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Evaluation of sperm DNA abnormality index as a indicator of male infertility

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Abstract

Aim and objective: To assess the prevalence of sperm DNA integrity in both normal and abnormal subjects by a simple and cost effective cyto-chemical method using toluidine blue stain, and to analyse the value of sperm DNA integrity as an efficient tool in male infertility.

Material and methods: Samples were collected from men visiting for evaluation of infertility. Routine semen parameter was assessed as per WHO manual 5th Edition and abnormal sperm DNA integrity index was calculated using toluidine blue method. Samples with abnormal semen parameter was taken as study group, sample from healthy proven voluntary donors were taken as control group. Abnormal sperm DNA integrity index was then correlated with the study groups.

Results: Abnormal sperm DNA integrity index also correlated well with the fertility potential.

Keywords: Toluidine blue, semen, DNA integrity

Introduction

Infertility is a Global social problem which is on the rise. It affects approximately 15% of all couples ^[1]. In the world of infertility, male factor forms one of the major components either solely or as contributory. Semen analysis forms the base line investigation and its results are taken as a surrogate measure of male fecundity ^[2]. However the parameters assessed by routine semen analysis provide only moderate predictive value, new parameters are needed for better prediction ^[1-5]. Sperm DNA integrity is now gaining importance as a new marker in the field of infertility ^[1-5]. Though studies favour role of sperm DNA integrity in infertility, a bit of controversy is still evident which requires further evaluation. This study is also undertaken in same view, to exploit the role of sperm DNA integrity in infertility. In this present study we have assessed the sperm DNA integrity of the infertility group and control group and have compared it. There are various methods to assess sperm DNA integrity, common being comet assay, TUNEL assay, acridine orange test and sperm chromatin structure assay ^[3]. But these are expensive and require specialised instruments and trained personals.

Presently many cyto-chemical tests are in the advent for the assessment of sperm DNA integrity, Toluidine blue is one of them. It is simple, inexpensive, does not require sophisticated instruments and is proven to be a sensitive method equivalent to TUNNEL assay and flowcytometry ^[6]. So we have assessed sperm DNA integrity with Toluidine blue.

Materials and methods

This was a prospective analytical study conducted in our institution. All the semen samples were obtained from men visiting our laboratory for the evaluation of infertility and informed written consent was obtained from each. Semen collected was evaluated for the all routine parameters like appearance, volume, liquefaction time, pH, motility, count and morphology as per the WHO guideline ^[7] and were grouped into infertile men with normal semen parameters and infertile men with abnormal semen parameter based on routine semen analysis as per the WHO criteria 2010. Semen from age matched fertile proven donors were collected and taken as control group. Thereafter 2 smears were prepared from each sample and were stained with 0.05% of toluidine blue for assessment of DNA integrity. Total of 200 cells were counted in different areas under light microscopy using oil emersion with x, 1000 magnification.

Sperm cell heads with good chromatin integrity were light blue, cells with abnormal integrity were dark violet, and few cells were of intermediate color. Dark violet cells were considered abnormal and percentage of it was determined which has been proved to be correlating with the TUNEL positive cells. Intermediate colored cells were also counted as light violet cells. The findings were then correlated within the groups.

Inclusion criteria: All patients who were investigated for infertility.

Exclusion criteria: Proven cases of malignancy, patients who have received chemotherapy, on steroids, azoospermic and aspermic patients were excluded.

Sampler size: our sample size was 100 on applying the exclusion criteria.

Study group: semen was grouped into infertile men with normal semen parameters and infertile men with abnormal semen parameter based on routine semen analysis as per the WHO criteria 2010.

Control group: samples were collected from 15 healthy voluntary proven donors of age above 25 years and below 35 years.

Collection of sample: samples were collected from the patients with minimum abstinence period of 3 days by masturbation technique in a wide mouthed proven nontoxic plastic container.

Semen analysis: Appearance, liquefaction time, volume, pH, motility, count and morphology are routinely assessed as per WHO 2010 criteria and documented.

Toluidine blue staining

- After liquefaction of semen at room temperature for 30 minutes, sperm was pelleted at 1,000 rpm for 10 minutes and re-suspended in its own supernatant to a approximate of 2×10^8 cells /ml.
- Thin smears are made from the suspension and air dried for 30-60 minutes.
- Dried smears were fixed with freshly made 96% ethanol: acetone (1:1) at 4°C for minimum of 30 min and air dried.
- Hydrolysis was done with 0.1 N HCl at 4°C for 5 min and was rinsed three times in distilled water for 2 min per rinse.
- Staining with 0.05% TB (COMPANY; LOBA CHEMIE TOLUIDINE BLUE FOR MICROSCOPY CI NO. 52040 LOT NO. S26701111) was applied for 5 min. The staining buffer consisted of 50% McIlvain's citrate phosphate buffer (pH 3.5).
- Slides were rinsed briefly in distilled water and lightly blotted with filter paper.
- Then dehydration was done 2×3 min with tertiary

butanol at 37 °C and 2×3 min with xylene, and mounted in DPX.

Preparation of control for staining: equal volume of patient semen sample and 20% hydrogen peroxide was mixed and incubated at room temperature for 15 minutes and then was proceeded with toluidine blue staining as stated above. Control slide was run in each batch of staining.

Analysis

In all 100 samples a total of 200 cells each were counted using light microscope (OLYMPUS CX 21) under oil emersion in different fields and Light blue sperm cell heads were scored as possessing DNA of normal integrity, and those with dark violet were scored as having damaged DNA. Only dark cells were taken as it was proved that only dark cells correlated with the TUNEL assay.

Statistical analysis

Statistical analyses were performed using SPSS version 17.0 for analysis. To compare independent samples, t-test was used. ROC curve was used to derive the cut-off value.

Results

Semen analysis and sperm DNA integrity assessment was done on 100 patients who were screened for infertility. Routine semen parameters and sperm DNA integrity was also assessed on 15 healthy proven (who fathered a child) donors as a control group. Sperm DNA integrity was assessed in 200 sperms using Toluidine blue and darker cells were scored as abnormal and lighter sperm was scored as normal. Intermediate coloured sperms were also scored separately.

Age distribution

The age ranged from 24 to 47 years. In this study, maximum number of patients were in the age group of 26 to 30 years which matched with our control group.

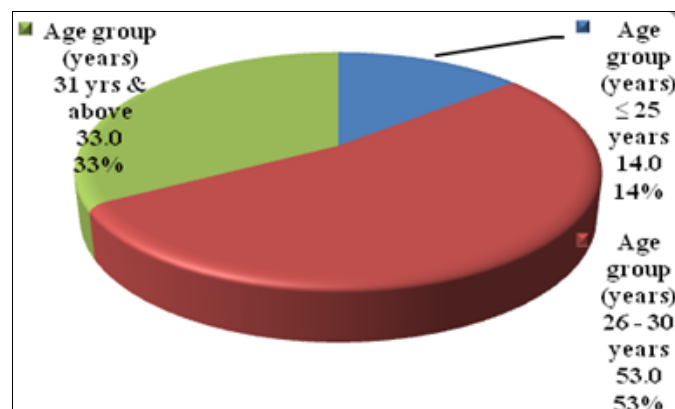


Fig 1: Age distribution in study group

In our study 37 out of the 100 patient samples had abnormal semen parameters and remaining 63 had normal semen parameters as per the recent WHO 2010 criteria.

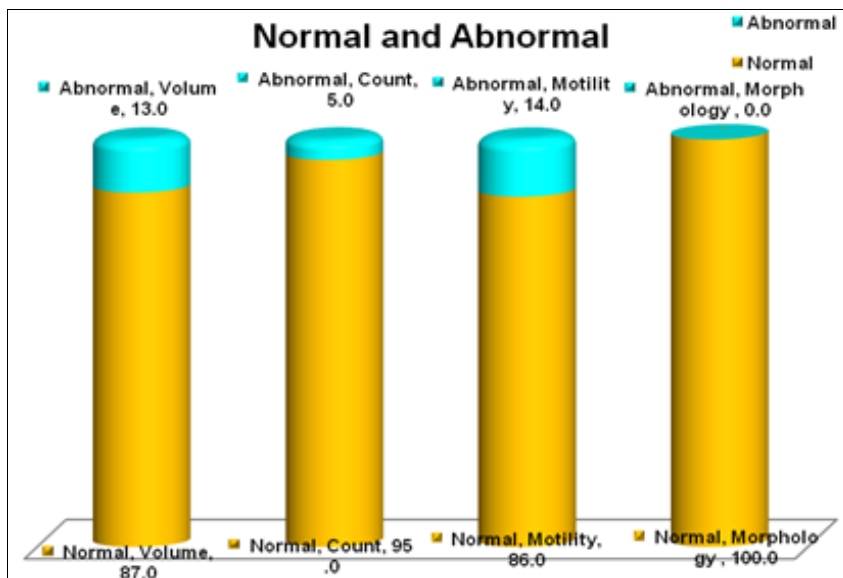


Fig 2: Distribution of semen Parameters in Normal and Abnormal Subjects

Normal-Normozoospermic infertile individuals Abnormal-infertile individuals with abnormal semen parameters

Abnormal DNA integrity index with respect to study groups

Mean value of DNA integrity index (dark violet colored sperm head) in our study was 22.73 in control group, 47.46 in normozoospermic infertile individuals and 63.05 in infertile individuals with abnormal semen parameters. The differences in the mean value was statistically significant with p=0.001.

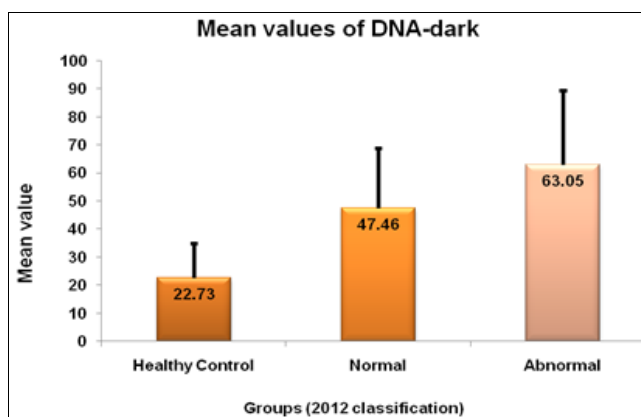


Fig 3: Distribution of abnormal DNA integrity in the study groups

Normal-Normozoospermic infertile individuals Abnormal-infertile individuals with abnormal semen parameters

Roc curve analysis for lower threshold

ROC curve analysis was applied utilising DNA integrity index of fertile control and infertile men screened for infertility to predict the lower infertility cut-off.

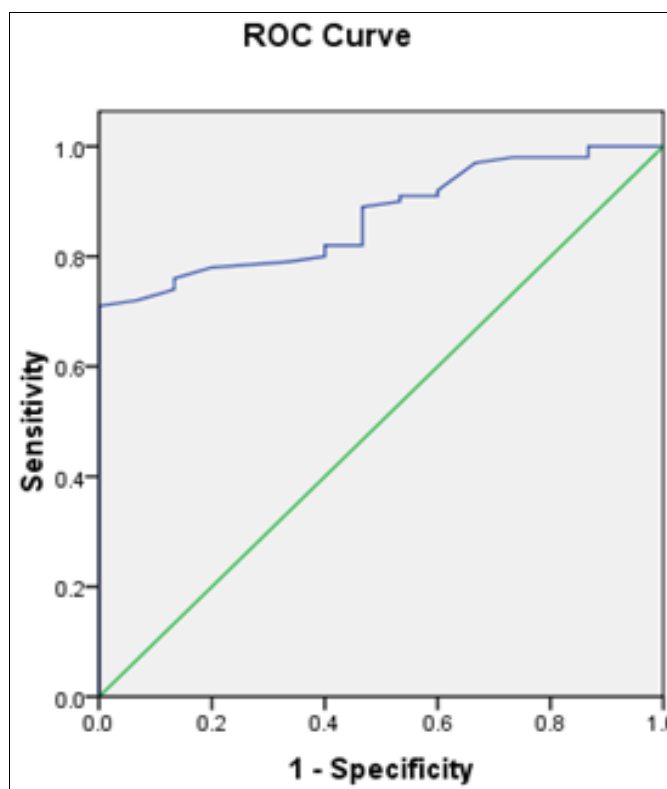


Fig 4: Roc curve analysis of lower threshold of abnormal DNA integrity index area under the curve = 0.872.

The ROC curve analysis suggests the cut-off value of DNA-dark ≥ 38.5 will be the best value to predict Infertility. Suggesting that subjects with DNA integrity index of less than 38.5 are fertile.

Sensitivity and specificity analysis

Table 1: Sensitivity and Specificity of lower threshold of abnormal DNA Integrity

		2012 classification		Total
		Abnormal infertile + Normozoospermic infertile individuals	Healthy control	
DNA dark	≥ 38.5	71	0	71
	< 38.5	29	15	44
Total		100	15	115

The determinant of lower cut off value of 38.5 to predict infertility is shown.

Table 2: Determinant of lower cut off value for abnormal DNA integrity

Parameter	Estimate	95% CIs Lower – Upper
Sensitivity	71.00%	61.46, 78.99
Specificity	100.00%	79.61, 100.00
Positive Predictive Value	100.00%	94.87, 100.00
Negative Predictive Value	34.09%	21.88, 48.86
Diagnostic Accuracy	74.78%	66.13, 81.83

Roc curve analysis for upper threshold

ROC curve analysis was also performed using the DNA integrity index between control, normozoospermic infertile

individuals and individuals with abnormal semen parameters.

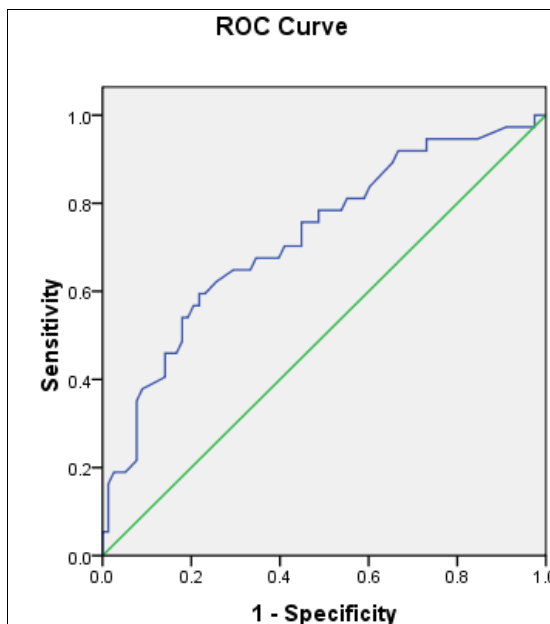


Fig 5: ROC Curve Analysis of upper threshold of abnormal DNA Integrity index Area under the Curve = 0.723

The ROC curve analysis suggested the cut-off value of DNA-dark ≥ 57.5 . Suggesting that patients with DNA integrity index more than 57.5 will be infertile with

abnormal semen parameters. Sensitivity and Specificity Analysis

Table 3: Sensitivity and Specificity of higher threshold of abnormal DNA Integrity index

	2012 classification		Total
	Infertile individual with abnormal semen parameters	Normozoospermic infertile individual + Healthy control	
DNA Dark ≥ 57.5	22	17	39
DNA Dark < 57.5	15	61	76
Total	37	78	115

Table 4: Determinant of higher cut off value for abnormal DNA integrity

Parameter	Estimate	95% CIs Lower-Upper
Sensitivity	59.46%	43.49, 73.65
Specificity	78.21%	67.84, 85.92
Positive Predictive Value	56.41%	40.98, 70.70
Negative Predictive Value	80.26%	69.96, 87.66
Diagnostic Accuracy	72.17%	63.37, 79.55

Discussion

DNA damage in spermatozoa occurs during late spermatogenesis as a consequence of endogenous factors or exogenous factors. If sperm with damaged DNA fertilize an oocyte it may result in various pathologies like infertility, spontaneous abortion [8, 9, 10] and childhood diseases [11, 12]. Sperm DNA damage is common amongst infertile men with normal and abnormal sperm parameters and may adversely affect the outcome of both natural and assisted conception [13].

Since sperm DNA damage may affect the outcome extensive studies are required to establish their role in infertility. To establish that, it is to be compared and analysed with semen analysis, which is the base line gold standard investigation of male infertility. So in this study we have assessed sperm DNA damage in both, infertile couples with normal semen and infertile couples with abnormal semen parameter and compared it with the control group (fertile person).

General distribution of cases

The majority of patients were between the age group of 26 to 30 years matching with our donor/control group. Out of 100 semen samples investigated from infertile couple 37% were considered as abnormal and 63% were considered as normal based on semen parameters as per WHO 2010 criteria. (Abnormality in even one parameter was considered as abnormal).

DNA integrity index

In the present study, mean abnormal DNA integrity index in control group was 22.73. Kishlay kumar *et al.* [5] evaluated the predictive value of DNA integrity in idiopathic pregnancy loss has reported a mean DFI of 21.40 by Sperm Chromatin Structure Assay method. In a study by Fernandez JL *et al.* (2005) [14] reported 16.3% of sperm DNA damage in his fertile control group by sperm chromatin dispersion method. Generally all the studies proposed a low value of DNA integrity index in fertile men, and emphasized the fact that fertile men have low abnormal DNA integrity index, but the differences can be explained by different evaluation methods applied.

Mean abnormal sperm DNA integrity index in normozoospermic individual of infertile men in our study was 47.46%. Piasecka *et al.* [15] studied 26 subjects and reported a mean value of 9.42(\pm 7.68%) by tunnel assay. In a similar study by Fernandez JL *et al.* [14] 27% sperm DNA damage was found in normozoospermic patients. Similar results were obtained by marchetti *et al.* [12] and carell *et al.* [16] 23(\pm 2%) and 11.9(\pm 1.0%) respectively. The mean abnormal sperm DNA integrity in normozoospermic individuals was slightly higher in our study, in comparison to other studies. Difference may be due to the variation in the sample size and evaluation method. Sperm DNA integrity is high in the normozoospermic group than control group as these are patient who were investigated for infertility and the high percentage of abnormal DNA may be the cause of negative pregnancy outcome. These cases are not picked up by the routine semen analysis, making it a modest predictor of infertility.

In the present study mean abnormal sperm DNA integrity index in men with abnormal semen parameter was 63.05% in contrary to 47.46% in the normozoospermic individual and 22.73% in control group, which was significantly different with a p value of 0.001 which was similar to the results obtained in previous studies. This implicates that abnormal sperm DNA integrity index increases with a decrease in fertility potential.

Evenson and colleagues [17] suggested that threshold of 0–15%, 16–29% and >30% DNA fragmentation index correlate to high, moderate and low fertility potential, respectively. Similarly Mona Bungum [1] states that in the interval of DFI 0–20%, the chance of spontaneous pregnancy was constant. When DFI was above 20% the chance of obtaining a spontaneous pregnancy was decreased and close to zero when the DFI level passed 30–40%. Benchaib *et al.* [18] found that the fertilization rate was significantly higher for DNA fragmentation <10% and no pregnancy was obtained when >20%. Similar results were also obtained in other previous studies.

In our study based on ROC curve analysis a threshold of <38.75%, 38.76–57.74%, >57.75% abnormal DNA integrity index was suggested for high, moderate and low fertility potential respectively. Differences in the cut-off values in different studies may possibly be explained by the different evaluation methods.

Henkel *et al.* [10] considered a threshold value of 36.5% by tunnel assay for pregnancy rate. And a value of 27–40% was proposed for SCSA method. Jekaterina Erenpreisa *et al.* [19] and Hee-Sun Kim *et al.* [20] proposed infertility threshold of 35% and 24.1 (\pm 14.5%) respectively by toluidine blue method correlating with our study.

As our results correlated well with other studies, our study also signifies that toluidine blue is a sensitive, equally

effective and economical method as compared to tunnel, comet assay to detect sperms with abnormal DNA integrity. Although sperm DNA damage is associated with lower pregnancy rates, a clinically relevant standard DNA damage test with a meaningful cut-off level needs to be standardized.

Limitations in the present study include, small sample size and lack of clinical outcome.

Conclusion

There appears to be a threshold of sperm DNA damage beyond which fertility potential decreases. Results of our present study also supported that. Abnormal DNA integrity index in control, infertile men with normal semen parameter and infertile men with abnormal semen parameter was 22.73%, 47.46%, and 63.05% respectively and the difference was significant with a p value of 0.001. Based on the ROC curve analysis a threshold of < 38.75%, 38.76 - 57.74%, >57.75% abnormal DNA integrity index was suggested using toluidine blue method for high, moderate and low fertility potential respectively. Although we propose a simple toluidine blue method to detect sperm DNA damage and a threshold to define low infertility a relevant standard DNA damage test with a meaningful cut-off value is to be standardised and established.

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