ASSISTED REPRODUCTION TECHNOLOGIES

Assessment of sperm chromatin condensation and ploidy status using flow cytometry correlates to fertilization, embryo quality and pregnancy following in vitro fertilization

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Abstract

Purpose Sperm flow cytometry (SFC) was used to evaluate the association of sperm chromatin condensation and ploidy with fertilization, embryo development, pregnancy and abortion rates following IVF.

Methods Conventional semen analysis was performed in one hundred fifty men, as well as SFC analysis, after acridine orange and propidium iodide staining, for the evaluation of sperm maturity and ploidy respectively. Conventional IVF was performed in all couples.

Results Couples with low percentages of mature spermatozoa presented with lower fertilization rates ($p<$ 0.005), lower rates of grade A embryos ($p<0.003$) and lower pregnancy rates ($p<0.006$), compared to couples with high percentages of mature spermatozoa. Couples with low total aneuploidy rates presented with higher fertilization rates ($p<0.007$), higher rates of grade A embryos ($p<0.004$) and higher pregnancy rates ($p< 0.003$), compared to couples with high total aneuploidy rates.

Conclusions Sperm chromatin condensation and ploidy constitute critical parameters for the evaluation of semen samples before IVF and for the identification of cases in need of ICSI application.

Keywords Flow cytometry · IVF outcome · Pregnancy rate · Sperm chromatin condensation · Sperm ploidy status

Capsule Sperm nuclear chromatin condensation and ploidy constitute critical parameters for the evaluation of semen samples before in vitro fertilization.

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Introduction

Fifteen percent of the couples worldwide face infertility problems, while half of these are affected by male factor infertility [1]. The development of assisted reproduction technologies (ART) has overcome the problem of male infertility, giving in infertile couples the possibility of a pregnancy. Sperm morphology, concentration and motility are mainly taken into account for the selection of the appropriate assisted reproduction technique. Despite normal semen analysis values, a high proportion of couples undergoing ART still fail to achieve a pregnancy. Thus, additional parameters should be used for the evaluation of sperm quality. Genetic and epigenetic

sperm abnormalities such as abnormal nuclear packaging, chromosome aneuploidy, sperm DNA damage, genomic imprinting errors and centrosome malformations might contribute to poor embryogenesis [2].

The structural organization of sperm DNA has been found to be vital for the proper functioning of spermatozoa [3], necessitating the characterization of sperm chromatin condensation and stability of semen samples before an ART application. Sperm nuclear chromatin condensation involves a cascade of transcriptional alterations, topological rearrangements, nucleosomal structure loss and histones to protamines substitutions, leading to spermatozoa with almost entirely condensed chromatin in their nucleus [4]. Flaws in the mechanisms that package and protect the sperm chromatin during spermiogenesis lead to sperm chromatin condensation abnormalities [5, 6]. Chromatin condensation and stability have been proposed as reliable markers of sperm maturity [7]. Indeed, the chromatin of spermatozoa from the distal cauda epididymis is more condensed than that from spermatozoa of the proximal sites of epididymis [8].

Chromosome aneuploidies are more frequent in patients suffering from spermatogenetic failures, such as severe oligospermia or azoospermia [9]. The development of fluorescent in situ hybridization (FISH), permitting the cytogenetic analysis of large numbers of spermatozoa, has revealed significantly increased sperm aneuploidy rates in azoospermic patients compared to normozoospermic men [10, 11]. Apart from men with aberrant karyotype, who are expected to have spermatozoa with chromosomal abnormalities, men with normal somatic karyotype are often presented with aneuploid gametes [12]. The aneuploidy of these gametes may be ascribed to an altered intratesticular environment that disrupts the chromosome segregation mechanisms [13]. Many studies have reported an inverse correlation between sperm aneuploidy rates and conventional semen parameters [14–19], confirming the association of chromosomal abnormalities with poor sperm quality.

Sperm flow cytometry (SFC) after Acridine Orange (AO) and Propidium Iodide (PI) staining has been proposed as a reliable method for the evaluation of sperm chromatin condensation [20] and ploidy [21], respectively. These parameters have been recently associated with sperm morphology and motility as well as with intrauterine insemination (IUI) success rates [22]. In the current study, we used SFC analysis to investigate the putative effects of sperm nuclear chromatin condensation and ploidy on spermatozoa fertilizing capacity, embryo quality, pregnancy and spontaneous abortion rates after conventional in vitro fertilization (IVF).

Materials and methods

Subjects

The study population consisted of 150 men, aged 28–46 years, who referred to the IVF Unit of the Department of Obstetrics and Gynecology of Ioannina Medical School for conventional IVF, following three or more failed IUI attempts.

A detailed medical history was obtained from all subjects. Physical examination was performed. Men carrying microdeletions of the long arm of the Y chromosome or karyotype abnormalities, men suffering from varicocele, hydrocele, hypogonadotropic hypogonadism and obstructive syndromes of the seminal tract as well as men under spermatogenesis-impairing medication were excluded from the study. Partners of women over 37 years old, or women suffering from endometriosis and severe congenital malformations of the reproductive organs were also excluded from the study.

Semen analysis was performed according to World Health Organization [23] guidelines. Men abstained from sexual activity for 3 days prior to semen analysis. Two independent investigators performed blind semen analysis at the day of oocyte pick-up. The average values of the two investigators were calculated. In the event of inconsistency (over 10% difference) a third assessment was done. The morphology was evaluated after Papanicolaou staining.

All sperm samples were processed with sperm gradient kit and sperm preparation medium (Medicult, Jyllinge, Denmark) for the IVF procedure. All women of the study population underwent a long GnRH agonist stimulation protocol as previously described [24]. Conventional IVF was performed in all couples.

The Institutional Ethics Committee approved the study protocol in accordance to the Helsinki declaration and all participants gave informed consent.

Primary IVF outcome measures

The fertilization rate, the embryo quality and the clinical pregnancy rate were the primary outcome measures. The fertilization rate, namely the percentage of oocytes with two formed pronuclears, was recorded 24 h after oocyte retrieval. Embryo development and quality were evaluated 3 days after oocyte pick-up. The number of blastomeres and the proportion of embryo volume occupied by fragments were used for the evaluation. Embryos with <10%, <10%–20%, <20%–30% and >30% fragments were recorded as grade A,B,C and D, respectively. Three embryos with the highest blastomere number and the best morphology were transferred the third day of each cycle.

Pregnancy was diagnosed by quantitative β-hCG 2 weeks after embryo transfer. Clinical pregnancy was confirmed by observing fetal cardiac activity on transvaginal ultrasound 4 weeks after a positive pregnancy test.

Flow cytometry analysis after Acridine Orange and Propidium Iodide stainings

The methods of AO and PI stainings in combination with SFC were used to study the abnormal chromatin condensation and the ploidy of human spermatozoa, respectively, according to protocols previously described [25, 26]. Briefly, two semen samples from each patient were used for the analysis, so as to ensure reliable results. The FACScalibur Flow Cytometer and the Consort 40 (Becton Dickinson, San Jose, CA, USA) were used for the resulting data acquisition and analysis. In case of AO staining, green fluorescence (BP 530/30 filter) and red fluorescence (BP 650LP filter) were measured for 30,000 cells per sample after excitation with a 488 nm argon laser, while in case of PI staining, red fluorescence (BP 650LP filter) emitted from individual cells was recorded for 10,000 cells per sample after excitation with a 488 nm argon laser.

Statistical analysis

Statistical analysis was performed using the chi-square test. Normal distribution of continuous parameters was tested by Kolmogorov-Smirnov test. Differences in continuous parameters were assessed by using *t*-test for independent variables. *P*-value of <0.05 was set as statistically significant. All analyses used the SPSS statistical package (version 14.0, SPSS Inc, Chicago, IL, USA).

Results

Study population characteristics

Our study population consisted of 150 men from equal number of couples undergoing IVF. These men had sperm concentration $75.2 \pm 32.7 \times 10^6$ spermatozoa/ml, sperm motility $54.1 \pm 10.9\%$ and normal morphology $28.3 \pm 7.9\%$.

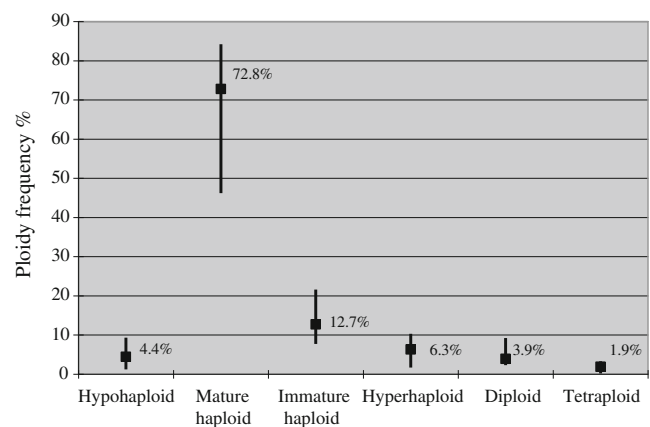### Sperm chromatin condensation analysis

All semen samples emitted both green and red fluorescence, at different intensities in each case, after AO staining and SFC analysis. AO staining produces green fluorescence when it binds double-stranded nucleic acids and red fluorescence when it binds single-stranded nucleic acids [27]. The intensities of green and red

fluorescence were significantly increased in semen samples characterized by increased percentages of spermatozoa possessing abnormal morphology such as head defects (large, small, round, pyriform, amorphous, small acrosomal area and tapered heads), midpiece defects (thick, abnormally thin and irregular midpieces) and tail defects (short, multiple, broken, hairpin and irregular wide tails) (data not shown). In contrast, no associations were observed between these fluorescence values and sperm concentration and motility.

Taking into account that green and red fluorescent intensities are inversely correlated with sperm chromatin condensation and maturation [25], we estimated for each semen sample the percentage of cells with fully condensed chromatin, namely the percentage of mature spermatozoa. This rate was calculated as 100(WINM)/(WINT), where window M corresponded to spermatozoa with completed epididymal sperm maturation; window T to the major sperm band, excluding cell debris and satellite populations [22, 25]. The median percentage of mature spermatozoa in our study population was 65.1%, while the rates ranged from 41.3% to 83.8%.

Sperm ploidy analysis

The spermatozoa ploidy analysis revealed peaks of hypo-haploid, normal haploid, immature haploid, hyperhaploid, diploid, and tetraploid cells in the histograms of all samples. These values were found to differ between semen samples. The median values and ranges of every ploidy are presented in Fig. 1. The median total sperm aneuploidy rate in our study population, which was estimated excluding immature haploid cells, was 16.7% while the frequencies ranged from 5.1 to 31.1%.

Fig. 1 The sperm ploidy status analysis of the study population. Data are shown as median value and range

Association of sperm chromatin condensation
with fertilization, pregnancy rates and embryo quality

To analyze the effects of sperm chromatin condensation on
IVF outcome, the study population was subdivided in
subgroups using as cut off the median percentage of mature
spermatozoa: men with percentage of mature spermatozoa
<65.1% (group A1) and men with percentage of mature
spermatozoa ≥65.1% (group A2).

Couples of group A1 presented with lower fertilization
and pregnancy rates compared to couples of group A2
(Table 1). Specifically, statistical significant differences
were observed in the fertilization rates between couples of
group A1 and couples of group A2 (group A1: 60.7±19.8%
vs. group A2: 79.4±19.1%, $p<0.005$) and in the pregnancy
rates (group A1: 19.4% vs. group A2: 39.7%, $p<0.006$).

In addition, couples of group A1 presented lower
embryo quality compared to couples of group A2 (Table 1).
Specifically, couples of group A1 had lower rates of grade
A embryos than couples of group A2 (group A1: 19.9±
8.4% vs. group A2: 35.7±10.1%; $p<0.003$) and higher
rates of grade C embryos (group A1: 38.1±11.9% vs. group
A2: 20.1±9.2%; $p<0.002$).

Finally, the spontaneous abortion rate, which was
estimated to be 25%, differed between the two groups. In
specific, couples of group A1 presented with higher
spontaneous abortion rate compared to couples of group
A2 (group A1: 33.3% vs. group A2: 21.7%). However, no
statistical significance was observed in the above difference
(Table 1).

Association of sperm ploidy with fertilization, pregnancy
rates and embryo quality

To analyze the effects of sperm ploidy on IVF outcome, the
study population was subdivided in subgroups using as cut

off the median total aneuploidy rate: men with total
aneuploidy rate <16.7% (group B1) and men with total
aneuploidy rate ≥16.7% (group B2).

Couples of group B1 presented higher fertilization and
pregnancy rates compared to couples of group B2 (Table 2).
Specifically, statistical significant differences were observed
in fertilization rates between couples of group B1 and
couples of group B2 (group B1: 77.2±20.9% vs. group B2:
58.7±18.8%, $p<0.007$) and in the respective pregnancy
rates (group B1: 42.1% vs. group B2: 20.2%, $p<0.003$).

In addition, couples of group B1 presented higher
embryo quality compared to couples of group B2 (Table 2).
Specifically, couples of group B1 had higher rates of grade
A embryos than couples of group B2 (group B1: 33.1±
11.3% vs. group B2: 18.3±6.8%; $p<0.004$) and lower rates
of grade C embryos (group B1: 19.8±9.5% vs. group B2:
38.4±12%; $p<0.002$).

Finally, the spontaneous abortion rate, which was
estimated to be 25%, differed between the two groups. In
specific, couples of group B1 presented with lower
spontaneous abortion rate compared to couples of group
B2 (group B1: 24% vs. group B2: 28.6%). However, no
statistical significance was observed in the above difference
(Table 1).

Discussion

The aim of the current study was to investigate the
contribution of sperm nuclear chromatin condensation and
ploidy on IVF primary outcomes. SFC analysis after AO
and PI staining was used in semen samples of couples
undergoing conventional IVF treatment. The fertilization
rates, the embryo quality and the pregnancy rates were
inversely associated with semen samples immaturity and
aneuploidy rates. In contrast, no significant associations

Table 1 Association of fertilization, pregnancy, abortion rates and embryo quality with sperm chromatin condensation

	Group A1	Group A2	p value
Number of patients	72	78	
Age (years)	35.1±6.7	34.4±6.5	ns[a]
Sperm concentration (×10⁶ spermatozoa/ml)	72.9±30.8	71.4±30.1	ns[a]
Sperm motility (%)	52.9±8.8	50.2±8.4	ns[a]
Normal sperm morphology (%)	27.2±6.3	30.1±8.7	ns[a]
Number of oocytes	14±6.1	12.5±5.7	ns[a]
Fertilization rate (%)	60.7±19.8	79.4±19.1	0.005[a]
Grade A embryos (%)	19.9±8.4	35.7±10.1	0.003[a]
Grade B embryos (%)	20.2±9.3	29.5±8.8	ns[a]
Grade C embryos (%)	38.1±11.9	20.1±9.2	0.002[a]
Grade D embryos (%)	18.2±6.6	11.3±5.4	ns[a]
Pregnancy rate (%)	19.4	39.7	0.006[b]
Abortion rate (%)	33.3	21.7	ns[b]

Group A1: percentage of mature spermatozoa <65.1%, Group A2: percentage of mature spermatozoa ≥65.1%.

[a] t-test analysis

[b] Chi square test analysis

Table 2 Association of fertilization, pregnancy, abortion rates and embryo quality with sperm ploidy status

	Group B1	Group B2	p value
Number of patients	76	74	
Age (years)	34.1±7.2	34.8±6.9	ns[a]
Sperm concentration (×10⁶ spermatozoa/ml)	69.3±34.9	74.8±33.1	ns[a]
Sperm motility (%)	51±9.1	52.7±8.9	ns[a]
Normal sperm morphology (%)	30.6±5.3	25.2±4.2	ns[a]
Number of oocytes	12.7±5.3	13.8±6.2	ns[a]
Fertilization rate (%)	77.2±20.9	58.7±18.8	0.007[a]
Grade A embryos (%)	33.1±11.3	18.3±6.8	0.004[a]
Grade B embryos (%)	28.9±9.1	21.1±9.8	ns[a]
Grade C embryos (%)	19.8±9.5	38.4±12	0.002[a]
Grade D embryos (%)	10±5.7	18.5±6.9	ns[a]
Pregnancy rate (%)	42.1	20.2	0.003[b]
Abortion rate (%)	24	28.6	ns[b]

Group B1: total sperm aneuploidy rate <16.7%, Group B2: total sperm aneuploidy rate ≥16.7%.

[a] t-test analysis

[b] Chi square test analysis

were observed between IVF primary outcomes and semen samples concentration or motility. Similar associations have been reported in a recent study of our group, examining men with "gray zone" sperm concentration undergoing IUI [22]. Namely, significantly lower pregnancy rates have been observed, when semen samples with increased aneuploidy rates and low sperm maturity have been used.

AO staining followed by SFC [25] was performed to estimate the percentage of sperm cells with complete epididymal maturation and totally condensed nuclear chromatin. The progressive chromatin packaging produces a reduction in DNA stainability relative to round spermatids and a marked decrease in AO binding to DNA as human spermatozoa traverse the epididymis [8]. Lack of appropriate sperm maturation results in an increased DNA stainability [28], frequently observed in many ejaculated spermatozoa from infertile men [6]. In our series, significant increases in green and red fluorescence values were positively correlated with increased percentages of spermatozoa with abnormal morphology due to their uncondensed nuclear chromatin. The increased intensity of green fluorescence is perhaps the result of an abnormal exchange of histone for transition proteins and protamines [9]. But an abnormal exchange would probably lead to immature spermatozoa, incapable to cause pronuclear formation after oocytes penetration. Indeed, Sakkas and co-workers [29], who examined the effects of human spermatozoan chromatin anomalies on fertilization after intracytoplasmic sperm injection (ICSI), suggested that abnormal chromatin might fail to decondense due to a physical or mechanical inability, leading to impaired fertilization. Furthermore, taking into account that protamine addition according to a specific ratio is crucial for fertilization and embryogenesis [30, 31] as well as that protamine deficiency [32] and absence [33] have been associated with significant impairments in fertilization, we

could explain why semen samples with abnormal chromatin packaging and consequently decreased maturity show lower fertilization rates after conventional IVF.

Abnormal sperm nuclear chromatin condensation, apart from fertilization, has been shown to affect embryo quality and pregnancy rates following IVF. In previous studies, similar associations have been observed. Specifically, the increased DNA breaks due to faulty replacement of histones by protamines [5] have been associated with decreased embryo morphology at early cleavage stages [34], whereas the improper protamine ratios have been proposed to be responsible for poor preimplantation stage embryo morphologies [30]. These embryos have been shown to arrest at the six- to eight-cell stage, coinciding with full activation of the embryonic genome. The impaired sperm maturation has also been associated with decreased pregnancy rates [35–37], failure of embryos to progress to the blastocyst stage in culture [38] and low pregnancy rates after ICSI treatment [29]. Furthermore, the spontaneous abortion rates of our study population, which were similar with those observed in previous studies [39], were increased in cases of semen samples with low percentages of mature spermatozoa. However, no statistical significance was observed in the above association. Larger series are needed to confirm or to disprove this possible inverse association. In conclusion, we could hypothesize that the increased sperm DNA breaks and the abnormal nuclear chromatin structure, markers of an altered sperm genetic constitution, are probably responsible for the impaired embryo quality and consequently for the low implantation rates after an IVF.

On the other hand, the primary IVF outcomes have been inversely associated with sperm aneuploidy. Sperm aneuploidy arises due to nondisjunctions during metaphase I or II of meiosis. FISH development has revealed high aneuploidy rates in human spermatozoa [40], mainly in

those obtained from subfertile and infertile men [15, 41], proving that sperm chromosomal abnormalities and aneuploidy rates constitute indicators of impaired sperm quality. In the current study, semen samples characterized by increased hypohaploid, hyperhaploid, diploid, and tetraploid rates led to low fertilization rates after conventional IVF. Similar results have been observed in a previous study [42], where semen samples with increased aneuploidy rates were used in ICSI. The impaired fertilization, which was observed [42], ensures the inverse association of sperm aneuploidy with fertilization.

Conventional IVF treatment of semen samples with increased aneuploidy rates also led to lower embryo quality and to decreased pregnancy rates. These results are reinforced by a previous study suggesting an association of sperm aneuploidy with an altered embryonic genetic constitution and morphology [43]. Additionally, sperm cell aneuploidy has been associated with low implantation rates [42, 44], low pregnancy rates [42, 45, 46] and increased possibilities of miscarriage in IVF cycles where the best morphologically embryos were transferred [47]. Indeed, in our study population, increased rates of spontaneous abortions were observed in cases of semen samples with high sperm cell aneuploidy. However, no statistical significance was observed in the above association. More cases are possibly needed to verify this finding. Finally, the fact that ICSI patients whose partners did not achieve a pregnancy had higher sperm aneuploidy rates compared to patients whose partners became pregnant [48], indicates that oocyte fertilization can be accomplished by aneuploid spermatozoa, but it results in lower pregnancy rates. Consequently, we could suggest that sperm aneuploidy probably does not affect the embryonic growth initiation, but seems to be responsible for a decreased implantation and live birth potential.

Taking into consideration the above analysis, we could assume that sperm nuclear chromatin condensation and ploidy constitute critical parameters for a successful conventional IVF outcome. Although swim-up and Percoll fractionation techniques generally increase the quality of semen samples used in IVF [25], a high proportion of attempts still fail to achieve a successful outcome. Given that SFC offers an aneuploidy screening of all chromosomes, while FISH technique analyses specific chromosomes overlooking a significant amount of aneuploidies [14, 49], as well as that SFC is a less time and money consuming method compared to FISH, we could suggest SFC for a better evaluation of sperm quality. This technique would help us discriminate which ART technique, conventional IVF or ICSI, is appropriate so as to increase the possibility of a successful outcome, even when sperm concentration and motility are within normal range according to WHO criteria.

This is the first study, to our knowledge, to associate simultaneously sperm chromatin condensation and ploidy

with the IVF outcome. However, larger series are needed to verify our preliminary results. Whereas the construction of 'Receiver Operating Characteristic' curves (ROC curves) for the percentage of mature spermatozoa and for the total aneuploidy rate, individually and combined, will ensure their value in predicting the fertilization rates, the embryo quality and the pregnancy rates. SFC analysis may help in the discrimination of semen samples with high fertilizing capacity and elevated pregnancy potential after a conventional IVF as well as in the identification of the cases in need of ICSI application, reducing the number of unsuccessful assisted reproduction treatments to a minimum.

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