# Using semen flow cytometry to evaluate association of ploidy status and chromatin condensation of spermatozoa with conventional semen parameters: Clinical application in intrauterine insemination

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**Objective:** To evaluate the association between the ploidy status and the nuclear chromatin condensation of spermatozoa with conventional semen parameters by using semen flow cytometry (SFC). The prognostic value of SFC on the successful outcome of intrauterine insemination (IUI) was examined.
**Design:** Prospective study.
**Setting:** Patients referred to the IVF Unit of Ioannina University School of Medicine.
**Patient(s):** Ninety-two men with sperm count between $15 \times 10^6$ and $40 \times 10^6$ spermatozoa/mL and one hundred normozoospermic men were analyzed.
**Intervention(s):** Conventional semen analysis and SFC analysis after acridine orange and propidium iodide staining. IUI performed in 92 couples.
**Main Outcome Measure(s):** Evaluation of sperm maturity and ploidy. Correlation with conventional semen parameters.
**Result(s):** An association of the spermatozoa ploidy status with sperm morphology and motility was revealed. The highest aneuploidy rates were observed when <15% of spermatozoa had normal morphology. An inverse relation was found between sperm morphology and maturity. The pregnancy rates were significantly lower when semen with <15% normal forms (9% vs. 25%), low percentage of mature spermatozoa, and increased total aneuploidy rate were used for IUI.
**Conclusion(s):** Ploidy status and sperm maturity are critical parameters for evaluation of the fertilizing capacity of spermatozoa. SFC could be used to evaluate semen samples before IUI and potentially prognose the outcome. (Fertil Steril® 2011;95:110–5. ©2011 by American Society for Reproductive Medicine.)

**Key Words:** Chromatin condensation, flow cytometry, IUI, ploidy status

Intrauterine insemination (IUI) is one of the most frequent treatments for couples with male subfertility. Although the treatment itself is less invasive and expensive than others, its efficacy has been challenged (1). Sperm concentration and motility are taken into account before an IUI procedure. However, increasing numbers of couples fail to achieve a pregnancy even though sperm parameters are normal, indicating that additional parameters should be evaluated for the determination of semen fertilizing capacity.

Reproductive failures have been associated with cytogenetic abnormalities in germ cells of infertile individuals with normal karyotype. It is well known that a normal somatic karyotype evaluated by standard cytogenetic analysis does not exclude the presence of aneuploid gametes (2). A significant correlation has been reported between the sperm aneuploidy rate and conventional semen parameters (3–6), suggesting that the ploidy status of spermatozoa might have a crucial role in their fertilizing capacity.

Sperm maturity constitutes another major factor for the proper functioning of spermatozoa and an important cause of infertility (7). A significant correlation exists between sperm maturity and nuclear chromatin stability (8). The spermatozoa nuclear chromatin stability increases during spermiogenesis, owing to substitution of histones by protamines (9), a process called "sperm chromatin condensation." Mature spermatozoa are those with totally condensed chromatin and complete epididymal maturation. Sperm maturity can be estimated by the degree of exclusion of the dye acridine orange, which produces green fluorescence when it binds double-stranded nucleic acids and red fluorescence when it binds single-stranded nucleic acids (10). Significant increases of fluorescence values were positively correlated with abnormal percentages of immature spermatozoa and a decreased fertilizing capacity of the semen samples (11).

Semen flow cytometry (SFC) is able to detect the ploidy status and the maturity of spermatozoa. SFC has been used for size, cell compactness, and cytoplasmic structure analysis of spermatozoa

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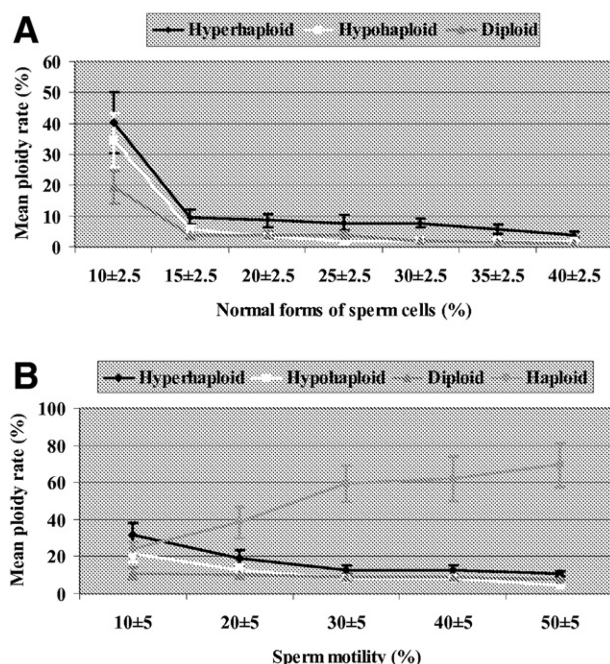
FIGURE 1

Association of mean ploidy rates in sperm cells with (**A**) sperm morphology and (**B**) sperm motility. Pearson correlation was used (**A**) between the hyperhaploidy, hypohaploidy, and diploidy rates and the percentage of normal forms of spermatozoa ($P<.01$) and (**B**) between the hyperhaploidy, hypohaploidy, haploidy, and diploidy rates and sperm motility ($P<.01$).




*Lazaros. Evaluating semen quality with flow cytometry. Fertil Steril 2011.*

(12, 13). The intensity of the fluorescent signal has been correlated with chromatin condensation (14) and the identification of haploid cells in semen (15). In the present study, we performed SFC to evaluate the ploidy status and the nuclear chromatin condensation of spermatozoa.

## MATERIALS AND METHODS

### Subjects

The study population consisted of 92 men aged 27–48 years, who were referred, prospectively, to the IVF Unit of the Department of Obstetrics and Gynecology, Medical School of Ioannina, Greece, for infertility treatment. A complete medical history was taken and physical examination performed. Men suffering from karyotypic abnormalities, hypogonadotropic hypogonadism, varicocele, or obstructive syndromes of the seminal tract and men under treatment with spermatogenesis/motility–impairing medication were excluded. Cases in which female infertility factor was found were also excluded. IUI was performed as first-line treatment of their infertility. The Institutional Ethics Committee approved the study protocol in accordance with the Helsinki declaration, and each participant gave informed consent.

Semen analysis was performed according to World Health Organization (WHO) guidelines (16). Men were asked to abstain from sexual activity for 2–5 days before IUI. Two independent investigators performed blind semen analysis. The average values from the two investigators were calculated. In the event of inconsistency (>10% difference) a third assessment was done. The sperm concentration was between $15 \times 10^6$ and $40 \times 10^6$ spermatozoa/mL. The morphology was evaluated after Papanicolaou staining. All samples were processed with sperm gradient kit and sperm preparation medium (Medicult, Jyllinge, Denmark) for the IUI procedure. A standard GnRH-antagonist protocol was used for ovarian stimulation as described by Zikopoulos et al. (17).

One hundred normozoospermic men (sperm concentration $73.3 \pm 13.5 \times 10^6$ spermatozoa/mL), age matched with the study population, participated in the study as a control group.

### Propidium Iodide Staining and Flow Cytometry

The method of propidium iodide staining and SFC was used to study the ploidy status of human spermatozoa, according to a protocol described previously (18). Two semen samples from each patient were used for the analysis to ensure reliable results. For the analysis, Facscalibur Flow Cytometer (Becton Dickinson, San Jose, CA) was used. Red fluorescence (BP 650LP filter) emitted from individual cells was recorded from 10,000 cells per sample after excitation with a 488 nm argon laser using a logarithmic scale to allow cells of all ploidy status to appear as peaks in the resulting histograms.

### Acridine Orange Staining and Flow Cytometry

The method of acridine orange staining and SFC was used to study abnormal chromatin condensation of human spermatozoa, according to a protocol described previously (11). Two semen samples from each patient were used for the analysis to ensure reliable results. The processed semen samples were screened using a Facscalibur Flow Cytometer (Becton Dickinson). Green fluorescence (BP 530/30 filter) and red fluorescence (BP 650LP filter) were measured for 30,000 counts/sample after excitation with a 488 nm argon laser. The Consort 40 (Becton Dickinson) was used to analyze the data.

### Statistical Analysis

Statistical analysis was performed using the chi-squared test. Normal distribution of continuous parameters was tested by Kolmogorov-Smirnov test. Differences in continuous parameters between genotypes were assessed with the nonparametric Kruskal-Wallis test. A $P$ value of $<.05$ was set as statistically significant. All analyses used the SPSS statistical package (version 14.0; SPSS, Chicago, IL).

## RESULTS

### Study Population Characteristics

Men with sperm count between $15 \times 10^6$ and $40 \times 10^6$ spermatozoa/mL and $33.6 \pm 24.1\%$ motility were examined (n = 92). All semen samples contained increased amounts of spermatozoa with abnormal morphology. Sperm concentration was inversely correlated with the percentage of abnormal forms ($P<.01$). When sperm concentration was $40 \times 10^6$ spermatozoa/mL, 35% of the sperm cells had abnormal morphology. When sperm concentration was $15 \times 10^6$ spermatozoa/mL, the prevalence of abnormal forms increased to 50%.

One hundred normozoospermic men, with sperm concentration $73.3 \pm 13.5 \times 10^6$ spermatozoa/mL and $67.4 \pm 9.35\%$ motility, age-matched with the study population, were examined. Their semen samples contained significantly lower numbers of spermatozoa with abnormal morphology compared with the study population.

### Association of Sperm Cell Ploidies With Sperm Morphology and Motility

The spermatozoa ploidy status analysis revealed peaks of hypohaploid, normal haploid, immature haploid (elongated spermatids), hyperhaploid, diploid, and tetraploid cells in the histograms of all samples. A significant correlation between these ploidies and sperm morphology was revealed (Fig. 1A). Hyper-, hypo-, and diploidies were observed more frequently in samples with high percentages of abnormal forms ($P<.01$). The highest rates of ploidies were observed when <15% of spermatozoa had normal morphology

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