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ICSI Outcome is not Associated with the Incidence of Spermatozoa with Abnormal Chromatin Condensation

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Abstract. *Background: The condensation of sperm chromatin during spermiogenesis and epididymal transport is of essential importance for fertilization. The main purpose of this study was to examine whether abnormalities of sperm nuclear condensation can influence the outcome of intracytoplasmic sperm injection (ICSI) cycles. Materials and Methods: Semen samples from 154 ICSI cycles were studied. Before semen preparation for ICSI, basic semen analysis was performed and a small portion from each sample was fixed. The condensation of sperm nuclear chromatin was evaluated with chromomycin A3 under a fluorescence microscope. Results: The incidence of spermatozoa with abnormal chromatin condensation was positively correlated with sperm concentration ($p=0.020565$), but was not correlated with other semen parameters such as morphology and motility. Abnormal chromatin condensation was also not correlated with fertilization rate, cumulative embryo score or pregnancy rate. Conclusion: The above results indicate that ICSI outcome is not influenced by the incidence of spermatozoa with abnormal chromatin condensation.*

Mammalian sperm DNA is the most tightly packed eukaryotic DNA. Sperm nuclear chromatin is at least six times more condensed than the nuclear chromatin of somatic cells during mitosis (1). The appropriate condensation of nuclear chromatin, taking place during the last stages of spermiogenesis and epididymal transport, is a critical factor that influences the fertilization potential of spermatozoa. The condensation of nuclear chromatin is related to biochemical changes such as the replacement of lysine-rich histones, which are connected with DNA, initially by transient proteins (testis-specific histones) and then by arginine-rich protamines. At the same time, bisulfidic bonds are formed between cysteine residues (1-3). In these ways, nuclear chromatin is tightly packed, also influencing the shape of the head. It appears that the small and compact shape of spermatozoa protect them from natural and chemical damaging factors, consequently increasing their fertilization potential.

Several studies have reported an association between abnormal sperm chromatin condensation and male infertility (4-11). Furthermore, it seems that this abnormality impairs the fertilization potential of spermatozoa in subzonal insemination (8) and conventional *in vitro* fertilization (IVF) attempts (12-15). Intracytoplasmic sperm injection (ICSI) is an assisted reproduction technique developed to bypass male factor infertility problems. The sperm concentration, motility and morphology are not always suggestive of male fertilization capacity in ICSI cycles (16). During ICSI, the artificial selection of motile and morphologically normal spermatozoa for fertilizing oocytes usually offers normal fertilization and pregnancy rates regardless of conventional semen parameters (16,17). However, as there is not a clear relationship between sperm chromosomal status and morphology or motility, there are concerns about the use of semen samples with a high incidence of chromosomal abnormalities in ICSI procedures. Lower condensation quality in morphologically normal spermatozoa is possibly a factor affecting the achievement of fertilization, *in vitro* development, implantation and pregnancy. Thus, the investigation of the relationship between abnormal sperm chromatin condensation and ICSI outcome is particularly important.

The condensation of nuclear chromatin can be examined using various stainings followed by either fluorescence microscopy or flow cytometry. The most frequently used

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Table I. *Descriptive statistics of the studied parameters. N=154.*

	Mean	Standard deviation	Median	Standard error
Semen volume (ml)	4.335	2.253	4	0.186
Sperm concentration (x 10^6)	73.844	62.839	60	5.183
Progressive motility (%)	43.748	21.431	50	1.768
Morphologically normal spermatozoa (%)	9.658	10.694	5	0.885
Incidence of spermatozoa with abnormal chromatin condensation (%)	19.136	9.773	18	0.788
MII oocytes	5.716	3.844	6	0.347
Fertilization rate (%)	76.585	22.128	80	1.825
CES	46.576	28.7	42	2.434

CES: Cumulative Embryo Score.

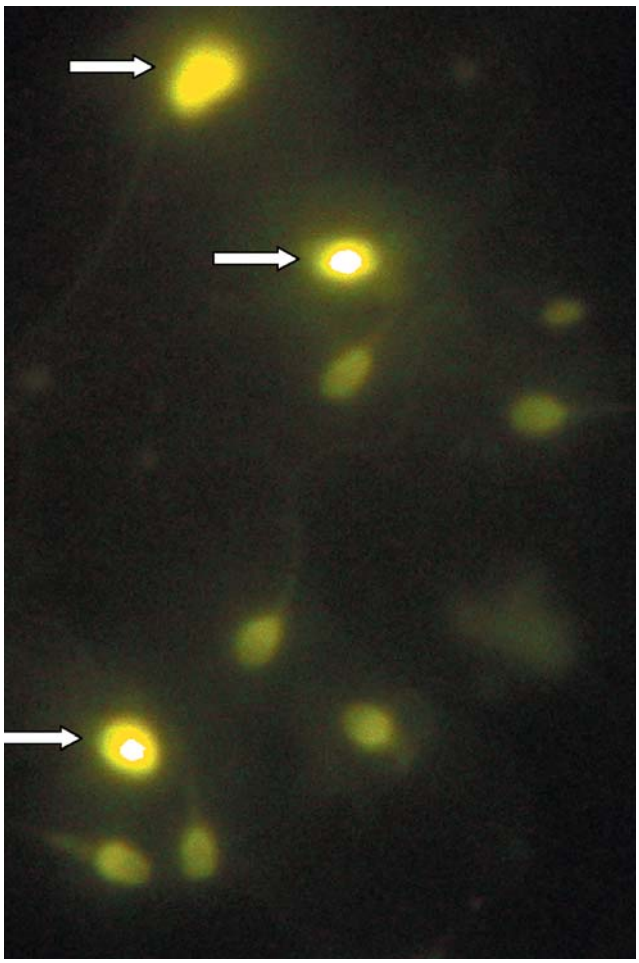

Figure 1. *Semen smear stained with CMA3 under fluorescence microscope. Spermatozoa with insufficient nuclear chromatin condensation fluoresce intensively (arrows).*

are: chromomycine A3 (CMA3), acridine orange, aniline blue, ethidium bromide and propidium iodide (4-15, 18-24). CMA3 is a fluorescent staining which is used to detect DNA regions rich in cytosine-guanine bases. In spermatozoa, such regions are those where the replacement of histones from protamines is insufficient or does not happen at all. CMA3 is attached to these regions, staining the affected spermatozoa, which can be visualized under fluorescence.

The present study aimed to examine whether the incidence of spermatozoa with abnormal nuclear chromatin condensation is correlated with basic semen analysis parameters and if this incidence can influence the fertilization and pregnancy outcome after ICSI.

Materials and Methods

In this prospective study, 100 couples were enrolled. All couples participated in ICSI treatment after controlled ovarian hyperstimulation with the long agonist protocol. ICSI was performed with freshly ejaculated spermatozoa. Thirty-four couples had more than one ICSI cycle. In total, the couples underwent 154 ICSI cycles.

The controlled ovarian hyperstimulation was applied, as previously described (25). Semen samples were obtained by masturbation after 3-4 days of abstinence. The semen was allowed to liquefy for 30 minutes at room temperature. Basic semen analysis was performed according to the ESHRE Manual on Basic Semen Analysis (26) and several smears were made from each semen sample. The semen smears were fixed with "Spray for cytodiagnosis" (Merck, Hamburg, Germany). Thereafter, the semen samples were prepared and ICSI was performed, as described elsewhere (27, 28). Embryo transfers took place on the second day after ICSI. Before each embryo transfer, the cumulative embryo score (CES) was calculated, as previously described (29). The confirmation of pregnancy was done by ultrasonography.

CMA3 staining. The slides with fixed semen smears were placed in a methanol/acetic acid solution (3:1) for 10-15 minutes. A solution of 0.25 mg CMA3 in 1ml phosphate-buffered saline (PBS) was prepared and 100 ml of it were placed in each slide. After 30 minutes, the slides were rinsed with PBS. On the next day, the slides were examined under a fluorescence microscope (Olympus BX40). From each slide, at least 100 spermatozoa were evaluated, the stained (fluorescent) ones were counted and the percentage of stained sperm heads was calculated.

Statistical analysis. Descriptive statistics were drawn for the following parameters: semen volume, sperm concentration, percentage of motile sperm, percentage of normal spermatozoa

Table II. *Descriptive statistics of semen parameters from 34 patients with more than one sample within the study population. Values are presented as mean±S.D. The differences were not statistically significant.*

	1st cycle (n=34)	2nd cycle (n=34)	3rd cycle (n=15)	4th cycle (n=5)
Semen volume (ml)	4.176±2.329	4.412±2.12	5.6±2.62	5±2.121
Sperm concentration (x 10^6)	58.618±55.083	57.324±48.807	71.467±53.613	66±37.98
Motility (%)	38.853±22.037	45±23.42	38±24.187	50±18.708
Morphologically normal spermatozoa (%)	8.394±10.161	11.242±13.711	14.133±12.557	7±2.739
Incidence of spermatozoa with abnormal chromatin condensation (%)	19.394±11.057	19.303±10.11	20.133±7.726	16.2±7.981

according to Tygerberg strict criteria, percentage of stained spermatozoa (with abnormal nuclear chromatin condensation), number of retrieved metaphase II (MII) oocytes, fertilization rate and pregnancy rate. Relationships were examined with the Spearman Rank Order correlation test and differences between groups with the *t*-test and Mann-Whitney *U*-test. Moreover, the subgroup of couples with more than one ICSI cycle was analyzed separately. Differences among the semen samples from successive ICSI cycles were examined by *t*-test for dependent samples and Wilcoxon matched-pairs test. Fertilization and pregnancy rates were compared with the χ^2 test. $P<0.05$ was considered as significant. The statistical analysis was performed with Statistica for Windows 5.0 (StatSoft Inc., Tulsa, OK, USA).

Results

In a total of 154 ICSI cycles, corresponding to 100 couples, the semen samples were evaluated for the incidence of spermatozoa with abnormal chromatin condensation (Table I). Abnormally condensed sperm nuclei were stained with CMA3 and exhibited intense green light under fluorescence (Figure 1). Thirty-four couples participated in more than one cycle; the statistical analysis did not reveal significant differences among the successive semen samples regarding the studied parameters (Table II).

In the overall population, the incidence of spermatozoa with abnormal chromatin condensation was not significantly correlated with any of the studied parameters, with the exception of sperm concentration (Spearman R=0.191531, $p=0.020565$). Forty-eight cycles resulted in pregnancy (31.16%). In the ICSI cycles that resulted in pregnancy, the semen parameters and fertilization rate were similar with those in cycles that did not result in pregnancy. The incidence of spermatozoa with abnormal chromatin condensation was slightly lower in cycles that resulted in pregnancy than in those that did not, although not at a statistically significant level (17.52% *vs*. 19.92%, $p=0.281$). However, the cycles that resulted in pregnancy had a significantly higher number of MII oocytes (7.195±4.654 *vs.* 5.184±3.136, $p=0.019804$) and a significantly higher CES (54.933±28.135 *vs*. 42.616±29.162, $p=0.0075$) (Table III).

Table III. *Comparison between ICSI cycles that resulted in pregnancy and those that failed. N=154. Comparison were made with the Mann-Whitney U-test.*

	Pregnancy	No pregnancy	*P*
Semen volume (ml)	4.12±1.777	4.475±2.52	0.9103
Sperm concentration (x 10^6)	66.446±63.274	78.181±63.671	0.184
Motility (%)	40.326±20.532	45.396±22.053	0.1577
Morphologically normal spermatozoa (%)	7.913±9.966	10.367±10.049	0.919
Incidence of spermatozoa with abnormal chromatin condensation (%)	17.521±9.505	19.924±10.252	0.281
MII oocytes	7.195±4.654	5.184±3.136	0.0198
Fertilization rate (%)	77.891±16.505	76.066±23.386	0.8409
CES	54.933±28.135	42.616±29.162	0.0075

CES: Cumulative Embryo Score.

Discussion

The results of the present study showed that the incidence of chromatin condensation is not correlated with sperm morphology and motility. On the contrary, there was a weak, but significant, positive correlation with sperm

concentration. It seems that semen samples with a high concentration of spermatozoa included a slightly higher percentage of spermatozoa with abnormal chromatin condensation. Previous studies have reported contradictory results; Hammadeh *et al.* found that sperm nuclear chromatin condensation is completely independent from conventional semen parameters (9) while, according to Esterhuizen *et al.*, low chromatin condensation quality is negatively-correlated with normal sperm morphology at a high statistically significant level (15). Molina *et al.* have also reported correlations with sperm morphology, motility and vitality (10). However, the presence of a higher percentage of spermatozoa with abnormal chromatin condensation in men with poor semen quality is well documented (4-8, 21). This fact raises concerns on the use of such sperm for oocyte fertilization. While in conventional IVF the natural processes of selection are partly maintained, in ICSI they are bypassed. Therefore, there is always a risk (higher in poor semen samples) of accidentally injecting spermatozoa with abnormal chromatin into oocytes.

The present study indicates that the incidence of spermatozoa with abnormal chromatin condensation is not correlated with the fertilization rate, CES and pregnancy rate in ICSI cycles. According to the results of the present study, the only significant determinant parameters for the ICSI outcome were the number of metaphase II (MII) oocytes and embryo quality. These results are in agreement with previous reports. In particular, Hammadeh *et al.* reported that neither chromatin condensation nor sperm morphology could predict the fertilization, implantation and pregnancy rate in ICSI cycles (21). On the contrary, Sakkas *et al.* postulated that poor sperm chromatin condensation may contribute to fertilization failure after ICSI (19). Razavi *et al.* also found that insufficient sperm chromatin condensation, assessed with CMA3, was negatively correlated with the fertilization rate in ICSI cycles (23).

ICSI is based on the artificial selection of morphologically normal spermatozoa to inject in MII oocytes. It is unanimously accepted that normal spermatozoa may have a low quality condensation of nuclear chromatin. It is also worth noting that various sperm preparation techniques, routinely used in ICSI, improve the percentage of spermatozoa with normal chromatin structure in semen samples of poor quality (20, 22). However, the possibility of injecting an oocyte with a poorly condensed spermatozoon still remains. On the other hand, it has been reported that human spermatozoa with abnormal chromatin are able to fertilize oocytes similarly to normal spermatozoa (30). Consequently, even when the incidence of spermatozoa with abnormal chromatin condensation is not correlated with fertilization and pregnancy rates, as in the present study, the concerns on the possible impacts remain. Therefore, we believe that sperm chromatin abnormality is a field requiring further research. Future studies should focus not only on the possible impact on fertilization and pregnancy outcome, but also on pregnancy complications, delivery rate and health status of the offspring.

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