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Original Article

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

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ABSTRACT

Background: Male infertility is on the rise globally. It contributes to about 40-50% of all infertility cases. Semen analysis forms the basic investigation for the assessment of male factors of infertility, but it has its pitfalls. Literature review has suggested newer parameters of semen analysis such as sperm quality, which can have a significant role in the outcome of pregnancy. This study aims to assess sperm DNA integrity and calculate the DNA fragmentation index, which is a measure of the quality of sperm, thereby highlighting the need for assessment of sperm quality along with sperm quantity.

Materials and methods: 459 cases underwent routine semen analysis along with the DNA fragmentation index. Sperm quality was assessed using Modified Acidic Aniline blue staining. The statistical analysis was done using SPSS using a t-test and Chi-square test.

Results: Advancing age did not affect all parameters. The DNA fragmentation index showed no effect on sperm count. Unpaired t-test correlation showed a negative correlation between DNA fragmentation index and sperm motility, viability, and morphology.

Conclusions: This study highlights the need for increasing awareness about the male factor of infertility with special reference to the quality along with the routine quantitative assessment of sperms. A conventional semen analysis along with DNA fragmentation index assessment can help the clinician to put forward the choice of artificial reproductive techniques for the couple and provide an accurate success rate of treatment.

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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.[1] 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.[2] 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.[3,4]

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 [5]. 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.[1] 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.[2] Slides were prepared by feathering method as described in the WHO manual.[1]

Modified Acidic aniline blue staining: Slides were air dried, fixed in 3% buffered Glutaraldehyde for 30 minutes and stained with AAB for 5 min.[5,6] 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:

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

DFI < 20% - Normal

DFI> 20 % - Abnormal or high DFI.

The results were compared by applying statistical tests as recommended by WHO 2010 manual.[1] 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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Table 1: Association of immature heads of sperms (%) with DNA Fragmentation Index

Immature heads of sperms (%)	n	DNA Fragmentation Index	
		DFI < 20 (n, %)	DFI > 20 (n, %)
< 20	305	303 (99.3)	2 (0.7)
20-40	122	122 (100.0)	0 (0.0)
40-60	22	0 (0.0)	22 (100.0)
>60	10	0 (0.0)	10 (100.0)
Chi square = 430.031, p<0.05			

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 as 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).

Normospermia and DFI:

On routine semen analysis, 256 normospermia cases were found. After estimating the DFI, the effective number of normospermia cases were reduced to 244 from 256, as 12 cases showing a DFI > 20, suggesting that these 12 cases have poor sperm DNA quality.

On the other hand, among other cases on routine semen analysis, 203 cases comprised asthenospermia, oligospermia, oligoasthenospermia, oligonecrozoopermia and necrozoospermia. After estimation of DFI, the number of cases significantly reduced from 203 cases to mere 22 cases, as only these cases with DFI>20.

Table 2: Comparing the results of routine semen analysis and the DFI

Result	Cases	DFI <20	DFI>20
Normal	256	244	12
Asthenospermia	118	108	10
Oligospermia	10	63	9
Oligasthenospermia	72	8	1
Oligonecrozoospermia	1	0	1
Necrozoospermia	2	2	1
	459	**425**	**34**

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]

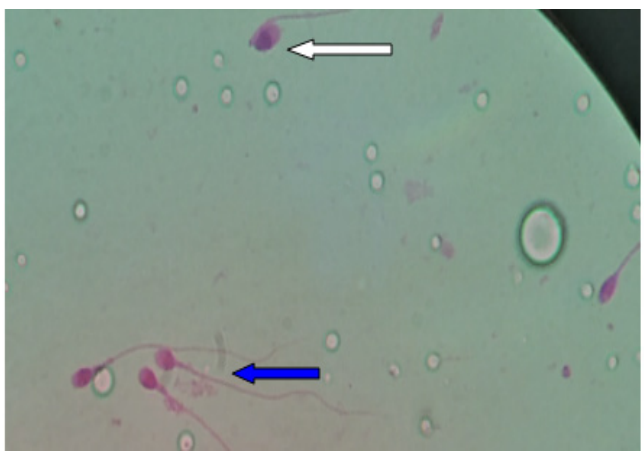

Figure 1: *Blue stained immature heads (white arrow) and pink stained mature heads (blue arrow) on modified aniline blue method (X100)*

This method was considered cost-effective and can be performed in a lab along with the routine semen analysis. It is said to have a positive correlation with the acridine orange

(AO) test. Patil et al[5], Hee sun kim et al [6], Sellami et al[7], and Hind abdulkhadim et al[8] 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.[9] 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).

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

Parameters	Group 1(DFI < 20)	Group 2(DFI > 20)	t-test	p-value
Age	32.896±6.226	34.294±6.668	1.253	0.211
Volume (ML)	2.413±1.055	2.382±1.000	0.163	0.870
Total Sperm Counts (M/ML)	55.432±42.449	49.709±47.263	0.750	0.454
Total Motility (%)	55.198±21.399	42.941±27.139	3.145	0.002
Progressive Motility (%)	34.984±19.813	23.529±22.003	3.217	0.001
Sperms with normal morphology (%)	65.274±17.615	56.471±28.164	2.657	0.008
Immature heads of sperms (%)	16.906±12.211	57.971±17.406	17.908	0.000

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 4: Comparison of semen parameters of various other studies based on DFI

Study	Parameters	Low DFI	High DFI
Patil et al[10]	Age(y)	35.6±42.6	35.1±6.3
	Semen volume (ml)	-	-
	Sperm count(m/ml)	35.21±14.3	29.8±15.4
	Sperm progressive Motility(%)	38.2±12.4	39.8±13.7
	Normal morphology(%)	9.6±8.4	4.6±7.9
Boushaba et al[12]	Age(y)	-	-
	Semen volume (ml)	2.37±0.51	3.03±0.29
	Sperm count(m/ml)	37.57±13.16	7.32±3.59
	Sperm motility(%)	54.17±7.90	43.13±3.26
	Normal morphology(%)	29.50±4.50	38.14±7.00
Yang et al[3]	Age(y)	30.5±4.8	32.2±6.7
	Semen volume (ml)	-	-
	Sperm count(m/ml)	67.9±39.3	52.65±34.5
	Sperm motility(%)	61.6±10.3	43.2±10.9
	Normal morphology(%)	8.5±3.2	4.3±3.3
Le et al[13]	Age(y)	-	-
	Semen volume (ml)	1.54±0.76	1.69±0.92
	Sperm count(m/ml)	31.74±13.31	34.38±13.63
	Sperm progressive Motility(%)	29.11±13.64	32.39±13.08
	Normal morphology(%)	-	-
Vinnakota et al[14]	Viability (%)	78.64±12.30	78.93±8.30
	Age(y)	37.6±6.7	44±9.5
	Semen volume (ml)	2.7±1.4	2.4±1.4
	Sperm count(m/ml)	48.4±46.2	52.4±35.9
	Sperm motility(%)	55.3±15.8	38.8±16.1
	Normal morphology(%)	-	-
Present study	Age(y)	32.896±6.226	34.294±6.668
	Semen volume (ml)	2.413±1.055	2.382±1.000
	Sperm count(m/ml)	55.432±42.449	49.709±47.263
	Sperm motility(%)	55.198±21.39	42.941±27.139
	Normal morphology(%)	65.274±17.615	56.471±28.164
	Sperm progressive Motility(%)	34.984±19.81	23.529±22.003

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.
2. The data does not include clinical outcomes including the rate of fertilization or pregnancy rate.

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