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Correlation of sperm DNA integrity with other semen parameters and analyse the role of abnormal sperm DNA in evaluation of infertility

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Abstract
Semen analysis forms the base line investigation and its results are taken as a surrogate measure of male fecundity.
Aim and Objective: To assess the prevalence of abnormal sperm DNA integrity index and to correlate it with the other semen parameters and analyse its role as an efficient tool in evaluation of 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 Toludine blue method. Samples were grouped into men with normal semen parameter and men with abnormal semen parameters based on WHO 2010 criteria. Sperm DNA integrity index was then correlated with the routine semen parameters and also within the study groups.
Results: Abnormal sperm DNA integrity index showed a significant negative correlation with sperm count, motility and morphology.

Keywords: DNA integrity, toluidine blue

Introduction
Integral sperm DNA is required for fertilisation and further embryo development, failure of which may result in early abortion. Its assessment also plays a role in in-vitro fertilisation [1]. A high level of DNA fragmentation in sperm may be a reason for male infertility. It cannot be detected by conventional examinations such as sperm concentration, motility analysis, and morphological assessment. Sperm DNA integrity is now gaining importance as a new marker in the field of infertility [2]. This study is also undertaken in same view, to exploit the role of sperm DNA integrity in infertility.

Aim: In this present study is to
1. To assess the sperm DNA integrity by toludine staining
2. Compare it with other semen parameters
3. To prove its causative association with male infertility.

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 [3]. Thereafter 2 smears were prepared from each sample and were stained with 0.05% of toludine 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 [4].

The findings were then correlated with other semen parameters and analyzed.

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.

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

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

Toludine 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 of2 x 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 toludine 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. Light violet colored cells were also counted

Statistical analysis

Statistical analyses were performed using SPSS version17.0 for analysis. To compare independent samples, t-test was used. To assess the relationship, co-efficient correlation was used.

Results

Age distribution & semen parameters

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.

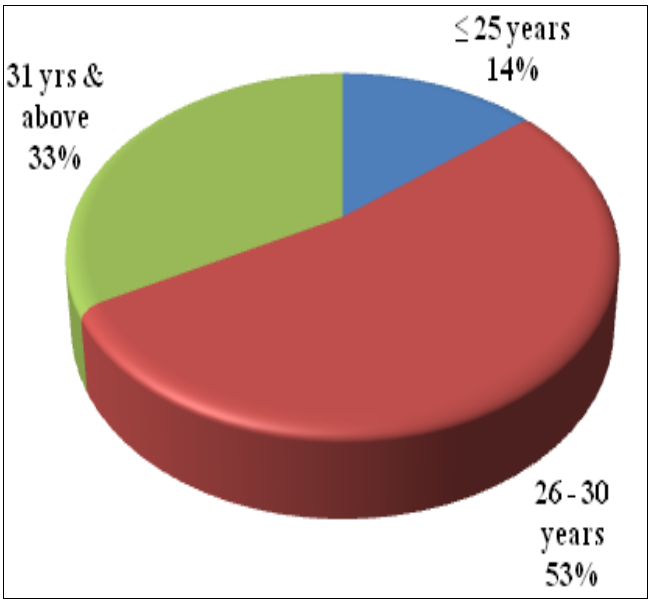

Fig 1; Age distribution

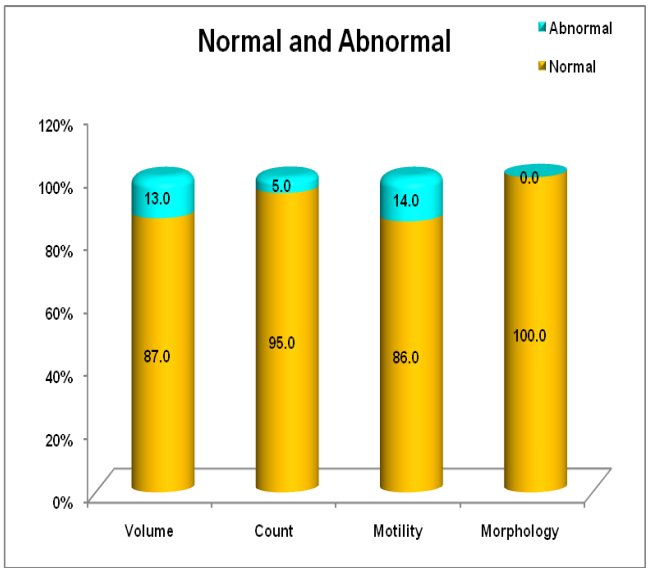

Fig 2: Distribution of semen Parameters in Normal and Abnormal Subjects

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.

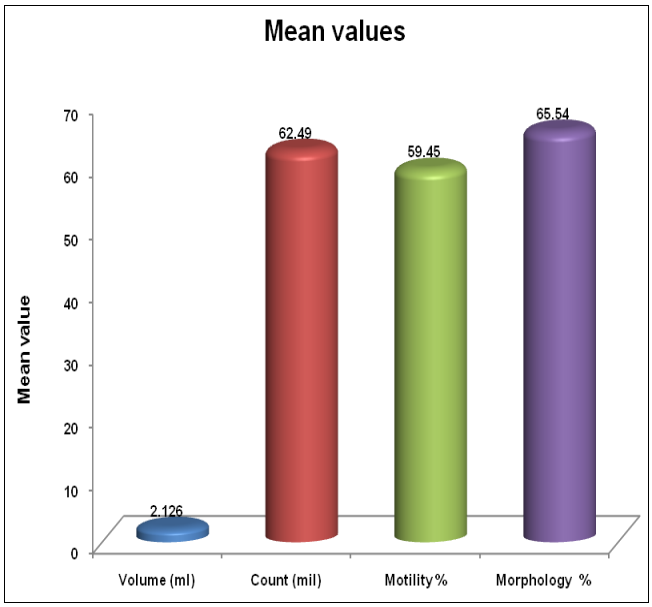

Fig 3: Mean Distribution of Semen Parameters in the Study

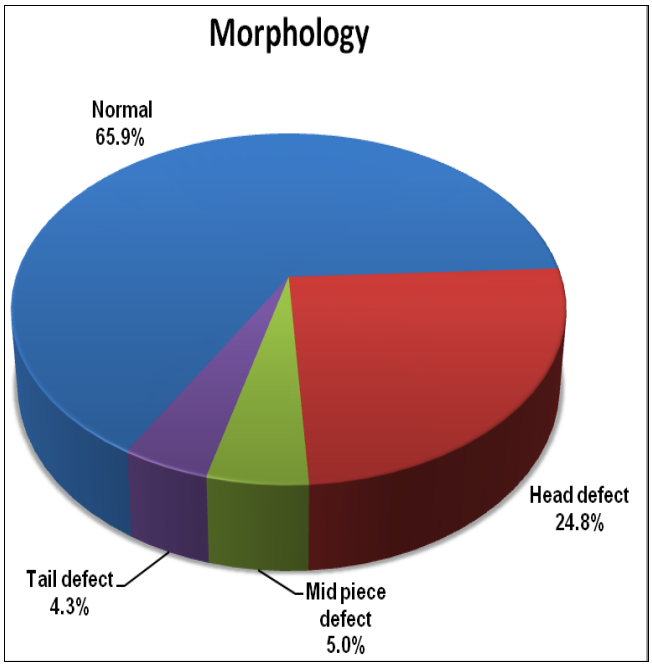

Fig 4: Distribution of Morphological Defects

Abnormal DNA integrity index with respect to staining pattern

Abnormal DNA integrity index was assessed by counting dark violet headed sperm and intermediate colored (light violet/ dark blue) sperm heads separately. Mean value of DNA integrity index of intermediate colored sperm head (light violet/dark blue colored sperm), dark headed (dark violet colored sperm) sperm and their total was compared in normozoospermic subjects and subjects with abnormal semen parameters.

There was a statistically significant difference in the mean DNA integrity index between normozoospermic patients and patients with abnormal semen parameter, when DNA integrity was assessed by counting dark headed sperms and in total (both dark and intermediate colored sperm heads). But no such significant difference was found when DNA integrity was assessed using intermediate colored sperm heads (Supported by other studies that only dark headed sperms correlate with tunnel positive DNA damaged sperm.).

Intermediate colored sperm head did not show any correlation with other semen parameters also, so in our study intermediate colored sperm head count was om itted and only dark colored sperm heads were considered.

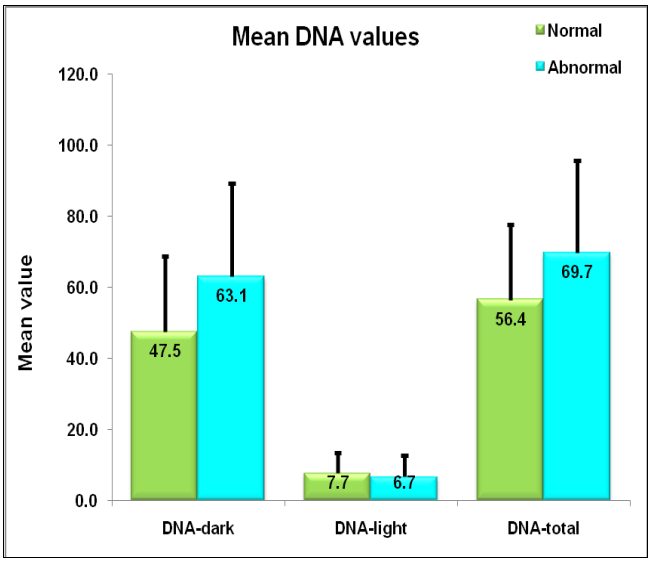

Fig 5: Abnormal DNA Integrity Index in Age Group

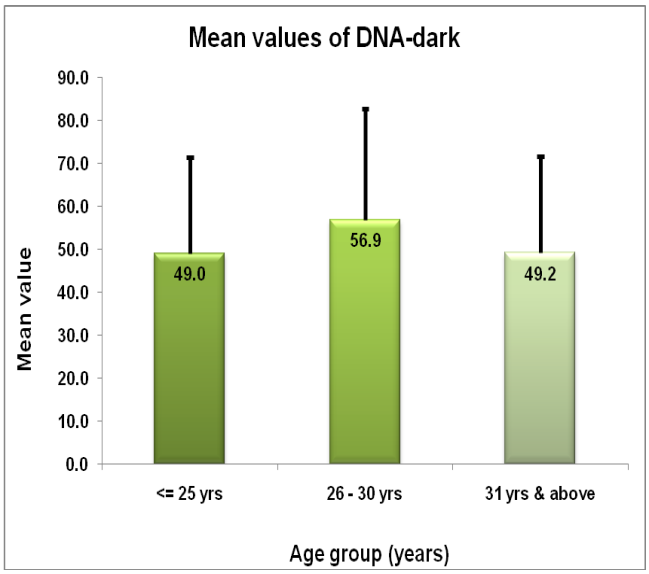

Fig 6: Distribution of Abnormal DNA integrity Index in Age Group

Distribution of mean DNA integrity according to age range is shown. Maximum mean value of 56.9 was observed in the age group of 26-30 years as compared to 49.0 in group <25 years and 49.2 in group >30 years, but the difference was not statistically significant as p value was 0.281.

Age versus abnormal dna integrity index

There was a negative correlation found between age and DNA integrity but not statistically significantly as p value was 0.624.

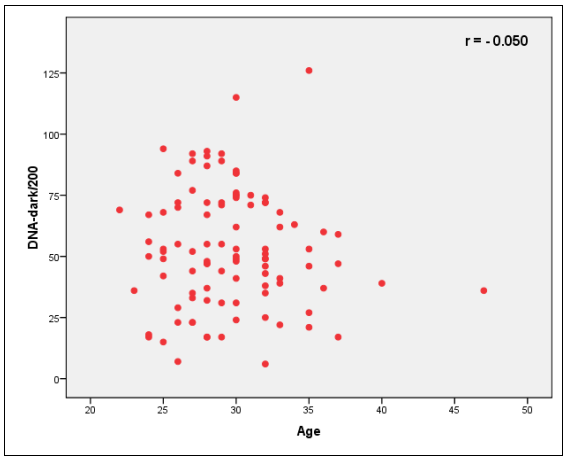

Fig 7: Scatter Diagram showing correlation of abnormal DNA Integrity index and Age

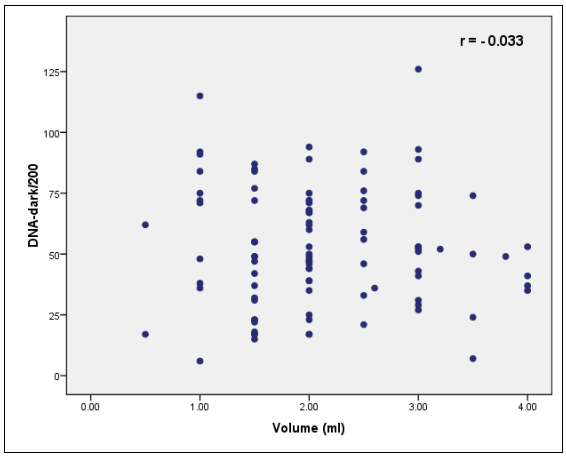

Fig 8: Scatter Diagram showing correlation of abnormal DNA Integrity index and volume

Volume versus abnormal DNA integrity index

In our study volume also showed a negative association but not statistically significant with a p value of 0.744.

Count versus abnormal DNA integrity index

A highly significant negative correlation was found between DNA integrity index and count with P = 0.00, and the strength of association was moderate (r =0.34).

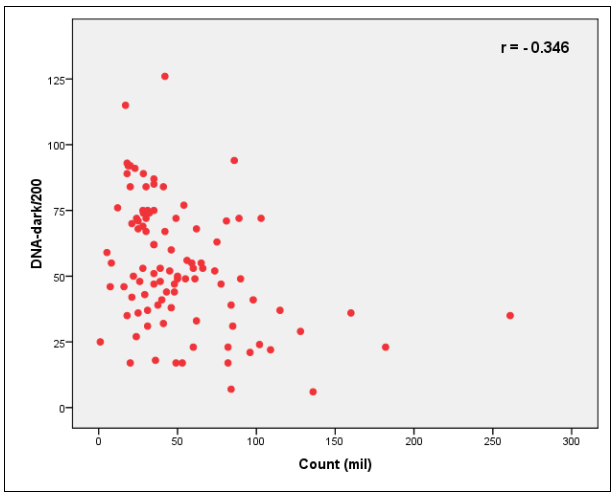

Fig 9: Scatter Diagram showing correlation of abnormal DNA Integrity index and count

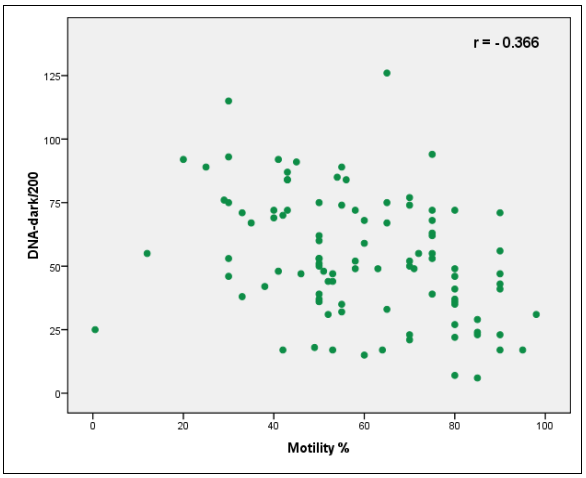

Fig 10: Scatter Diagram showing correlation of abnormal DNA Integrity index and motility

Motility versus abnormal DNA integrity index

Our study also showed a negative correlation between motility and DNA integrity with a significant p (0.000) value. Strength of association was moderate with r =o.366.

Morphology versus abnormal dna integrity index

Present study also observed a negative correlation between DNA integrity and morphology with a highly significant p value (0.000) and moderate strength of association with r = 0.46.

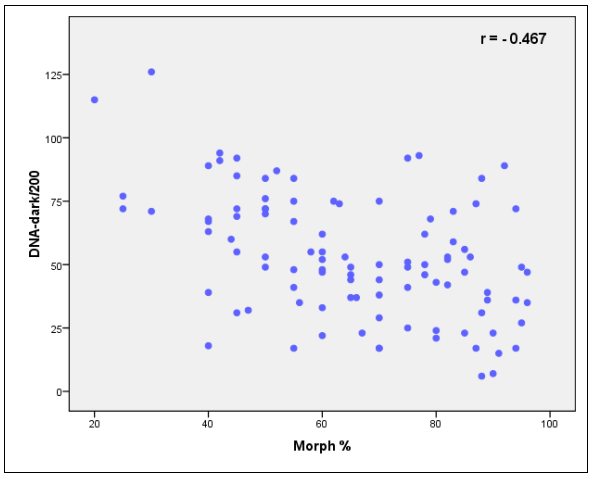

Fig 11: Scatter Diagram showing correlation of abnormal DNA Integrity index and morphology

Discussion

Since sperm DNA damage may affect the outcome extensive studies are required to establish their role in infertility. To establish that, it has to be compared and analysed with semen analysis, which is the base line gold standard investigation of male infertility.

In this study we have assessed sperm DNA damage in both, infertile couples with normal semen and infertile couples with abnormal semen parameter and analyzed its association with routine semen parameters.

Different methods are suggested for sperm DNA damage assessment. We have assessed using toludine blue, which is a simple, cost effective, sensitive, rapid and reproducible method. Making it feasible and easy, to be adopted in all laboratories.

Intermediate colored sperm head did not show any correlation with other semen parameters. So in our study, intermediate colored sperm head count was omitted and only dark colored sperm heads were considered.

General distribution of cases

The majority of patients were between the age group of 26 to 30 years.

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).

The mean value of volume, count, motility and morphology are 2.1ml, 62.49million, 59.45% and 65.54% respectively.

Age and abnormal DNA integrity index

In this present study subjects were grouped as <25 years, 26-30 years, and >30 years and maximum subjects were in the age group of 26-30 years.

The mean DNA integrity index in the three age group was 49.0, 56.89 and 49.1 respectively. Though index was high in age group between 26-30 years, but the difference was not significant as p = 0.281.

Age related increase in sperm DNA damage was observed by L Vagnini *et al*. Similar results were obtained by Morris *et al.* who studied 60 men participating in IVF by comet assay [6].

In contrast to these previous studies our study showed no significant association. Similar to present study zuying Chen *et al.* (p-0.78) and few other studies also showed same results [7].

Volume and abnormal DNA integrity index

In the present study, abnormal sperm DNA integrity was correlated with the volume and was found to have negative correlation but not significant as p=0.744.Hee-Jun Chi *et al.* studied 498 samples using neutral comet assay and found no significant correlation (p = 0.53) [7]. Our results were comparable with this study

Count and abnormal DNA integrity index

Our study showed a significant inverse correlation between the count and abnormal DNA integrity index with a p value of 0.000 and modest correlation coeeficient of 0.3. Oosterhuis *et al.* also found the same. But Zuying Chen *et al.* (p = 0.65) [8] and Hee-Jun Chi *et al.*
(p = 0.91) [9] showed no significant association in contrast to our study. It is hypothesized that Sperm DNA damage causes initiation of apoptosis resulting in decreased count [10].

Motility and abnormal DNA integrity index

Zuying Chen *et al.* studied 23 subjects by TUNNEL assay and found a significant negative correlation between motility and sperm apoptosis [11]. This correlated well with our results (P = 0.000, r = 0.3). Similar results were reported by several other studies [12]. They hypothesized that sperm DNA damage affects motility of the sperm which also correlates with our findings [13].

Morphology and abnormal sperm DNA integrity index

A significant inverse association was found between morphology and abnormal sperm DNA integrity index with p=0.000, and a modest correlation with correlation coefficient of 0.46. Few studies do not show any correlation [14], but many studies show inverse association between morphology and abnormal sperm DNA integrity index [15]. It is hypothesized that alteration in sperm chromatin compaction, (protamine deficiency or incomplete sulfhydryl oxidation) may contribute to defects in sperm head morphology. This is attributed to increased morphological defects in subjects with high abnormal sperm DNA integrity index.

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, which was significantly different with a p value of 0.001 which was similar to the results obtained in previous studies (diagnos, comet). This implicates that abnormal sperm DNA integrity index increases with a decrease in fertility potential.

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

Conclusion

Successful human reproduction depends in part on the inherent integrity of the sperm DNA. There appears to be a threshold of sperm DNA damage beyond which fertility potential decreases. Results of our present study also supported that. Our data also shows a significant relationship between sperm DNA damage and sperm count, motility and morphology high lightening the role of sperm DNA integrity in infertility causation. We also conclude that the Toludine blue method to assess abnormal DNA integrity index is a simple, effective, economical and potentially useful tool in the evaluation of infertility.

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