Effect of Sperm DNA Fragmentation Index on Clinical Outcomes of Intra-Uterine Insemination Patients

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To study the effect of sperm chromatin condensation (DNA fragmentation index (DFI)) using aniline blue-eosin (AB-E) staining on pregnancy outcomes in patients facing unexplained infertility undergoing intra-uterine insemination (IUI). Our initial hypothesis states that if DNA fragmentation is high then chances of pregnancy are low/NIL hence these patients should be recommended with advanced ART procedures like IVF and ICSI. Prospective study. Tertiary care infertility centre. A total of 185 patients with age less than 40 years, non-smokers and without history of any pathogenic infection in the past 2 months facing unexplained infertility i.e., males with normal semen analysis reports and females with normal ovulation and hysterosalpingography (HSG) reports were selected for the study. Patients were undergoing their first or second IUI treatment cycle between the period of June 2016 to December 2016. DNA fragmentation index (DFI) using aniline blue-eosin staining method was studied in semen samples provided on the day of IUI procedure. The patients were separated into 3 groups: low DFI (DFI< 10%), medium DFI (DFI=11% - 20%), and high DFI (DFI >= 21%) and clinical pregnancy outcomes of IUI were recorded. Statistical analysis was performed using Pearson correlation co-efficient, ANOVA and Shapiro Wilk Test on the above groups. DNA fragmentation index (DFI) (%), Clinical pregnancy rate (%). The overall clinical pregnancy rate for the selected patient pool was 21.08% with an average DFI of 8.84% in the pregnant female group and 14.65% in the non-pregnant female group. Sperm DFI % and clinical outcomes in IUI treated patients were statistically significant and negatively correlated with correlation coefficient (r) of -0.1, -0.3 and -0.3 in low DFI%, medium DFI and high DFI% groups respectively. Our study demonstrated that DFI (%) and clinical pregnancy rate (%) are significantly and negatively correlated in patients with normal semen parameters undergoing IUI. The higher the DFI% the chances of clinical pregnancy become very low, therefore, these patients should not be recommended IUI but with advanced ART procedures like IVF and ICSI.

Keywords: Aniline blue- eosin, Clinical pregnancy rate, DNA fragmentation index (DFI), IUI, Sperm chromatin compaction

Infertility is observed in around 15% of males with a normal semen analysis report1. This proposes that a simple semen analysis may not reveal sperm DNA integrity and cannot predict the fertilization capability of the sperm.

Several mechanisms suggesting the cause of sperm DNA damage have been reported like...
abnormal chromatin packaging and oxidative stress 
2,5, but the exact causes have not yet been fully 
understood.

Therefore, assessment of sperm chromatin 
abnormalities is important in treating male 
infertility as shown in various studies1,6. Sperm 
DNA assessment not only affect natural conception 
but also assisted reproductive technology (ART) 
success rates.

Intrauterine insemination (IUI) is a 
common procedure performed for treatment of 
moderate male factor and unexplained infertility. 
IUI is a very cost-effective and non-invasive 
procedure in which the semen washing step removes 
prostaglandins, immotile sperms, leukocytes and 
immature germ cells, thereby decreasing free 
oxygen radical formation and improving the quality 
of sperm for fertilization.

Elevated levels of sperm DNA 
fragmentation in men with poor semen parameters 
have been observed in previous studies7-8, however 
few studies show that men with normal semen 
parameters undergoing their IUI procedure cycles 
have abnormal sperm chromatin integrity9.

Assessment of sperm DNA can be done 
by various methods like, flow cytometric-based 
sperm chromatin structure assay (SCSA) and 
terminal deoxynucleotidyl transferase mediated 
deoxyuridine triphosphate nick end labeling 
(TUNEL) assay which provide better diagnostic 
results than the standard semen analysis. Also there 
are simple, sensitive and inexpensive cytochemical 
assays which don’t need specialized laboratory 
setup10.

In this study we evaluated sperm 
chromatin compaction or maturation by aniline 
blue staining, which detects sperm chromatin 
defects related to their nucleoprotein content11-12. 
Lysine rich histones that are retained in the 
immature sperms are bound by the acidic aniline 
blue in the sperm head making it appear dark blue 
whereas, arginine and cysteine rich protamines of 
mature sperm are not stained by Aniline blue12.

This method is simpler and cost effective 
which could be utilized by all ART laboratories for 
reporting sperm DNA damage in semen analysis 
to improve treatment outcome in ART.

The aim of the present research was to 
study the effect of DNA fragmentation index (DFI) 
for sperm chromatin compaction using Aniline 
blue-Eosin staining on clinical outcomes in patients 
undergoing IUI.

MATERIALS AND METHOD

Patient Selection
A total of 185 patients undergoing their 
first or second IUI cycle were studied between 
the period of June 2015 to Dec 2016. All patients 
selected in the study were less than 40 years old 
having unexplained infertility where the couples 
facing infertility have all standard investigation 
tests for ovulation, hysterosalpingography (HSG) 
and conventional semen parameters are reported 
as normal. Patients with history of any pathogenic 
infection in past 2 months and smokers were ruled 
out.

DNA fragmentation index (DFI) using 
aniline blue- eosin staining method was studied 
in semen samples provided on the day of IUI 
procedure.

The semen samples were washed and 
prepared, and the female partner underwent IUI 
procedure and clinical pregnancy outcomes were 
recorded.

Based on sperm DFI results, the patients 
were divided into the following 3 groups: low DFI 
(DFI<= 10%), medium DFI (DFI=11 % - 20%), 
and high DFI (DFI >= 21%) and clinical outcomes 
were compared among the 3 groups. This study 
was carried at a tertiary care infertility centre and 
permission to conduct the experiments was taken 
from the hospital ethical committee, also informed 
written consents were obtained from each patient.

Semen Analysis
Patients provided semen samples for 
analysis by masturbation after an abstinence of 
3 to 6 days. After 30 minutes of liquefaction at 
room temperature, every sample is analysed for 
conventional semen parameters13 and DFI using 
Aniline blue- eosin staining.

In this technique, semen samples in 
stained with Aniline Blue- eosin as previously 
described14-16. 10 ìL of raw semen sample was 
smeared on a slide and subsequently air dried for 
each patient. Slides were fixed at room temperature 
in 4% formalin for 5 minutes, then rinsed with 
water and air dried. 5% aqueous aniline blue 
solution (HIMEDIA) (pH 3.5) was used to stain the 
slides for 5 minutes followed by rinse with water
and air drying. Slides were then counter stained for 1 minute in 0.5% eosin (Merck), again rinsed and air dried. A modification of Aniline Blue staining method i.e. a counter stain eosin when used after aniline blue enhances the staining. Slide examinations were carried under bright field microscope at X 1000 magnification using oil immersion. Immature sperms stain dark blue, whereas the eosin counter stain, stained the mature sperms red pink. The percentage of abnormal sperm chromatin condensation or DNA fragmentation index (DFI) was recorded as the ratio of the number of dark-blue sperm to total number of sperm cells observed and multiplied by 100. A minimum of 200 sperm cells were observed for every slide.

IUI Treatment plan

In the selected pool of patients, the female partners underwent ovulation induction using Clomiphene citrate. An injection of human chorionic gonadotropin (hCG) 10000 IU was given to the female partner when at least 1-2 follicles reached the size of 18 mm in diameter. IUI procedure was scheduled 36 hrs after the injection was given. The male partner provided a fresh semen sample on the day of IUI treatment and the sample was prepared by density gradient centrifugation (DGC) method using sperm grade solutions of 40% and 80% (Vitrolife, Sweden). 1 ml of the prepared semen sample was then deposited using an IUI catheter inside the uterine cavity of the female partner. Serum â-hCG was assessed 15 days after the treatment to detect positive pregnancy (>50mIU/ml). Ultrasound examination was done after 6 weeks to confirm clinical pregnancy.

Statistical Analysis

Statistical analysis was performed using Pearson correlation co-efficient, ANOVA and Shapiro Wilk Test on the above groups.

RESULTS

The 185 patients selected for the study had an average male age of 32.19 years and average female age of 30.30 years. The overall pregnancy rate of this group was 21.08%.

The pregnant group had an average DFI of 8.84% and the non-pregnant group had 14.65% average DFI.

The patients were divided according to their DFI results into 3 groups:

- Low DFI % (<= 10%), Medium DFI % (11% - 20%), High DFI % (>=21%), with an average female age of 29.83yrs, 30.61yrs & 31.22yrs respectively; and an average male age of 31.81yrs, 32.33yrs & 33.31yrs respectively which were not statistically significant

In this study, a negative correlation was observed between percentage of sperm DFI and clinical outcomes among the three groups i.e. low DFI gave high pregnancy results.

Table 1 shows that DFI and clinical outcomes in IUI treated patients are significantly and negatively correlated with correlation coefficient (r) of -0.1, -0.3 and -0.3 in low DFI%, medium DFI and high DFI% groups respectively.

A normality test (Shapiro Wilk test) was performed i.e. a test to compare our distribution of data with normal distribution, we found that the p value was >0.05 hence we do not reject

| ITEM              | Cycle (n) | DH (%)   | Clinical pregnancy rate (%) | Correlation co-efficient (r) | P-value |
|-------------------|-----------|----------|----------------------------|-----------------------------|--------|
| Low DFI (<= 10%)  | 91        | 8.42     | 35.16                      | -0.1                        | <0.05  |
| Medium DFI (11%-20%) | 72       | 15.26    | 9.72                       | -0.3                        | <0.05  |
| High DFI (>= 21%) | 22        | 28.09    | 0                          | -0.3                        | <0.05  |
the null hypothesis that the data follow a normal distribution as shown in graph 1 and 2.

As we can clearly see that our dataset is positively skewed i.e. most people have a lesser DNA fragmentation % in our dataset. Hence to do further analysis we need to do log transformation of our values as provided in the below graph.

Hence we can say that age of male patients is not a predictor of DFI % and clinical outcomes of IUI.

Graph 1 & 2. Plotting DNA Fragmentation % with normal distribution curve
DISCUSSION

Sperm DNA contributes to 50% of the embryo’s genetic material and any damage to the sperm DNA affects conception rates naturally as well as in ART procedures. Unexplained infertility in males show higher incidence of sperm DNA damage9, recently assessment of sperm DFI is being carried along with routine tests18-19, also its importance has been stated in American Urology Association20.

Sperm chromatin abnormalities could occur at the time of spermatogenesis which takes place within several structures of the male reproductive system. Sperm chromatin is a compact structure, which protects the male genetic material against damages during its passage from testis to the female fallopian tubes21.

Sperm chromatin compaction involves the replacement of histones by protamines which help in the final condensation and dense packaging of the sperm DNA into unique tightly coiled “doughnut-loop” subunits22-23.

The chromatin condensation process may sometimes lead to breaks and nicks in the sperm DNA, a failure to repair these breaks results in DNA damage24.

In this study, the aniline blue -eosin staining evaluates the degree of sperm chromatin compaction25-26, where the lysine rich histones that are retained in the immature sperms are bound by the acidic aniline blue in the sperm head making it appear dark blue, as compared to the arginine and cysteine rich protamines of the mature sperm DNA which are stained pink with eosin counter stain, thereby enhancing the immature sperm heads for better observation14,15,17.

In our study we used the density gradient centrifugation method for sperm preparation which has shown lower sperm deformity and DFI in comparison to unprocessed semen in previous studies27.

Our study though based on a smaller patient group, demonstrated that higher the DFI% the chances of clinical pregnancy become very low, therefore, these patients should not

Graph 3. Plot of DNA fragmentation % with respect to age of male patients and categorising it with outcome
be recommended IUI but with advanced ART procedures like IVF and ICSI. High DFI % group of \( \geq 21\% \) did not report any pregnancy whereas low DFI % group reported a pregnancy rate of 35.16% making DFI a good predictor for IUI outcomes.

Also, on doing normality distribution test we clearly observed that our dataset was positively skewed i.e. most people had a lesser DNA fragmentation % in our dataset. Hence to do further analysis we did a log transformation of our values which showed that age of male patients is not a predictor of DFI % and clinical outcomes of IUI. Therefore, more studies should be carried out with a vast patient pool of different age groups at multiple centres so as to have a bell-shaped normal distribution curve.

Previous studies by Duran et.al.\(^2\) reported that higher the DNA fragmentation levels, lower the pregnancy rates in IUI cycles where DFI was significantly higher in failed cycles and no pregnancy was achieved with sperm DFI > 12% by TUNEL method. Yang et.al.,\(^2\) in their study observed a significantly low pregnancy rate in IUI cycles where male samples had a DFI > 25% compared to those where DFI<25%. In another study, Bungum et.al.\(^3\) lower biochemical pregnancy rates, clinical pregnancy rates and delivery rates were observed in IUI patients with \( \geq 30\% \) DFI. Hui et.al.\(^4\) reported that couples with elevated DFI levels should choose ICSI treatment instead of IUI and DFI should be used as a routine screening marker. In a meta-analysis, it was observed that the conception rate in females was 7.3 times higher where the male partner had a normal DFI than those with higher DFI\(^2\)\(^\text{2},\)\(^3\)\(^\text{3}\). In our study, the male age and female age were statistically significant in all the 3 groups of low DFI, medium DFI and high DFI, indicating that age is not a predictor of DFI, also IUI outcomes were independent of age of male patients.

**CONCLUSION**

DNA fragmentation index (DFI) based on sperm chromatin compaction/condensation is a very important parameter for the assessment of male fertility and for predicting reproductive outcome. Our study demonstrated that DFI (%) and clinical pregnancy rate (%) are significantly and negatively correlated in patients with normal semen parameters undergoing IUI. The higher the DFI% the chances of clinical pregnancy become very low, therefore, these patients should not be recommended IUI but with advanced ART procedures like IVF and ICSI. Further assessment of sperm DNA status and different types of damages is required to establish their overall importance in natural as well as ART conceptions. We suggest this test for analyzing sperm DNA fragmentation be a part of the routine semen analysis for patients suffering infertility, as it is simple method to establish in an ART laboratory and also is cost-effective and would not put financial pressure on patients who are already socially, psychologically and economically disturbed.

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**Conflicts of Interest**

The authors have no conflicts of interest to declare.  
**Ethical Study**

Jov IVF Center, Jain Hospital’s Ethical Committee approved the study.

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