Sperm Nuclear DNA in Ejaculates of Fertile and Infertile Men
Correlation with Semen Parameters

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Introduction: Our aim was to compare the nuclear DNA integrity of the spermatozoa from infertile men with abnormal semen parameters with that from normospermic fertile men, and to evaluate the relationship between the sperm DNA integrity and semen parameters.

Materials and Methods: Thirty ejaculate samples with abnormal semen analysis and 30 ejaculates with normal semen parameters were randomly collected from infertile and fertile men, respectively. The acridine orange test was used to assess the integrity of sperm DNA.

Results: The number of ejaculates with a DNA Fragmentation Index (DFI) above 30% were 16 (53.3%) and 22 (73.3%) in fertile and infertile subjects, respectively ($P = .10$). The mean DFI was 37.7 ± 19.6% and 46.1 ± 16.7% in the fertile and infertile subjects, respectively ($P = .24$). The DFI of the fertile men with normal sperm morphology (12 patients) ranged from 1% to 80%. In the samples with oligoasthenospermia, mean DFI was 52.7 ± 17.9%. There were 2 samples with severe teratospermia (normal morphology less than 4%) and DFIs of 70% and 87%. There were no significant correlations between the DNA integrity and the 3 parameters of semen quality in our 60 subjects.

Conclusion: Our results failed to show any significant difference in the DNA integrity of the spermatozoa between infertile and fertile men. Also, no correlation was noticed between the DNA abnormality and the semen parameters in the studied samples.

Keywords: DNA, spermatozoa, acridine orange, semen analysis

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INTRODUCTION

Conventional semen analysis is still one of the most popular tests for the evaluation of male fertility. However, in many circumstances, semen analysis per se is unable to predict the fertilizing capacity of ejaculated spermatozoa, because it does not assess some factors such as the integrity of sperm nuclear DNA.[1-3] It has been reported that some of the infertile men are normospermic; while fertilization has occurred in others with an abnormal semen parameters.[4] Therefore, sperm function tests like sperm nuclear maturation assay have been developed to assess the fertility capacity of individuals in assisted reproductive technologies (ART). It is now well understood that the integrity of sperm DNA is an essential factor for normal transmission of genetic materials during the process of fertilization as well as embryo development.[1,3,4] Therefore, it is essential to develop accurate diagnostic tests that provide better prognostic capabilities than the conventional sperm assessments. At present, several assays are available for

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the evaluation of the nuclear chromatin integrity or the maturity of human spermatozoa.[4,5] One of these tests which was first introduced by Evenson and colleagues in 1980 is acridine orange test (AOT).[6]

In human testis, the histone nucleoprotein is replaced by protamine during spermiogenesis. Protamine is rich in cysteine and also other basic amino acids. The structure of the sperm nuclear chromatin becomes stable following transformation to DNA-protamine complex.[2,3,7] During the epididymal passage, the thiols of cysteines in protamine are oxidized to disulfide bonds (S-S). This process will make the spermatozoa more stable than testicular sperm. At ejaculation, sperms are matured enough for the next step to occur—fertilization. The maturity of sperm nuclei (rich in S-S) can be determined by the use of AOT. The acridine orange molecules are intercalated into double-stranded DNA and green fluorescence is emitted from the sperm nuclei. However, with immature nuclei (poor S-S), sperm DNA is easily denatured into single strands. Consequently, the acridine orange molecules aggregate in the nuclei and the color of the fluorescence changes into orange-red.[8]

It has previously been demonstrated that significant differences exist in the proportion of the spermatozoa with impaired DNA integrity between fertile and infertile populations.[3,9] Also, it has been demonstrated that human spermatozoa with impaired chromatin structure, detected by AOT, are not able to penetrate the zona pellucida and fuse with the oolemma of the hamster oocytes.[10] This indicates that a sperm chromatin abnormality seems more likely to affect the fertilization process. Also, in another clinical study, it has been shown that in individuals with immature sperm nuclei, DNA is easily denatured into single strands, thus reducing the chance of a successful in vitro fertilization (IVF).[1] A correlation between the staining of sperms by acridine orange and sperm morphology has also been found.[11]

Although most studies have supported the significant correlation between the acridine orange fluorescence capability and the in vitro fertilizing ability of human spermatozoa, a negative correlation has also been reported.[12] In addition, there have been controversial issues in terms of the relationship between the integrity of sperm DNA and semen parameters and whether measurements of DNA integrity can differentiate the spermatozoa of fertile and infertile men.[1,3] It is still unclear whether the widely used DNA integrity tests are sensitive enough to reveal the differences in baseline sperm DNA integrity between the semen collected from fertile and infertile populations. The purpose of this cross-sectional study was first, to compare the nuclear DNA integrity of spermatozoa using AOT in ejaculates of infertile patients with abnormal semen parameters with that of normospermic fertile people, and second, to evaluate the correlation between the integrity of sperm DNA and semen parameters (sperm concentration, motility, and morphology) in both groups of fertile and infertile men.

MATERIALS AND METHODS

Patients

This cross-sectional study included a randomly selected group of 30 Iranian men with a history of infertility presented to our clinics. They were diagnosed with male factor infertility, and their wives were reported to have normal reproductive function following gynecological and endocrinological examinations. Infertile patients with normospermia were excluded. Also, 30 randomly selected married fertile men were served as controls. They had normal semen parameters and presented with other complaints. All subjects provided informed consent and the study was approved by our university's research and ethics committees. Every single patient was asked to collect a fresh ejaculated semen sample into a sterile wide-mouth container.

Semen Analysis

All semen analyses were performed after the liquefaction of the semen samples within 15 to 20 minutes. Using the WHO criteria (sperm count, $> 20 \quad 10^6$/mL; normal sperm motility, $\geq 50\%$; and normal sperm morphology, $\geq 30\%$), the samples were categorized into normospermic and those with single, double, and triple defects in spermatozoa.[13] Following macroscopic evaluation of each specimen, sperm count and motility were evaluated using Makler Chamber and light microscopy at $\times$ 200 magnification. Motility was expressed as the percentage of progressive and nonprogressive spermatozoa. Sperm morphology was assessed on

smears with the Giemsa staining (Merck Chemical Co, Darmstadt, Germany). The percentage of spermatozoa with a normal morphology was determined by assessing 100 sperms under oil immersion with magnification of × 1000 under bright-field illumination.[2,14]

Acridine Orange Staining

For assessment of sperm DNA integrity, the smears were air-dried for 1 hour and then fixed overnight in freshly made Carnoy's solution (one part glacial acetic acid, three parts methanol) at 4ºC. The slides were rinsed 2 times with distilled water and dipped in McIlvaine phosphate-citrate buffer (pH = 4) for 5 minutes. Each sample was then stained with freshly prepared acridine orange (0.19 mg/mL; Sigma Chemical Co, St Louis, USA) in McIlvaine phosphate-citrate buffer for 10 minutes in the darkness.[15] The preparations 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).[16] 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 Analyses

For statistical analyses, SPSS software (Statistical Package for the Social Sciences, version 9, SPSS Inc, Chicago, Ill, USA) was used. The chi-square test and the Fisher exact test were used to compare the frequencies and Student *t* test to compare quantitative values. The Pearson correlation test was used to evaluate the relationship between sperm DNA integrity and sperm parameters. A *P* value less than .05 was considered significant.

RESULTS

Overall, semen samples of 30 infertile and 30 fertile men were assessed. The mean sperm concentration, motility, and normal morphology were significantly higher in the fertile subjects. The mean values of semen parameters are presented in Table 1. Also, the viscosity of the seminal specimens were abnormally high in 4 (13.3%) and 2 (6.7%) patients in the fertile and infertile groups, respectively (*P* = .67). Nineteen men (63.3%) in the infertile group had asthenoteratospermia, 7 (23.3%) had asthenospermia, and 4 (13.4%) had oligoasthenoteratospermia. Therefore, the majority of our patients suffered from abnormal progressive motility.

The number of ejaculates with denatured sperm DNA (DFI) above the normal value of 30% were 16 (53.3%) and 22 (73.3%) in fertile and infertile subjects, respectively (*P* = .10). The mean DFI was 37.7 ± 19.6% and 46.1 ± 16.7% in the fertile and infertile subjects, respectively (*P* = .24). The mean percentage of green fluorescent sperm in AOT was 62.4 ± 28.8% and 53.8 ± 24.3% in the fertile and infertile subjects, respectively (*P* = .22). A total of 4 (13.3%) and 1 (3.3%) ejaculates were presented with over 97% double-stranded-DNA sperms (green staining) in the fertile and infertile groups, respectively (*P* = .35). Interestingly, the mean DFI of the fertile men with a normal sperm morphology (12 patients) was 32.2 ± 8.3% (range, 1% to 80%), and 6 of them had a DFI greater than 30%.

Table 1. Semen Parameters and Sperm DNA Integrity for 60 Ejaculates of Fertile and Infertile Men*

Semen Parameters	Fertile Group	Infertile Group	*P*
Volume, mL	3.8 ± 1.6 (1 to 8)	3.5 ± 1.8 (1 to 7)	.46
Sperm concentration, × 10^6	81.5 ± 36.0 (38 to 180)	55.1 ± 36.3 (3 to 170)	.006
Progressive motility, %	60.3 ± 7.5 (50 to 73)	19.3 ± 16.4 (2 to 47)	< .001
Nonprogressive motility, %	10.3 ± 2.6 (5 to 17)	16.3 ± 7.1 (7 to 38)	< .001
Normal morphology, %	52.5 ± 11.7 (38 to 78)	22.6 ± 12.4 (2 to 48)	< .001
Round cells, × 10^6	0.3 ± 0.1 (0 to 0.4)	0.52 ± 0.9 (0 to 0.3)	.36
Normal DNA integrity, %	62.4 ± 28.0 (19 to 100)	53.8 ± 24.3 (12 to 98)	.22

*Values are demonstrated as means ± standard deviations (ranges).

In the samples with oligoasthenospermia, the mean percentage of green sperms was 48.25 ± 6.8% (range, 28% to 71%) and the mean DFI was 52.7 ± 17.9%. In addition, there were 2 samples with severe teratospermia (normal morphology less than 4%) and DFIs of 70% and 87%.

There were no significant correlations between the DNA integrity (demonstrated by the green staining of the sperm nucleus in AOT) and the 3 parameters of semen quality in our 60 subjects (Table 2).

Table 2. Correlations Between DFI and Sperm Parameters in Fertile and Infertile Men*

	DFI	
Semen Parameters	**Fertile Group**	**Infertile Group**
Sperm concentration	-0.23 (.21)	-0.29 (.12)
Progressive motility	-0.02 (.91)	-0.05 (.80)
Normal morphology	-0.06 (.74)	-0.15 (.44)

* Values are correlation coefficient (*P*). DFI indicates DNA Fragmentation Index.

DISCUSSION

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 made solely on the basis of semen analysis results. Although in many circumstances the sperm parameters are impaired in infertile population, there is a significant overlap between the semen parameters of infertile and fertile men.[3,16] Thus, to upgrade the prognostic and diagnostic ability of semen analysis, an sperm function assay should be included. Nowadays, 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 or pregnancy outcome following ART is still a matter of controversy. One of the standard assays is AOT, which is quick and easy to be performed at clinical andrology laboratories. Application of acridine orange fluorescent staining allows the nuclear chromatin integrity of the human spermatozoa to be analyzed under either fluorescent microscope or by flowcytometry. This can be used for testing the sperm maturity and predicting the fertilization capacity of ejaculated spermatozoa.[16] Hoshi and colleagues showed that men who had ejaculates with more than 50% green sperms in AOT (double-stranded DNA) had a significantly higher fertilization capacity for IVF compared with men with immature sperms.[17] Therefore, it seems that sperm chromatin abnormality affects the fertilization process in clinical setting.

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 populations.[9,16,18] Zini and coworkers studied the prevalence of sperm DNA denaturation by AOT among 13 fertile and 88 infertile men. They observed that the rate of DNA denaturation was significantly lower among infertile men with normospermia compared with infertile patients with abnormal spermatozoal parameters (11.1% versus 23.1%, respectively). Thus, a negative correlation was reported between the DNA integrity and semen parameters.[16] However, our results disagree with the aforementioned studies; the proportion of sperms with a normal DNA integrity in our fertile (normospermic) subjects was slightly higher than that in infertile 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. The rate of sperm DNA integrity was not consistent among the fertile men with normal semen parameters. For example, 54% of the fertile men had a sperm DFI over 30%. In addition, samples with normal sperm morphology revealed a 32.2% DFI which is above the normal range. Evaluation of sperm nuclear integrity with AOT and fluorescent microscopy, as used in our study, is a routine procedure in andrology units; however, usage of flowcytometry is more efficient as stated by Erenpreiss and coworkers.[9] But, flowcytometry is an expensive technique and also time consuming, thus, less practical for daily evaluation of sperm DNA in ART.

In our previous studies, we noticed that 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 from 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.[3,19] Therefore, it can be concluded that mature sperms from ejaculates 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.

It should be emphasized that normal DNA integrity of spermatozoa is very important in intracytoplasmic sperm injection (ICSI), because the sperm selection is performed by the embryologist on the basis of the motility and morphology status. Bungum and colleagues observed significantly higher DFI levels in successful ICSI cases compared with those in successful IVF or intrauterine insemination cases.[20] Therefore, the highest level of sperm DNA damage was detected in men with the poorest semen quality (ICSI candidates). The authors concerned the safety of the ICSI procedure since damaged DNA is often observed more frequently among ICSI candidates. Developing assays for detecting and selecting spermatozoa with intact DNA for microinjection during ICSI procedure will be the future challenge. Our results showed that none of the sperm parameters correlated with DNA integrity of the spermatozoa;[18] therefore, we cannot predict the outcome of the ICSI cycles solely on the basis of sperm parameters such as sperm motility and nonstained sperm morphology.

Acridine orange test has been successfully used by some laboratories in an attempt to improve the male fertility evaluations; however, the predictive value of AOT is still controversial.[1,12,16,20-22] Most 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.[12] 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.[23] 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.[11] We, however, have not investigated the DNA integrity of such cases so far.

CONCLUSION

Our study showed that the relationship between DNA integrity status and the sperm quality is a rather complex issue. Although more DNA abnormalities were detected among infertile cases with abnormal sperm parameters, the difference was not significant when compared with ejaculates of fertile controls. However, defects in sperm DNA integrity were associated with severe cases such as oligoasthenospermia or severe teratospermia. Additional large-scale trials are needed to confirm the predictive value of sperm chromatin integrity using AOT for the outcome of ART cycles.

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CONFLICT OF INTEREST

None declared.

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