

# Effects of Sperm Chromatin Integrity on Fertilization Rate and Embryo Quality Following Intracytoplasmic Sperm Injection

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## Abstract

Sperm chromatin integrity has been being recognized as an important factor in male fertility. During normal fertilization, high quality sperm with intact chromatin are selected through natural selection in journey from vagina to fallopian tube. However, using Assisted Reproductive Techniques, particularly ICSI, the natural selection is bypassed. Therefore sperm with DNA breakage have the opportunity to fertilize the egg which may lead to decreased embryo quality and implantation rate. The aim of this study was to evaluate the effects of sperm chromatin integrity on ICSI outcomes. A total of 200 semen samples were collected from couples undergoing ICSI and were analyzed according to WHO criteria. Each sample was evaluated for sperm chromatin integrity using four cytochemical assays and semen processing by swim up method. The ICSI was carried out according to a long-term pituitary down-regulation protocol. The correlation between sperm parameters, sperm chromatin integrity and ICSI outcomes (fertilization rate and embryo quality) was examined. The mean number of oocyte, fertilization rate and cleavage embryos per cycles was  $7.5 \pm 5.0$ ,  $74.06\% \pm 25$  and  $5.4 \pm 3.6$ , respectively. There was not significant correlation between the results of chromatin assays (AO, AB, TB, and CMA3) and fertilization outcomes following ICSI. The fertilization rate was significantly higher for a group with less than 10% chromatin abnormality ( $p < 0.05$ ). Sperm chromatin integrity is essential for successful fertilization, embryo development and normal pregnancy. A protamine deficiency appeared to affect fertilization rate and embryo quality. However, the presence of confounding factors such as selection of spermatozoa according to normal morphology may influence the effect of sperm chromatin status on ICSI outcomes.

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## Introduction

The field of Assisted Reproductive Technology (ART) including ICSI, IVF and IUI has been significantly developed in the last two decades and consequently demand for such treatments have dramatically increased. In Europe, the number of ART treatment is more than 270,000 per year <sup>(1)</sup> and in United state the number of ART treatment

cycles rose from approximately 59,142 cycles in 1995 to 115,392 cycles in 2002 <sup>(2)</sup>.

Among various assisted reproductive techniques, ICSI has become a preferred and widely applied method for helping infertile couples to achieve conception <sup>(3,4)</sup>. There is also an increased tendency for ICSI than IVF, according to Human Fertilization and Embryo-

ology Association (HFEA) statistics <sup>(5)</sup>. However, despite the incredible success and broad use of ICSI, these techniques carry some risks. It is argued that through bypassing the natural selection of sperm-zona penetration, the risk of oocyte fertilization by defective sperm will be increased which then can lead to malformations in the offspring <sup>(6)</sup>. During ICSI, the embryologist attempts to select the best spermatozoa according to its morphology and motility as markers of normality and viability. However, according to the recent studies the routine sperm analyses such as sperm count, motility and morphology, are not reliable indicators of sperm function and chromatin integrity <sup>(7,8)</sup>. Therefore sperm with normal motility and morphology, but containing chromatin abnormality will be given the chance to penetrate and fertilize the eggs.

Sperm functional assay is considered as the valuable diagnostic tool in evaluation and prediction of male infertility <sup>(9-13)</sup>. Different studies have assessed the diagnostic potential of this assay in the outcome of assisted reproductive technologies (ART). Sperm chromatin is tightly packaged to protect DNA during the transit that occurs before fertilization. Any abnormality in chromatin structure and integrity could lead to failure in fertilization. A variety of tests have been described to assess sperm chromatin status that are considered as valuable predictive tools for *in vivo* and *in vitro* fertilization outcomes. However, there were widespread controversies over the relationships between sperm chromatin status and ICSI outcomes. Based on various studies of different cases subjected to ICSI program, abnormal sperm factors lead to low fertilization rate and poor embryo quality <sup>(14-17)</sup>. In contrast there are studies indicating that fertilization rate, embryo cleavage and pregnancy rates are independent of sperm parameter in ICSI <sup>(18)</sup>. To further assess the importance of semen routine parameters and sperm chromatin integrity on ICSI outcomes, we conducted an experimental study to determine the relationship between sperm chromatin status using four cytochemical staining assays [Chromo-

mycin A3 (CMA3) for protamine deficiency, Aniline Blue (AB) for abnormal persistence of histones, Toluidine Blue (TB) and Acridine Orange (AO) for DNA abnormality] and ART results (fertilization rate, cleavage rate and embryo quality) during the ICSI treatment.

## Materials and Methods

### Patients

A total of 200 semen samples were collected from couples undergoing ICSI at Avicenna Infertility Clinic (AIC), Tehran, Iran. The informed written consent for use of the surplus semen in this research was obtained from the couples in accordance with the Avicenna Research Institute Medical Ethics regulation in research involving the human subjects. The cryopreserved semen samples and also samples with less than  $1 \times 10^6$  sperm/ml were excluded from the study. The inclusion criteria for the female partner was age <35 yrs.

### Semen collection and preparation

Briefly, on the day of oocyte aspiration, semen sample was produced on-site by masturbation and allowed to liquefy at 37°C for 20 min prior to analysis. Each sample was divided in two aliquots to be used for analysis of sperm parameters and another part was used for sperm processing to inseminate oocytes via ICSI. The sperm parameters of counts, motility and morphology were analyzed according to WHO guidelines using raw semen sample <sup>(19)</sup>. Swim up processing was performed as previously described <sup>(20)</sup>.

### ART procedure

The Controlled Ovarian Hyperstimulation (COH) was performed according to the long luteal suppression protocol, using a GnRHa (Superfact, Ferring, Germany) and combination of HMG (Menogon, Ferring, Germany) or recombinant FSH (Gonal-F, Serono, Switzerland). The ovulation was induced when at least three follicles had a diameter of  $\geq 18$  mm, using 10,000 IU HCG (Choriomon, IBSA, Switzerland). Transvaginal oocyte retrieval was performed by ultrasound guidance. The oocytes containing cumulous cells were col-

lected from clear follicular fluid. Granulosa cells were separated from collected oocytes using enzymatic (Collagenase, Sigma) and mechanical digestion. The ICSI was performed as previously described<sup>(21)</sup>. Luteal phase support was performed using intravaginal and intramuscular progesterone administration (Osveh, Iran).

### **Embryos**

The oocytes were assessed for fertilization at 16-18 *hrs* after microinjection (two pronuclear stage, 2PN). The embryos were graded according to their morphology, 48 *hrs* after insemination. The classification was as follows: grade A: no fragmentation and four regular cells; grade B: <25% fragmentation; grade C: between 25 and 50% fragmentation and grade D: >50% fragmentation<sup>(22)</sup>. The transfer of the embryos took place either at 48 or 72 *hrs* after the ICSI, according to the treatment plan of the center.

### **Acridine Orange (AO) staining**

The method for Acridine Orange staining was the modified protocol previously described<sup>(23,24)</sup>. A raw semen sample was washed three times in Phosphate Buffered Saline (PBS). Thick smears of washed spermatozoa were prepared on pre-cleaned degreased slides (product: 7201, Surwin Plastic Enterprises, China) and allowed to air-dry for 10 *min*. The smears were fixed for 1 *hr* in ethanol-acetone (1:1) at 4°C and allowed to dry for a few minutes before staining for 7 *min* with AO (0.19 *mg/ml*) at room temperature. The AO staining solution was prepared as follows: 10 *ml* of 0.1% AO stock solution in distilled water was added to a mixture of 40 *ml* of 0.1 *M* citric acid and 2.5 *ml* of 0.3 *M* Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, pH=2.5. After staining, the slides were gently rinsed in a stream of distilled water and air dried. This was followed by evaluation under fluorescence microscope (Zeiss, Germany) with a 100×oil immersion objective. Sperm heads with intact chromatin had green fluorescence, and those with denatured chromatin had orange-red staining. Each sample was scored within 1 *hr* after staining. At least 200 sperm per slide were evaluated to

estimate the number of sperm stained with green and red fluorescence.

### **Toluidine Blue (TB) staining**

The air-dried smears were fixed according to AO staining and allowed to dry for a few minutes before staining with 0.05% Toluidine Blue (Merck, Germany) in staining buffer for 10 *min*. The staining buffer consisted of 50% citrate phosphate (McIlvaine buffer, pH=3.5)<sup>(25)</sup>. After staining, the slides were gently rinsed in distilled water and air dried. Finally slides were viewed under light microscope using a 100×oil immersion objective. Sperm heads with intact chromatin were light blue and those of denatured chromatin and abnormal packaging were deep violet (purple). At least 200 sperm per slide were counted to calculate the number of sperm with blue and violet head.

### **Chromomycin A3 (CMA3) staining**

The air-dried fixed smears were treated for 20 *min* with 100  $\mu$ l CMA3 solution (0.25 *mg/ml* CMA3 in McIlvane buffer, containing 10 *mmol/L* MgCl<sub>2</sub>). After staining, the slides were gently rinsed in distilled water and air dried. At least 200 spermatozoa were scored as abnormal chromatin (bright green) or intact (dull green or colorless) for CMA3, using fluorescence microscope (Zeiss, Germany) with a 100×oil immersion objective<sup>(26)</sup>.

As a positive control, semen samples were treated with hydrogen peroxide 100  $\mu$ M for 10 *min*, prior to staining with Toluidine Blue, Chromomycin A3 staining and Acridine Orange.

### **Aniline Blue (AB) staining**

The air-dried fixed smears were stained for 7 *min* with 0.5% Aniline Blue in PBS buffer. The pH was adjusted to 3.5 using acetic acid<sup>(27)</sup>. Slides were gently rinsed in distilled water and air dried. Finally samples were viewed under light microscope using a 100×oil immersion objective. Sperm heads with intact chromatin were colorless and immature sperm with excessive histone were blue. At least 200 sperm per slide were counted to estimate the number of sperms with blue and colorless

head. The semen samples were assessed individually by two expert laboratory staff. Both intra-assay and inter-assay coefficient of variation of AO, TB, AB and CMA3 staining and semen analysis were less than 10 percent.

#### Statistical analysis

Fertilization rates were calculated for each cycle as the number of cleavage embryos divided by the number of metaphase II oocytes. The correlations between the sperm parameters, sperm chromatin assay results, fertilization rate and embryo quality were evaluated by calculating Pearson's correlation coefficient (*r*). To test the statistical significance of the results, a non-parametric method of the Mann-whitney U test was used. All statistical analyses were carried out using SPSS Version 11.5 (SPSS Inc, USA, IL).

### Results

For this study, 200 semen samples were collected. A sample of 28 were excluded from the study due to insufficient volume of semen sample, lack of oocyte retrieval following puncture and due to anomalies and low quality of oocytes. The mean age of male participant was  $33.9 \pm 6$  years. The results of semen analysis, chromatin assay and the ICSI cycle results are summarized in Tables 1 and 2. According to results of semen analysis, there were 64 (37.2%) normozoospermia, 12(7%) oligoasthenoteratozoospermia, 48 (27.9%) asthenoteratozoospermia, 44 (25.6%) teratozoo-

Table 1. Summary of semen analysis and sperm chromatin assay results in 172 men undergoing ICSI treatment

	Mean±SD
<b>Volume</b>	2.4±1.1
Sperm Concentration ( $\times 10^6$ /ml)	83±59
Total sperm Count	260±226.8
Normal Morphology (%)	24.71±9.4
<b>Motility</b>	
Rapid progressive Motility (a) (%)	11.18±8.46
Slowly progressive Motility (b) (%)	21.83±8.9
Non progressive Motility (c) (%)	16.06±6.2
Immotile Sperm (d) (%)	50.9±14.4
<b>Sperm chromatin assay</b>	
Acridine Orange % <sup>a</sup>	26.5±13.0
Aniline Blue %	27.1±13.0
Toluidine Blue % <sup>a</sup>	31.4±13.6
Chromomycin A3 % <sup>a</sup>	36.2±12.9

<sup>a</sup> Percentage of sperm with abnormal chromatin

Table 2. Summary of embryology results in 172 couples undergoing ICSI cycle

	Mean±SD
Mature oocyte (MII)	7.5±5.0
No. cleavage oocyte	5.4±3.6
No. embryos transferred	3.5±1.0
Fertilization rate (%)	74.06±25
<b>Embryo quality (%)</b>	
Grade A	58.1±33
Grade B	37±31
Grade C	4.9±1.4

spermia, 3 (1.7%) astenozoospermia, and 1 (0.6%) oligozoospermia. The mean age of female participant was  $30.2 \pm 5$  yrs.

#### Semen parameters and fertilization rate

The mean fertilization rate was  $74.06 \pm 25$  and there were no significant correlations between sperm function tests and fertilization rate. However, when chromatin integrity was measured by aniline blue divided in two groups according to a threshold of 10%, the fertilization rate was significantly higher for the group with less than 10% chromatin abnormality ( $85 \pm 22.3$  vs.  $73.4 \pm 25.4$ ) ( $p < 0.05$ ). However this was not significant for grouping according to thresholds of 20% or 30%. Also, the fertilization rate following ICSI was not influenced by sperm parameters of motility, morphology and concentration ( $p > 0.05$ ) (Table 2). Grouping was according to the previously studied threshold for chromatin assays<sup>(28)</sup>.

#### Sperm chromatin integrity and embryo quality

The average percentage of embryo quality (grade A, B, C) is summarized in Table 2. When cases were divided according to the threshold of 30% abnormal chromatin using Chromomycin A3<sup>(29,30)</sup>, a significant difference was observed for embryo quality (grade A) between the two groups ( $p < 0.05$ ). The embryo quality grade A was 71.3% when CMA3 results were less than 30% and 57.2% when CMA3 results were greater than 30%. There were significant negative correlations between embryo quality grades A, B and percentage of abnormal chromatin using CMA3 staining (Table 3).

Table 3. Correlation between sperm function test results and embryo quality

Test	Grade A		Grade B		Grade C	
	r	p-value	r	p-value	r	p-value
<b>Sperm function parameters</b>						
Acridine Orange % <sup>a</sup>	0.05	NS	-0.06	NS	-0.006	NS
Aniline Blue % <sup>a</sup>	0.074	NS	-0.03	NS	-0.09	NS
Toluidine Blue % <sup>a</sup>	-0.071	NS	0.05	NS	0.042	NS
Chromomycin A3 % <sup>a</sup>	0.207	0.017*	-0.2	0.01*	-0.71	NS

\* p<0.05, a: Percentage of sperm with abnormal chromatin, NS= not statistically significant; r = Pearson's correlation coefficient

## Discussion

In the present study, four different cytochemical staining methods including Toluidine Blue, Aniline Blue, Chromomycin A3 and Acridine Orange assays were applied in order to assess different aspects of sperm DNA integrity<sup>(31)</sup>. The Chromomycin A3 (CMA3) staining was applied for the evaluation of protamine deficiency, Aniline Blue staining to detect excessive presence of histones; Toluidine Blue and fluorescence Acridine Orange, as a sensitive structural probes, were used for assessing sperm chromatin structure and packaging<sup>(23,32,33)</sup>. The TB is a classic nuclear dye used for external metachromatic and orthochromatic staining of chromatin, which overall is negatively charged<sup>(23,25)</sup>. It becomes heavily incorporated in damaged dense chromatin and can be used as a fairly reliable method for assessing the integrity of sperm chromatin. The AO assay measures the susceptibility of sperm nuclear chromatin to acid induced denaturation *in situ* by quantifying the metachromatic shift of AO fluorescence from green (native chromatin) to red (denatured chromatin). This stain is a sensitive structural probe for chromatin structure and packaging, assessed either by microscopy or flow cytometry.

Our results showed that when the cases were divided in two groups according to chromatin abnormality, fertilization rates were significantly increased in the group with less than 10% abnormal chromatin threshold using Aniline Blue. Such finding may indicate that protamine deficiency has considerable affect on fertilization rate. However in case of grouping by 20% or 30% thresholds of abnormal chromatin, no significant differences were found between the two groups. We also found no significant correlation between other

chromatin staining assays and fertilization rate. This was in agreement with Sakass and Zini<sup>(34,35)</sup> that found no significant correlation between Chromomycin A3, Acridine Orange and Toluidine Blue with fertilization rate in infertile couples undergoing IVF/ ICSI. Also, our results were in agreement with the finding of Hammadeh and his colleagues, that reported no significant correlation between sperm with abnormal chromatin and fertilization rate and embryo quality with regard to 30% cut off for DNA fragmentation using Aniline Blue staining<sup>(27)</sup>. In contrast, there are broader studies showing negative associations between frequency of sperm DNA fragmentation and fertilization rate and embryo development<sup>(36-39)</sup>. Therefore, there are still controversies over the affect of sperm chromatin status on fertilization rate and pregnancy outcomes<sup>(40)</sup>.

Normal sperm chromatin structure and integrity is essential for the accurate transmission of paternal genetic information to the next generation and also appropriate function of sperm in the fertilization process<sup>(41,42)</sup>. It has been shown that sperm with protamine deficiency and increased histone remnants, lead to premature chromatin condensation that is the cause of failures in fertilization and embryo development<sup>(43-45)</sup>. Moreover, the chromatin of sperm with reduced amounts of protamine was observed to be susceptible to chemical disruption<sup>(46)</sup>. Considering all these studies, it is most probable that sperm with abnormal chromatin can affect fertilization rate and embryo quality following ICSI procedure.

A reason for the current findings of "no significant correlation between fertilization rates and most of the sperm parameters" may be due to the fact that assays for sperm chro-

matin abnormalities were performed on raw semen samples that contained large numbers of immotile, nonviable or degenerated sperm with abnormal chromatin. However, through pre-ICSI processing techniques (gradient centrifugation, swim up or glass wool, etc.) most abnormal sperms are removed and the resultant processed semen contain motile sperm with normal morphology<sup>(47-49)</sup>. Because that such processed sperm possess greater quality, assessment of the correlation between sperm parameters in raw semen sample and fertilization ability of the processed semen samples adds further to the controversies. In other words, detected sperm in raw semen does not reflect the population of normal selected sperm applied in the ICSI treatment. This is especially true for oligoasthenoteratozoospermic (OAT) samples that contain largely non-viable and immotile sperm. Indeed, spermatozoa of OAT samples recovered after processing techniques are significantly improved sperm parameters<sup>(50)</sup>.

Indeed the aim of pre-ICSI processing techniques is to select the best quality spermatozoa during ICSI and as far as possible to avoid the transfer of the genetic abnormalities to oocyte that is similar to the natural selection during *in vivo* fertilization. Therefore it is suggested that in order to assess the role of sperm parameters in ICSI outcomes, further studies should be performed on pre-ICSI processed samples.

Another reason that may explain the lack of relationship between sperm parameters and fertilization rate in our study and related studies could be due to the fact that during ICSI procedure, the operator's attempt to select motile and morphologically normal spermatozoa may affect the ICSI outcomes as a confounding factor. This could be the second level of sperm selection in ICSI procedure substituted by the natural selection in normal pregnancy that may slightly reduce the risk of transfer of genetic abnormalities in ICST.

We also found a significant difference in embryo quality grade A between groups

below and above 30% abnormal chromatin using Chromomycin A3 staining. The finding indicates that chromatin abnormality as well as fertilization rate can affect embryo quality. Although the impact of chromatin abnormality remains controversial, there is a wider agreement about its negative affect on embryo development<sup>(51)</sup>.

In conclusion, considering the importance of sperm chromatin structure in protecting sperm genome, abnormal structures of chromatin could have negative influences in the ART outcomes. The present study indicated that protamine deficiency could affect fertilization rate and embryo quality. Thus improving methods for selecting sperm with intact chromatin is suggested. However, there are confounding factors such as pre-ICSI processing techniques and selecting spermatozoa with normal morphology and motility that may influence the assessment of the affect of sperm chromatin status on ICSI outcome.

Indeed the sperm pre-selection process during ICSI procedure, that is substitute to the natural selection barrier in normal pregnancy, could affect the validity of the studies performed to find the correlation between sperm quality and fertilization outcomes. More precise studies with reduced confounding factors are needed to evaluate the correlation between sperm parameters and ICSI.

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