Sperm Chromatin Maturity Assay by Aniline Blue Dye and It’s Correlation with The Result of Intracytoplasmic Sperm Injection

Hind abdulkadim Al-Ebrahimi¹  Yahya K. Al-Sultani²
¹ College of Medicine, Kufa University, Najaf, Iraq
² College of Pharmacy, Kufa University, Najaf, Iraq

Received 20 October 2014      Accepted 16 November 2014

Abstract
Immature chromatin condensation is a type of DNA damage in human spermatozoa may adversely affect reproductive outcomes.
To assess sperm chromatin maturity in infertile men and to evaluate the relationship between sperm chromatin status and ICSI outcome.
Fifty six infertile couple attending the clinics of fertility center in Al-sader teaching hospital were included in this study. Male partner were classified according to their spermiogram, their semen sample tested by AB for chromatin maturity.
The percentage of positive AB stain is significantly higher in patients with abnormal spermiogram than normozoospermic group.
No significant difference in ICSI outcomes between patients with high proportion of abnormal chromatin and apposite group.
Male with abnormal spermiogram show high percent of sperm chromatin immaturity which have no significant influence on the result of ICSI procedure.

Key words: Aniline blue, sperm chromatin, ICSI.

Introduction
Several investigators have considered optimizing conventional routine methods to improve male infertility diagnoses, over the last two decades, the main investigation areas have been focused on sperm function, morphology and assessment of sperm chromatin quality and DNA fragmentation(1). The DNA integrity of human spermatozoa contributes significantly to embryonic...
growth and fetal health (2). The paternal genome is transferred to the oocyte in a balanced physical and chemical condition to complement genetic division during embryo development. Although the paternal genome is not effective in the human embryo until day 3, four- to eight-cell stage (3).

Sperm with damaged DNA are released in the semen, and despite the likely result is infertility, these defective cells may still retain the ability to fertilize. Sperm DNA damage has been associated with several infertility phenotypes including unexplained infertility, idiopathic infertility, repeated IUI and IVF failure, and recurrent miscarriage and disease in offspring, such as childhood cancers and autism (4).

Assessment of sperm DNA damage provides a relatively independent measure of fertility that yields diagnostic and prognostic information complementary to, but distinct and more significant than, standard sperm parameters (5).

The selection of a spermatozoon with nucleotide or DNA damage during ART procedures may influence the genetic quality of the embryo. These genetic modifications contribute to impaired implantation and poor embryogenesis.

Chromatin of mature spermatozoa has been shown to possess a varying binding capacity for many nuclear dyes and stains. This binding capacity reflects anomalies in the chromatin packaging quality (6).

Therefore, tests have been developed for the evaluation of sperm DNA packaging and maturity. These tests include DNA fluorescence stains or fluorochromes, such as chromomycin A3 (CMA3) and aniline blue (7).

Aniline blue staining is used to assess the nuclear maturity of spermatozoa. This test is especially helpful for the detection of extra lysine-rich histones which may be an indication of lower amounts of protamines in the sperm nucleus, as well as immature chromatin condensation, so AB distinguishes between lysine-rich histones and protamines. Spermatozoa with immature chromatin condensation will stain positive blue, whereas spermatozoa containing mature chromatin will not be susceptible to the stain (8).

**Materials and Methods**

This study included 56 infertile couple complaining from either primary or secondary infertility attended the clinics of infertility treatment center and underwent intracytoplasmic sperm injection throughout period from April 2013 to June 2014. All patients were of at least 1 year duration history of regular unprotected intercourse, mean duration of infertility period was 7.53±0.59. All females included in this research were less than 35 years old; the mean age of female partner was 31.31±0.86 years. Those infertile couples were divided into three groups according to spermiogram of male partner and include: Oligoasthenoteratozoospermia, asthenoteratozoospermia and normozoospermic group. Chromatin condensation evaluated in each group by mean of AB stain.

The procedure of AB stain is as follow:

We spread 10uL of fresh semen on glass slides, and sperm smears are allowed to dry in air. Smears were fixed with a solution of 3% buffered glutaraldehyde in 0.2M phosphate buffer (pH = 7.2) for 30 minutes. Slides were then stained with 5% aqueous aniline blue solution mixed with 4% acetic acid (pH = 3.5) for 5 minutes. For each stained smear, 200 spermatozoa were evaluated with light microscope in oil immersion magnification (100x objective).

Chromatin condensation evaluated in each group by mean of AB stain.

**Results**

For each group ICSI outcome represented by fertilization rate, cleavage
rate, embryo quality and pregnancy rate were evaluated and compared for all groups.

**Results**

Figures (1) and (2) show the percentage of positive aniline blue stain in studied groups. Figure (1) shows the difference in percentage of positive aniline blue stain between OAT and normozoospermic group which is highly significant with lowest reading seen in normozoospermic group, 11.90±2.51 versus 49.90±2.84 in OAT, P = 0.0001.

There is significant difference in aniline blue stain percent between AT and normozoospermic group with highest reading seen in AT, 25.44±3.76 versus 11.90±2.51 in normozoospermic group. 

This is illustrated in figure (2).

The studied groups also sub classified according to the percent of aniline blue stain. 95% (19) of OAT group show high values of aniline blue stain that exceed 30% of sperm in a given sample.

Regarding AT group 50% (8) of them show high values of aniline blue stain that exceed 30% of examined sperm. While most of normozoospermic group (95%) show low values of aniline blue stain, 19 of studied samples show readings less than 30% and only (5 %) one of normozoospermic group show high values of aniline blue stain that exceed 30% of studied sperm. The above details are explained in table (1).

There is significant difference among studied groups in regard to the percent of aniline blue stain P=0.0001.

**Figure (1):** Mean of positive aniline blue stain percent of OAT group in comparison with normozoospermic group. 

OAT: Oligoasthenoteratozoospermia. 

N: Normozoospermia. 

*Highly significant difference with corresponding group, P=0.0001.
Figure (2): Mean of positive aniline blue stain percent of AT group in comparison with normozoospermic group.
AT: Asthenoteratozoospermia. N: Normozoospermia. *Highly significant difference with corresponding group, P=0.004.

Table (1): Sub classification of studied groups according to percent of positive aniline blue stain to those less than 30% and those more than 30%.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Chromatin stain &lt;30%</th>
<th>Chromatin stain ≥30%</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAT (n=20)</td>
<td>1 (5%)</td>
<td>19 (95%)</td>
</tr>
<tr>
<td>AT (n=16)</td>
<td>8 (50%)</td>
<td>8 (50%)</td>
</tr>
<tr>
<td>N (n=20)</td>
<td>19 (95%)</td>
<td>1 (5%)</td>
</tr>
</tbody>
</table>

OAT: Oligoasthenoteratozoospermia. AT: Asthenoteratozoospermia. N: Normozoospermia. *Highly significant difference among the studied groups, P=0.0001.

Table (2) shows the differences in fertilization rate, cleavage rate, embryo quality and number of arrested embryos between samples in which stained sperm (chromatin positive) ≥30% and samples in which stained sperm is <30%. There is no significant difference in fertilization rate between first and second group, P=0.729. No significant difference in cleavage rate between our groups, P=0.501. Also there is no significant difference in embryo quality between them, P=0.398 for good quality embryo and P=0.956 for bad quality embryo. No significant difference in the number of arrested embryos between the above two groups, P=0.789. No significant difference in pregnancy rate P=0.842.

Figure (3) shows the differences in positive aniline blue stain percent between pregnant and non-pregnant group. There is no significant difference between them, P=0.149.

In our study, the predictive value of sperm DNA fragmentation was assessed in a cohort of 56 couples using AB staining when the association between sperm DNA maturity and pregnancy was analysed, the results of the ROC curve analysis showed that DNA abnormality assessment can be
used as a predictive parameter to distinguish between potentially pregnant and not potentially pregnant population. The cutoff point was at 40% sperm DNA abnormality with a sensitivity of 53% and a specificity of 68% as shown in figure (4) i.e., this study was concluded that pregnancy rate start to regress when positive AB staining ≥40% of sperm in studied semen sample.

**Table (10):** Differences in fertilization rate, cleavage rate, embryo quality, number of arrested embryos and pregnancy rate between samples in which stained sperm ≥30% and samples in which stained sperm is <30%.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Samples with ≥30% stained sperm (n=27)</th>
<th>Samples with&lt;30% stained sperm (n=29)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertilization rate</td>
<td>68.423±4.709</td>
<td>71.035±5.769</td>
<td>0.729</td>
</tr>
<tr>
<td>Cleavage rate</td>
<td>93.961±2.825</td>
<td>90.607±3.984</td>
<td>0.501</td>
</tr>
<tr>
<td>Good quality embryo</td>
<td>58 (26.68)</td>
<td>93 (30.32)</td>
<td>0.398</td>
</tr>
<tr>
<td>Bad quality embryo</td>
<td>18 (28.29)</td>
<td>26 (28.61)</td>
<td>0.956</td>
</tr>
<tr>
<td>Embryonic arrest</td>
<td>14 (28.98)</td>
<td>14 (28.02)</td>
<td>0.789</td>
</tr>
<tr>
<td>Positive pregnancy%</td>
<td>52.9%</td>
<td>50%</td>
<td>0.842</td>
</tr>
</tbody>
</table>

**Figure (3):** Difference in aniline blue percent between pregnant and non pregnant. No significant difference P= 0.149.
Figure (4): Roc curve show the best cut off point of chromatin abnormality percentage associated with pregnancy

**Discussion**

This study revealed that sperm chromatin compaction defect (as assessed by aniline blue staining) is significantly higher in semen samples with impaired sperm parameters than normozoospermic group.

Several studies demonstrated a significant negative correlations between CMA3 positivity and sperm count, motility, and morphology (10). Infertile males with protamine deficiency show a lower sperm counts, motility, and higher abnormal sperm morphology (11). This association between defective sperm parameters and DNA abnormality refer to inherent disorders of protaminization anomalies which are probably related to a generalized defective spermiogenesis with abnormal semen parameters in result (12).

In contrast other studies disagree with us (13,14,15) and proved no connection between the level of sperm chromatin condensation assessed by AB staining and sperm parameters, including motility, viability, and sperm concentration (9).

Truly, it seem to be a threshold of sperm chromatin damage (i.e., fragmentation, defective chromatin compaction, and protamine deficiency) beyond which embryo development and pregnancy are compromised (16). This threshold is a matter of debate and there is no exact or certain cutoff point for it. Depending 30% as a threshold in this study reflect no significant difference in ICSI outcome.

There is a controversy on the correlation between sperm nucleus maturity evaluated with AB staining and sperm fertilization capacity and embryo quality after ICSI. In a study regarded the threshold for sperm DNA damage 29%, the fertilization rate with regard to chromatin condensation was almost the same in the two groups, the cleavage rate was also the same, while the pregnancy rate unexpectedly seem to be higher in the group with more immature spermatozoa (>29% spermatozoa positively stained) but this was not
statistically significant (17), these results did not show any correlation between chromatin condensation and fertilization, cleavage and pregnancy rate after ICSI and it compatible with our results. These findings may be due to the fact that neither pronucleus formation nor initial embryogenesis is depend on sperm DNA normality, because the embryonic genome is not expressed until after the second mitotic cleavage i.e., at the four cell stage embryo (18) and early stages of embryonic development regulated and controlled by the maternally-derived mRNA, also good-quality oocytes have the ability to repair nuclear damage in the male gamete (19).

Other studies confirm an association between DNA integrity and reproductive potential, showing that natural pregnancy diminished significantly when more than thirty percent of the spermatozoa are recognized as having DNA defect (20) and the chance of achieving ongoing pregnancy was very low with sperm samples containing more than 30% DNA fragmentation (21), and a DNA damage > than 20 % cause a higher rate of repeated ART failures (9).

According to some literatures when DFI was 30%, the result of ICSI was significantly better than that of IVF (22), this findings could be explained by the presence of confounding factors such as ICSI procedure operator’s selection of spermatozoa according to normal morphology that may influence the effect of sperm chromatin status on ICSI outcomes. Furthermore, sperm selection techniques, like swim-up migration or density gradient centrifugation, increase the proportion of sperm with normal chromatin Structure (23).

**Conclusion**

Spermatozoa from infertile males with defective sperm parameters possess significantly more chromatin damage than do those of males with normal spermiogram.

Depending 30% as a threshold for sperm chromatin integrity for patients included in this study reveal no significant difference in ICSI outcome between couples with sperm chromatin defect in their semen samples ≥ 30% and those with chromatin defect <30%.

This study concluded that the best cut off point of abnormal chromatin percentage associated with pregnancy was 40%.

**References**


