immature sperm were

## FIGURE 1

Panels of double treated spermatozoa with aniline blue staining (*left*) and FISH (*right*). Arrows on left side indicate staining intensity patterns.



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stained more extensively (intermediate, with slightly or partially stained head, moderately immature), and severely arrested mature sperm with substantial degrees of persistent histones stained darkly (dark, darkly filled head and in some midpiece, severely immature sperm) (5).

### FISH Procedures

The DNA decondensation treatment of destained spermatozoa was performed as described (12, 14, 28–30). The cells were treated with 10-mmol/L 1,4-dithiothreitol (Cleland's reagent; DTT, Sigma) in 0.1 mol/L Tris-HCl buffer, pH 8.0 for 30 minutes on ice. After, the sperm slide was covered with 4 mmol/L lithium 3,5-diiodosalicylic acid (Sigma) in 0.1 mol/L Tris-HCl buffer, pH 8.0 for an additional 90 minutes at room temperature. Slides were rinsed in 0.1 mol Tris-HCl, pH 8.0 buffer, allowed to air dry, then fixed in methanol/acetic acid (3:1).

For FISH, directly labeled alpha satellite centromere-specific probes were used, for chromosome X (Vysis, Downers Grove, IL; Spectrum Green CEP X) and chromosome Y (Vysis; Spectrum Orange CEP Y). Chromosome 17 was hybridized by an indirectly, biotin-labeled centromere-specific probe (SPOT-Light chromosome 17 centromeric probe; Zymed, San Francisco, CA). The sperm DNA was denatured in 70% formamide and two times standard saline citrate (2X SSC) at 75°C for 6 minutes. The slides were snap cooled and dehydrated in an ethanol series at -20°C, air dried, then placed onto a slide warmer at 37°C. The hybridization solution, prepared according to manufacturer's instructions was added and the slide was covered with a cover slip and sealed with acid-free rubber cement (Ross, Columbus, OH).

Hybridization was conducted in dark humidity chamber for overnight at 37°C. After hybridization washing was

accomplished with 50% formamide in 2X SSC at 42°C for 15 minutes followed by treatment in 2X SSC for 15 minutes at 42°C. After a blocking step of 3% BSA for 30 minutes, to visualize the indirect biotin-labeled probe 17, the slides were incubated with avidine–rhodamine (fluorescent red; Roche Biochemicals, Indianapolis, IN), avidine–fluorescein isothiocyanate conjugate (FITC) (fluorescent green; Roche Biochemical). Slides were washed with a solution of 4X SSC, 0.1% Tween 20 at 42°C for 15 minutes, and counterstained with 6-diamino-2-phenylindole (DAPI; Roche Biochemicals), mounted with an antifade solution (Vectashield; Vector Scientific, Torrance, CA), and a coverslip was applied.

### Scoring Criteria and Data Collection

For FISH pattern evaluation, an Olympus AX 70 epifluorescence microscope (Olympus, Center Valley, PA) was used with a triple pass filter for DAPI, FITC, and tetramethyl-rhodamine isothiocyanate (rhodamine). To improve signal resolution, separate single pass filters were also used for DAPI, FITC, and rhodamine.

Sperm nuclei were scored according to published criteria (14). Nuclei were not scored if they overlapped or if they lacked signals, indicating hybridization failure. A spermatozoon was considered disomic when it showed two fluorescent domains of the same color, comparable in size and brightness in approximately the same focal plane, clearly positioned inside the edge of the sperm head, and at least one domain apart. Diploidy was recognized by the presence of two double-fluorescence domains with the same criteria. Nullisomies were not directly scored, and were considered as frequency equivalent to disomies (12, 14, 17, 28–30).

We have detected nuclear maturity by aniline blue, and the presence of disomy 17, XX, YY, XY and diploidy, and scored at least 8,000 spermatozoa per man. [Figure 1](#), the double panel, demonstrates the same sperm fields treated with aniline blue and FISH.

### Statistical Analysis

For analysis of significance the Mann-Whitney *U* test and Pearson correlation test were done (Sigmastat 2.0; Jandel Corporation, San Raphael, CA).

## RESULTS

### Aniline Blue Staining Results

In the seven moderately oligozoospermic men, we have evaluated a total of 58,793 spermatozoa ([Table 1](#)). Regarding nuclear maturity, the proportion of the light, intermediate, and dark aniline blue-stained sperm (representing mature, moderately immature, and severely arrested maturity spermatozoa) were  $73.1\% \pm 2.3\%$ ,  $21.8\% \pm 2.1\%$ , and  $5.0\% \pm 0.5\%$ . It is of interest that the sperm concentrations showed a larger variation compared with the proportions of sperm with light, intermediate, or dark staining. This idea of independence between sperm concentration and sperm maturity was described previously with various other biochemical markers ([5](#), [14](#), [31](#)). The three groups of spermatozoa with various levels of cellular maturation served as a model for exploring the potential relationship between a defect of the histone transition protein–protamine replacement cycle and chromosomal aneuploidies, as both may be related to defects of HspA2 action during meiosis or nucleoprotein transport.

### FISH Results

Assessment of the sperm population with FISH indicated that the mean frequencies of aneuploidies were as follows: disomy X:  $0.07\% \pm 0.01\%$ , disomy Y:  $0.09\% \pm 0.02\%$ ; disomy XY:  $0.18\% \pm 0.03\%$ , and disomy 17:  $0.16\% \pm 0.03\%$ . The frequencies of diploidy were  $0.17\% \pm 0.04\%$ .

In the sperm groups of various aniline blue staining intensity or nuclear maturity, the frequency of each of the four disomies and total disomy was higher in the intermediate compared with the light staining (mature) sperm ([Table 2](#)). The increase of frequencies were 3.9 times in X disomy, 5.2 times in Y disomy, 6.1 times in XY disomy, 4.5 times in 17 disomy, and 5.3 times higher in the aggregate or total disomy rates (all comparisons, except X disomy, are  $P < .001$ ). There was also a higher incidence of diploidy in the intermediate compared with the light cells, but this comparison did not reach significance.

Regarding the correlation between the variations of disomy frequencies versus the aniline staining attributes, the findings were confirmatory. [1] There was a relationship between the frequencies of various disomies and sperm with light and intermediate aniline blue staining, particularly between the proportions of intermediate sperm and frequencies

of disomy XY ( $r = 0.82$ ,  $P < .05$ ), disomy 17 ( $r = 0.70$ ,  $P < .05$ ), and total disomy frequencies ( $r = 0.76$ ,  $P < .05$ ). [Figure 2](#) is a graphic presentation of the relationship between aniline blue staining intensity and aneuploidy frequency. However, there was no correlation between aniline blue staining and diploidies or sperm concentrations.

There was an unexpected finding with respect to the FISH patterns in the case of aniline blue dark, the severely arrested mature spermatozoa. There were no chromosome signals with the centromere-specific FISH probes ([Fig. 1](#), left and right panels). Furthermore, some of the dark staining sperm also lacked DAPI nuclear staining, indicating a diminished presence of DNA.

## DISCUSSION

Attributes of sperm with arrested maturity may be detected by various nuclear and cytoplasmic probes ([5](#), [6](#), [22](#), [28](#)). Regarding the double probing technique, it was developed for a previous study, in which approximately 10,000 individual spermatozoa were treated with double markers for aniline blue staining and cytoplasmic retention, aniline blue and caspase 3 content, as well as for aniline blue and DNA fragmentation. There was a substantial 70% agreement between these cytoplasmic and nuclear markers with respect to the degree of maturity within the same spermatozoa. This indicated that arrested sperm maturation is not restricted to a single sperm compartment ([22](#)). Thus, the nuclear and cytoplasmic aspects of arrested maturity (e.g., persistent histones, cytoplasmic retention, DNA fragmentation, chromosomal aneuploidies, caspase 3 activity, Tygerberg abnormal morphology) may occur concurrently and are related ([4](#), [6](#), [8](#), [9](#), [11](#), [12](#), [21](#), [22](#), [28](#)). Substantial evidence supports that the relationship between cytoplasmic and nuclear features is based on a common contributory factor—the diminished level of HspA2 chaperon protein. HspA2 is expressed in two waves, in spermatocytes and elongated spermatids, and HspA2 chaperone action supports multiple cellular mechanisms ([1](#), [6–8](#), [32](#)).

The recognition that the aniline blue stain may be removed from sperm by destaining with acetic acid was very fortuitous, as acetic acid is also used as a solvent for aniline blue staining and is a component of fixatives for several sperm biochemical markers, such as FISH, immunocytochemistry, and DNA nick translation. Regarding the study design, the sequence of aniline blue staining and FISH was important, as the FISH procedures of decondensation and denaturation disrupt the nucleoprotein structure.

In earlier studies an association was reported between sperm developmental arrest, as demonstrated by aniline blue staining of persistent histones, and the number of chromosomal aberrations in semen samples ([23](#), [24](#), [33](#)). These data lead us to a more detailed study of the relationship between sperm nuclear maturity and chromosomal aneuploidies as we pursued aniline blue staining and FISH within the same spermatozoon.

**TABLE 2****FISH results.**

Aniline blue staining pattern		Disomy X		Disomy Y		Disomy XY		Disomy x,y,xy		Disomy 17		Total disomy X,Y,XY,17	
		L	IN	L	IN	L	IN	L	IN	L	IN	L	IN
Sample no.	1	0.029	0.11	0.043	0.430	0.058	0.75	0.130	1.290	0.058	0.543	0.19	1.82
	2	0.029	0.16	0.043	0.210	0.072	0.42	0.144	0.79	0.072	0.418	0.22	1.20
	3	0.033	0.11	0.033	0.170	0.066	0.56	0.132	0.84	0.104	0.335	0.21	1.17
	4	0.035	0.10	0.017	0.15	0.104	0.45	0.156	0.700	0.155	0.403	0.26	1.11
	5	0.034	0.14	0.052	0.18	0.086	0.36	0.172	0.54	0.120	0.501	0.33	1.18
	6	0.046	0.24	0.046	0.18	0.092	0.54	0.184	0.960	0.120	0.423	0.31	1.39
	7	0.040	0.13	0.060	0.21	0.100	0.43	0.200	0.770	0.100	0.515	0.30	1.29
Mean		0.035	0.14	0.035	0.22	0.082	0.50	0.152	0.860	0.099	0.447	0.26	1.31
Increases (fold-x)			3.9x		5.2x		6.1x		5.6x		4.5x		5.3x
Significance		$P = .028$		$P < .001$		$P < .001$		$P < .001$		$P < .001$		$P < .001$	

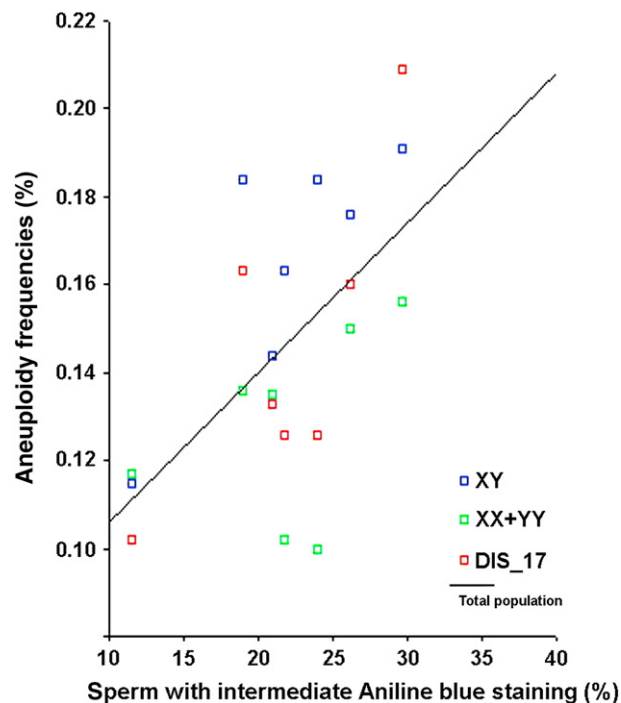
Note: The aneuploidy frequencies in the light (L) and intermediate" (IN) spermatozoa fractions. All disomy frequencies are expressed in percentage. The increases of aneuploidy frequencies in the L versus IN groups, and the degrees of statistical differences are noted in the two bottom lines.

Óvári. Sperm aneuploidy and persistent histones. Fertil Steril 2010.



**FIGURE 2**

Relationship between proportion of sperm with intermediate aniline blue staining and disomy frequencies ( $r = 0.6$ ,  $P = .002$ ).



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The relationship between diminished maturation of the nucleoprotein replacement pathway and occurrence of aneuploidies may be expected based on two aspects. [1] In spermatozoa with arrested maturity, the low expression of HspA2 would adversely affect histone transition protein–protamine replacement as well as the meiotic process (1, 6, 7, 9, 32–38). A central role for HspA2 in the nucleoprotein replacement has been recently demonstrated based on a global proteomic approach (8). [2] In mature sperm that underwent plasma membrane remodeling and are able to selectively bind to solid state hyaluronic acid, there are no persistent histones or increased frequency of chromosomal aneuploidies (5, 28).

In the present study, the aniline blue staining and FISH results of >58,000 individual spermatozoa have confirmed the relationship between nuclear maturity and aneuploidies. In sperm with light versus intermediate staining patterns (or mature or moderately arrested mature sperm, respectively), there was a four- to sixfold increased frequency of chromosomal aneuploidies (Table 2), affecting the sex and 17 autosomal chromosomes. There was also a correlation between disomy frequencies and aniline blue staining of disomy 17, disomy XY, and total disomy (Fig. 2). These findings agree with previous work in which an increased frequency of chromosomal aneuploidies was found in sperm with surplus cytoplasm (14).

There was also a slight increase in the frequency of diploidies in sperm with arrested maturity, but it did not follow the relationship of disomies and arrested sperm maturity because diploidies arise from a mechanism unrelated to sperm maturation (14, 16).

A further interesting finding was related to sperm with severe arrest of nuclear maturity and dark aniline blue staining, thus high level of persistent histones. These cells exhibited no FISH signal, and sometimes the DAPI staining of sperm DNA was also missing (Fig. 1). We propose two potential mechanisms for the lack of FISH probe binding. [1] The combined effects of arrested sperm maturation—cytoplasmic retention and increased production of reactive oxygen species production may cause higher levels of DNA chain degradation (34, 35). The fragmented DNA is held in place by the secondary helical structure in intact sperm. However, after the decondensation and denaturation steps necessary for FISH, the DNA fragments are released from sperm. [2] The second mechanism is related to low levels of HspA2 expression, which causes a combination of retarded histone transition protein–protamine replacement and delayed delivery of DNA repair enzymes, thus sperm DNA chain integrity is diminished (1, 6, 7, 22). This second point is well supported by Govin et al., a report that actually defines HspA2 as a transition protein chaperone (8).

Our experimental findings suggest that in sperm with severe maturation arrest, both of these proposed mechanisms may lead to the loss of FISH signal. Thus, one may surmise that disomy frequencies in oligozoospermic men, with a higher proportion of arrested maturity sperm, have been historically underestimated.

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