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ROLE OF SPERM NUCLEAR DNA INTEGRITY STATUS AND THE SEMEN QUALITY OF FERTILE AND INFERTILE MEN

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ABSTRACT: The aim of the study is to evaluate the diagnostic DNA profiles of the assessment of semen analysis data with respect to male fertility potential. Seman analysis taken form 50 patients of known fertility and 50 suspended infertility were studied by conventional semen analysis by suitable methods. The acridine orange test was used to assess the integrity of sperm DNA. The data throw doubt upon the validity of an approach based on the number of deviations for the normal standard values defined by the WHO. The alternative approach as specific semen characteristics (particularly morphology) as the major predictor of fertility produced no beneficial results. The means of sperm concentration in fertile males was significantly higher than infertile males (62.7±18.7 million vs. 36.7±25.40 million). Total sperm motility of fertile males also was significantly higher compared to infertile (56.32±0.07% vs. 28.8±10.7%). Normal sperm morphology of fertile males was also significantly higher compared to infertile (24.5±8% vs. 12.87±4.6%). According to these results we can conclude that testing sperm DNA damage is helpful in selection of spermatozoa with the least amount of damage for use in assisted conception.

INTRODUCTION: The management of infertility problems has become an increasingly important part of health services during the past two decades in the most of the countries. Recent studies have focused on semen quality of men in the general population. However, most studies of semen quality and reproductive hormones in unselected populations have little previously reported [1]. It is established that sperm DNA quality is important in maintaining the reproductive potential of men [2].

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The fertilizing potential of sperm depends not only on the functional competence of spermatozoa but also on sperm DNA integrity [3]. Classical semen analysis, which include sperm concentration, motility and morphology gives an approximate evaluation of the functional competence of spermatozoa, but does not always reflect the quality of sperm DNA. Men with normal spermograms may still the infertile; the cause could be related to abnormal sperm DNA [3].

Sperm DNA integrity has an important role not only for fertilization but also for normal embryo and fetal development [4]. Sperm with compromised DNA integrity, regardless of the degree of DNA damage, appear to have the capacity to fertilize oocytes at the same rate as normal sperm [2].

However, the embryos produced by fertilization of an oocyte with DNA damaged sperm cannot develop normally. Therefore, the evaluation of sperm DNA integrity, in addition to routine sperm parameters, could add further information on the quality of spermatozoa and improved predictive values could be obtained from validated sperm DNA fragmentation assays [5].

A suitable sperm DNA integrity assay relies not only on its discriminative power to predict fertilization failure but also on its capacity to help clinicians in the choice of therapeutic procedures [5].

A number of studies have investigated the relationship between human sperm DNA damage and semen parameters, such as concentration, morphology, and motility [6]. In several different study populations, using different assays to measure DNA damage, investigators found associations between some semen parameters and sperm DNA integrity whereas several others did not find associations [7].

The analysis of human semen has been formerly carried out mainly when a couple had infertility problem and the women had been found more or less free form pathology that could explain the barren union. This and other forms of bias in the selection of men have given most clinician and scientists a wrong vie of the normal values for various semen variables.

Only analysis of semen specimens from men who are representative of the normal male population should be used to assess the normal limits.

Such limits are generally defined as equal to the 95% confidence limits which can be calculated in different ways. There is no reason to believe that the limits for normal are the same for semen from young and older men.

Nor should we accept the normal limits to the same in all countries or among all ethnic groups. The limits for normal should not be linked with fertility since it is well documented that an abnormal semen sample is not necessarily incompatible with fertility.

Hence, it is programmed the role of sperm nuclear and DNA interfrity status and semen quality of fertile and infertile men.

MATERIALS AND METHODS:

Study groups: The study was done on 50 infertile men and 50 fertile men was selected from couples attending the infertility clinic with a history of infertility of ≥1 years and 75 healthy volunteers of proven fertility (initiated a successful pregnancy) served as the control group. The study protocol was approved by the ethics committee of Nandita Fertility and Research Centre. Written informed consent was obtained from each participant. All the clinical diagnosis was analyzed in Nandita Fertility and Research Centre, Chetpet, Chennai, Tamil Nadu, India.

Standard semen analysis: In all cases, after 2-6 days of sexual abstinence, semen samples were collected. All samples for evaluation, were allowed to liquefy for at least 30 minutes at 37°C and then evaluated for sperm concentration, motility, and morphology, Round cells, Normal DNA integrity and leukocyte concentration according to the guidelines of the World Health Organization guidelines [8].

Morphology smears were scored using the Kruger's Strict Criteria [9]. Sperm parameters were considered normal when sperm concentration was ≥20×106/ml, motility was ≥50% and normal sperm forms were ≥15%. Motility was expressed at the percentage of progressive and Non-progressive spermatozoa. Sperm morphology was assessed on smears with the Giemsa staining (Merck Chemical spermatozoa with a normal morphology were determined by assessing 100 sperms under oil immersion with magnifications of X1000 under bright field illumination. The exclusion criteria were the presence of < 10 × 106 / mL total motile spermatozoa in the original (post–liquefaction) sample, azoospermia and severe oligospermia. No subjects in either group were smokers, on medication, had a history of exposure to chemotherapy or radiation, or a varicocele.

Acridine Orange staining: For assessment of sperm DNA integrity, the smears were air-dried for 1hour and then fixed overnight in freshly made Carnoys solution (One part glacial acetic acid, three parts methanol) at 4^{o}C. The slides were rinsed 2 times with distilled water and dipped in McIvaine phosphate citrate- buffer (pH 4) for 5 minutes.

Each sample was then stained with freshly prepared acridine orange (0.19mg /ml Sigma Chemical Co., St. Louis, USA) in McIvaine phosphate citrate- buffer for 10 minutes in the darkness. The preparation were washed with distilled water, covered with glass cover slips and assessed on the same day using fluorescent microscope (Zeiss, Oberkochen, Germany,) with a 460 nm-filter. The duration of illumination was limited to 40 seconds per field.

The percentage of green (normal DNA integrity) and orange-red (abnormal DNA integrity) spermatozoa per 100 spermatozoa in each sample was calculated by a same single person. An abnormal integrity of sperm nuclear DNA was considered as more than 30% denaturation (orange –red spermatozoa on acridine orange staining).The DNA Fragmentation Index (DFI), which is the ratio of the orange-red to the total (orange red+ green) fluorescence intensities of spermatozoa, was also calculated for the samples.

Statistical analysis: Data are reported as Mean ± S.E. The comparisons between two groups were tested by Students *t*-test using SPSS13. Correlation between two continues outcomes were evaluated using Pearson correlation co-efficient. $p<0.05$ was considered as statistically significant.

RESULTS: The mean of participants' age, semen analysis parameters in two groups are summarized in **Table 1**. As shown in the Table 1, There are no significant differences in the mean of participants' age in two groups but the mean of sperm concentration and sperm motility of fertile males were significantly higher than that of infertile males ($p<0.001$).

The means of sperm concentration in fertile males was significantly higher than infertile males (62.7±18.7 million vs. 36.7±25.40 million). Total sperm motility of fertile males also was significantly higher compared to infertile (56.32± 0.07% vs. 28.8±10.7%).

Normal sperm morphology of fertile males was also significantly higher compared to infertile (24.5±8% vs. 12.87± 4.6%). Also, the correlation between DNA fragmentation Index and Sperm parameters in fertile and unfertile men were shown in **Table 2**.

TABLE 1: SEMEN PARAMETERS AND SPERM DNA INTEGRITY FOR 550 EJACULATES OF FERTILE AND INFERTILE MEN

***Semen parame*ters**	**Fertile group (n= 50) (Mean± SE)**	**Infertile group (n=50) (Mean ±S.E)**	**p-value**
Age (y)	35.8 ±0.067	37.6±0.98	NS
Volume (mL)	3.5±0.98	3.2 ±0.08	NS
Sperm concentration x 10^6	62.7±18.7	36.7±25.40	<0.001
Porgressive motility %	56.32±0.07	28.8±10.7	<0.001
Normal morphology %	24.5±0.08	12.87±4.6	NS
Round cells x 10^6	0.4 ±0.07	0.6 ± 0.23	NS
Normal DNA integrity %	65.7 ±0.08	54.5 ±0.45	NS

TABLE 2: CORRELATION BETWEEN DFI AND SPERM PARAMETERS IN FERTILE AND UNFERTILE MEN.

	DFI (DNA Fragmentation Index)	
Semen parameters	**fertile group**	**infertile group**
Sperm concentration	-0.24 (.15)	-0.26 (.22)
Porgressive motility	-0.7 (.75)	-0.9 (.75)
Normal morphology	-0.02(.540	0.12(.35)

Values are Correlation Coefficient (p)

DISCUSSION: This study indicate that sperm DNA damages in infertile males is significantly higher than fertile males and sperms with abnormal morphology and low levels of motility has more abnormal DNA damages than motile and normal sperms. Our results have provided evidence of an important relationship between some of semen parameters and sperm DNA damage. Normozoospermics had a lower percentage of sperm with DNA damage compared with infertile group. Our finding of negative relationship between DNA damage and semen quality is supported by data from other laboratories. For example, the evaluation between semen parameters

and DNA integrity among a group of infertile patients and a group of normozoospermic donors [7], they showed that semen parameters, especially sperm concentration, were inversely correlated with the comet assay parameters and demonstrated a significant negative correlation between semen parameters and DNA damage in sperm [16]. However, this relationship was not found by who examined DNA fragmentation rates in the spermatozoa of normozoospermic fertile donors and asthenozoospermic infertile patients with modified single-cell gel electrophoresis assay [6], possibly because of differences in composition of their study group [12].

Although the extent of DNA damage is closely related to sperm function and male infertility, the origin of such damage is still largely controversial. It is believed that despite of improper packaging and ligation during sperm maturation and germ cell apoptosis, oxidative stress is an important factor in sperm DNA damage [13]. This is important as mature spermatozoa with DNA damage may exhibit lower functional potential and this may explain the patients' subfertility status.

In clinical practice, the conventional subjective semen analysis using light microscopy still plays the central role in the assessment of fertility in men. However, a definitive diagnosis of infertility cannot often be male solely on the basis of semen analysis result. Although in many circumstances the sperm parameters are impaired in fertile population, there is significant overlap between the semen parameters of infertile and fertile men [14]. Thus, to upgrade the prognostic and diagnostic ability of semen analysis, a sperm function assay should be included. Now days, there are several assays available to assess the sperm nuclear integrity in clinical setting.

However the efficacy or prognostic value of each assay for prediction of fertilization of pregnancy outcome ART (Assisted Reproductive Technologies) is still a matter of controversy. Application of acridine orange fluorescent staining allows the nuclear chromation integrity of the human spermatozoa to be analyzed under either fluorescent microscope or by flow cytometry. This can be used for testing the sperm maturity and predicting the fertilization capacity of ejaculated spermatozoa.

It has been previously reported that a significant difference exists between the proportions of the sperms with impaired DNA integrity in the fertile and the infertile population [15]. There are the negative correlation was reported between the DNA integrity and semen parameters. However our results disagree with the aforementioned studies, the proportion of sperm with a normal DNA integrity in our fertile (normospermic) subjects with impaired semen parameters, but the difference was not significant. Neither could we find any correlation between the status of sperm DNA integrity and the sperm parameters in our patients.

In the earlier studies, the sperm parameters were more defective in samples extracted from the testis comparing with the ones extracted from the ejaculates. This is due to the fact that sperms are immature in the testis and should reach the epididymis to achieve the maturity. Also the fertilization rates were lower with sperms retrieved form testis than either epididymal or seminal spermatozoa. In addition, the fertilization and pregnancy rates were higher in samples with normospermia compared with the abnormal samples with 2 or 3 defects in the sperm parameters [14]. Therefore, it can be concluded that mature sperms from ejaculated of infertile patients are capable of fertilizing oocytes at higher rates comparing with the immature spermatozoa that are retrieved from the testis of infertile subjects, and also, the seminal samples with normal sperm parameters have higher levels of fertilizing potentials than abnormal samples regardless of the source of collection.

Acridine orange test has been successfully used by some laboratories in an attempt to improve the male fertility evaluations; however the predictive values of AOT (Acridine Orange Test) is still controversial [16, 17]. Most of the investigators have noticed a significant relationship between the AOT and the sperm parameters or sperm fertilizing ability, while a negative correlation has also been reported.

As our results showed, AOT may be associated with severe teratospermia or oligoastheno teratospermia. Therefore, we do not recommend the application of AOT as a part of the routine fertility workup.

On the other hand, it should only be used in selected infertile men with severely abnormal sperm parameters. It should be emphasized that the application of other chromatin integrity assays may reveal the precise quality of the spermatozoa [14]. Thus, it may be necessary to apply several chromatin or DNA integrity assays for the elucidation of the precise pathophysiology of male infertility. In addition, as Katayose and associates stated, acridine orange fluorescent staining is very useful in cases with unexplained infertility [15].

This study indicate that sperm DNA damages in infertile males is significantly higher than fertile males and sperms with abnormal morphology and low levels of motility has more abnormal DNA damages than motile and normal sperms. Our results have provided evidence of an important relationship between some of semen parameters and sperm DNA damage.

CONCLUSIONS: In summary, we have demonstrated that there was a negative correlation between sperm motility and morphology in infertile males and sperm DNA damage. Also there was negative correlation between sperm motility and DNA damage in infertile males. According to these results, it is concluded that testing sperm DNA damage is helpful in selection of spermatozoa with the least amount of damage for use in assisted conception. In turn, this may alleviate the financial social and emotional problems associated with failed ART attempts.

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