Semen analysis revisited- Qualitative assessment of sperms using cytochemical stains- the new norms of male infertility workup

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INTRODUCTION

Semen analysis is used as the first investigation in any infertility workup in general and in the determination of male factor infertility in particular, wherein the semen is analyzed for concentration, motility, and morphology of the spermatozoa according to WHO criteria. However, these standard parameters do not reveal qualitative sperm defects, of which the nuclear status of human sperm cells is of great importance in assessing the fertilizing capacity of sperms. The loss of structural integrity of sperm chromatin results from the influence of endogenous factors like sperm...
maturation, and from exogenous factors, like infectious or toxic agents. Spermatozoa comprise of an extraordinary high percentage of polyunsaturated fatty acids in their plasma membrane. Due to an extremely low content of cytoplasm, sperm cells have a particularly low potential to scavenge reactive oxygen species (ROS), and are therefore highly sensitive to oxidative stress, which lead to sperm nucleus DNA damage/fragmentation. Clinically, it has been noted that the high percentage of spermatozoa with impaired chromatin structure in ejaculates is predictive not only of infertility, but also of fetal anomalies and recurrent abortions, irrespective of the sperm count.

In order to detect the sperm chromatin abnormalities, several techniques like cytochemical assay, comet assay, and terminal deoxynucleotidyl transferase mediated deoxy uridine triphosphate nick end labeling (TUNEL) assay have been used. Among these the cytochemical assays are sensitive, simple, and inexpensive since they do not require special instruments such as flow cytometry. The stains commonly used are Acridine orange, Toluidine Blue, Aniline Blue etc.

Aniline blue (AB) staining was used for visualization of sperm chromatin integrity and condensation. The stain evaluates sperm chromatin defects by differential staining of lysine rich histones and arginine-cysteine rich protamine in the sperm nuclei. A modification of Acidic Aniline Blue (AAB), staining method is carried out in this study. A counter stain Eosin is used after Aniline Blue to make the identification and differentiation of mature condensed sperm heads from abnormal immature heads more feasible. This study aims to assess sperm DNA integrity by cytochemical assay using Modified Acidic Aniline Blue stain.

**MATERIALS AND METHODS**

The study was conducted in the Department of Pathology in a tertiary care center in South India, with approval of Institutional Ethics Committee and informed consent obtained from all the patients. The study was an observational, prospective and laboratory based spanned over one and half years, from January 2018 to May 2019. During this period, we examined 500 men who visited the outpatient departments of Obstetrics & Gynecology and Surgery for the assessment of infertility.

Exclusion criteria were cases with azoospermia and patients with history of orchitis, varicocele and those who had family history of genetic disease. In this study, 41 cases meeting the above exclusion criteria were excluded.

All the rest 459 cases underwent routine semen analysis, performed according to the WHO 2010 Manual. The protocols described in WHO laboratory manual for the sample collection, examination and processing of human semen were followed. Initial macroscopic and microscopic examination was done and findings recorded. Sperm DNA Integrity and DNA fragmentation index was assessed in this study using Modified Aniline blue method. Slides were prepared by feathering method as described in the WHO manual.

Modified Acidic aniline blue staining: Slides were air dried, fixed in 3% buffered Glutaraldehyde for 30 minutes and stained with AAB for 5 min. The AAB slides were further dipped in aqueous Eosin for 3 min. After the counter staining, the immature / damaged sperm heads remained dark blue while mature sperm heads (normal) stained red-pink as in image I. The slides were mounted, observed under oil immersion lens and 200 sperms counted. The DFI was recorded as percentage.

Calculation of sperm DNA fragmentation index (DFI) as per studies:

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\text{DFI} = \frac{\text{No. of sperm with fragmented DNA (Sperms stained blue)}}{\text{No. of sperm evaluated (Sperms stained blue and pink)}} \times 100
\]

DFI < 20% - Normal

DFI > 20% - Abnormal or high DFI.

The results were compared by applying statistical tests as recommended by WHO 2010 manual. The results were tabulated on daily basis in an excel sheet. The statistical analysis was done with SPSS software using chi square test and unpaired t test. The sperm count, the percentage of progressive motility, percentage of normal forms and DFI were compared for the test of significance.

**RESULTS**

In the present study, 459 samples from patients who had come for evaluation of infertility were studied. The patients included were between the age of 19 years and 55 years. Majority of patients were below the age of 35 years followed by patients between the age of 35-45 years.

Two types of samples were studied, washed semen samples (3.5%) and unwashed semen samples (96.5%). We found that 7.7 percent of unwashed samples had DFI of more than 20 and all of the washed samples had a DFI of less than 20. Washed semen samples were from patients undergoing ART and unwashed samples were of patients who had come for routine semen analysis.

Association of percentage of immature/damaged sperm heads found with AAB staining with the DNA fragmentation assessed using chi square test shows significant p value of <0.05. The semen samples with DFI >20 was seen in samples with more than 40% of immature/damaged sperm heads (table 1).

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DNA fragmentation index was calculated using the immature/damaged sperm heads, for all the 459 semen samples. The samples were grouped as DFI < 20 and DFI > 20. Of the total sample size, 34 cases (7.4%) alone had DFI > 20 and the rest of them that is 93% (425) had DFI < 20.

The entire study group was divided into two, patients with DFI < 20 and DFI > 20, further were compared to each of the routine semen parameters (table 2). The parameters with p value of < 0.05 was considered significant correlation with the DFI. As per the statistical analysis- age, volume of the sample, total sperm count did not have any significant correlation with DFI, whereas p value was significant with total motility, progressive motility, percentage sperm with normal morphology and the immature sperm heads identified using the modified AAB stain.

On comparing the routine semen analysis results with DFI the following results were obtained (table 2).

**DISCUSSION**

An infertile couple is offered treatment in the form of many different assisted reproduction techniques (ART). The success rates of ART procedures as discussed earlier depend predominantly on the quality and maturity of the sperm and ova. The DNA in the sperm nucleus plays an important role in the formation of the embryo. Hence, damaged DNA is responsible for the inability to fertilize or even if there is fertilization, early fetal loss or lead to a fetus born with congenital anomalies. The present study was taken up as recent research suggested that sperm DNA describes the quality of the sperm in the semen, as it is important for the outcomes of ART. To study the association of sperm DNA fragmentation with all the routine semen parameters of the given semen samples, we assessed the sperm DNA maturity and DNA fragmentation index using AB method. Terquem and Dadoune founded this technique.2 They showed that Acidic AB on staining the smear reacts with lysine amino acids in the sperm heads and under the light microscope, the sperm nucleus with high levels of histone is seen in blue (fig.1). Studies have shown that adding Eosin color as a contrast color to AB staining (Modified aniline blue method) can increase the chance of detecting immature sperm with an additional histone and thus increase the sperm chromatin density estimation.2
(AO) test. Patil et al, Hee sun kim et al, Sellami et al and Hind abdulkhadim et al assessed the sperm chromatin integrity using the AB staining technique, similar to this study. A literature search showed variability in the consensus on cut-off values of DFI. To know the impact of DFI on sperm parameters, the semen samples obtained were divided into two groups, and the results were tabulated (table 3).

The results obtained after semen analysis and AB staining in the present study and several other studies (table 4), indicate that along with the other routine semen parameters, one has to be cautious about DFI.

### Table 3: Comparison of semen analysis with DFI (%)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (DFI &lt; 20)</th>
<th>Group 2 (DFI &gt; 20)</th>
<th>t-test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>32.806±6.226</td>
<td>34.294±6.668</td>
<td>1.253</td>
<td>0.211</td>
</tr>
<tr>
<td>Volume (ML)</td>
<td>2.413±1.055</td>
<td>2.382±1.000</td>
<td>0.163</td>
<td>0.870</td>
</tr>
<tr>
<td>Total Sperm Counts (M/ML)</td>
<td>55.432±42.449</td>
<td>49.709±47.263</td>
<td>0.750</td>
<td>0.454</td>
</tr>
<tr>
<td>Total Motility (%)</td>
<td>55.198±21.399</td>
<td>42.941±27.139</td>
<td>3.145</td>
<td>0.002</td>
</tr>
<tr>
<td>Progressive Motility (%)</td>
<td>34.984±19.813</td>
<td>23.529±22.003</td>
<td>3.217</td>
<td>0.001</td>
</tr>
<tr>
<td>Sperms with normal morphology (%)</td>
<td>65.274±17.615</td>
<td>56.471±28.164</td>
<td>2.657</td>
<td>0.008</td>
</tr>
<tr>
<td>Immature heads of sperms (%)</td>
<td>16.906±12.211</td>
<td>57.971±17.406</td>
<td>17.908</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Cases with low sperm count and motility cannot be rejected, as Sperm DNA could be of better quality and such cases are favourable for ART. In cases where the sperm concentration is within the range of normalcy and with normal morphology according to the criteria set by WHO, but the patient is having failure of ART procedure, to a high DFI indicative of damaged sperm DNA. It may be the cause for failure of pregnancy after embryo transfer leading to repeated abortions or failure to continue the pregnancy through the term as also a cause for congenital anomalies as seen in the 12 normospermia cases in our study.

Sperm wash and DFI Sperm washing procedure showed better yield of normal sperms with good DNA integrity (DFI<20), suggesting that washed samples tend to have more success rates with ART.

### CONCLUSIONS

Sperm DNA is a fundamental element in the success of human reproduction. The sperm DNA integrity test helps in strengthening the importance of lifestyle modification like cessation of smoking and alcohol consumption, predicting fertility, and monitoring the patient’s response to intervention. A thorough semen analysis along with DFI can help the clinician explain the couple in a better way about the choice of ART and the success of treatment. A sperm washing procedure is advisable as it not only removes chemicals from semen but also removes sperms with low motility or damaged sperms, improving the success rates of assisted reproductive procedures with healthier sperms. To assess sperm nuclear maturity in the exploration of male infertility we can use simple techniques, such as AAB staining, like in the present study, which can be used as a screening method. The use of more sophisticated techniques like chromomycin A3 assay and transmission electron microscope image cytometry provides more accurate and specific results in the evaluation of sperm DNA maturation but has many technical constraints to use in routine day-to-day practice. Therefore, there is a need to study sperm chromatin quality in infertility by using simpler cytological techniques to understand their role in the fertilizing capacity.
and study their impact on the results of in vitro fertilization and embryo development. This study highlights the need to incorporate the aspect of qualitative assessment of sperm in routine semen analysis along with quantitative analysis.

Limitations

In the present study, there are a few limitations.

1. The sample size of the study is small and does not constitute all of the infertile men’s representative population.
2. There are chances of interobserver variability (subjective), inherent to the procedure.
3. The data does not include clinical outcomes including the rate of fertilization or pregnancy rate.

REFERENCES


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