Comparison Between Different DNA and Conventional sperm parameters in Infertile Men

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Abstract

Background: The discriminative power of the classical WHO parameters in relation to male fertility is quite low, because they only address few aspects of sperm quality and function. This has led investigators to focus their attention on the male gamete and in particular its genome.

Objective: To explore which of the sperm DNA damage parameters measured by comet assay are more reliable, and their relations with the standard semen parameters.

Methods: Study was done on 40 infertile men selected from couples attending the Institute of Embryo Reasearch and Infertility Treatment at Al-Kadhimiya City/ Baghdad in the period between February 2009 and May 2009, with a history of infertility of ≥1 years; and 15 healthy volunteers of proven fertility serving as control. Samples were allowed to liquefy for at least 30 minutes at 37°C and then evaluated according to the guidelines of the World Health Organization, 1999. The single cell gel electrophoresis (comet) assay was studied in the ejaculated spermatozoa. The exclusion criteria were the presence of azoospermia and severe oligospermia. Results were compared with the standard semen characteristics (concentration, motility and morphology).

Results: Compared to healthy volunteers, infertile patients had highly significantly higher values of comet parameters (P = 0.00001). In all infertile patients, comet extent did not correlate with any of the classical semen parameters; whereas, tail length (μm), % DNA in tail, tail moment and olive moment had all significant negative correlations with the standard parameters. Significant positive correlations were observed between the studied comet parameters, except for the comet extent which was not significantly correlated with tail length and % DNA in tail (P = 0.06, P = 0.7; respectively).

Conclusion: Comet assay is a very useful technique in assessing sperm DNA damage. Comet tail parameters and comet extent, may clarify different aspects of DNA damage, and together give a better insight to the integrity of the male genome.

Keywards: sperm, comet extent, % DNA in tail, tail moment and olive moment.

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Introduction

Diagnosis of male infertility has mainly been based on the traditional semen parameter's concentration, motility and morphology; although none of which are sufficient for the determination of male fertility capacity or for evaluation of the fertility potential of a couple. These WHO parameters only address few aspects of sperm quality and function and this may explain why the discriminative power in relation to fertility is quite low[1]. New markers are needed that might better discriminate infertile from fertile men and predict pregnancy outcome and the risk of adverse reproductive events[2].

Together with improvements in our understanding of the cell and molecular biology of human sperm, this situation has led investigators to focus their attention on the male gamete and in particular its genome[3]. During the last couple of decades, numerous sperm DNA integrity tests have been developed. These are claimed to be characterized by a lower intraindividualvariation, less intralaboratory and interlaboratory variation and thus less subjective than the conventional sperm analysis[1]. A variety of assays have been developed to measure sperm DNA damage. The use of these tests has been driven largely by the growing use of assisted reproductive technologies (ARTs) and the concern that the integrity of the sperm genome is of importance in this context[4]. Even though during micromanipulative IVF treatment, oxidative damage to sperm DNA does not preclude fertilization[5], several authors have reported significant correlations between sperm DNA damage and fertilization[6]. Moreover, sperm DNA status is an essential prerequisite to the achievement of a successful pregnancy[7].

Sperm DNA damage can be measured directly (fragmentation, oxidation) or indirectly (sperm chromatin compaction). Direct assessment of

DNA damage can be obtained by means of single-cell gel electrophoresis assay or "Comet" assay. Comet assay is a fluorescence microscopic test in which sperm cells are mixed with melted agarose gel and then placed on a glass slide. The cells are lysed and then subjected to horizontal electrophoresis. DNA is visualized with the help of a DNA-specific fluorescent dye. Electrophoresis causes DNA fragments to migrate away from the central DNA core, revealing a "comet", and DNA damage is quantified by measuring the displacement between the genetic material of the nucleus comet head and the resulting tail[1, 2].

There is evidence to suggest that markers of sperm DNA integrity may be better measures of male fertility potential than conventional measures, but larger studies are needed to define the clinical value of testing sperm DNA integrity[2]. The aim of the present study is to explore the relations between different DNA damage parameters measured by comet assay(Comet Length, Tail Length, %DNA in Tail, Tail Moment and Olive Moment), and standard semen parameters (Concentration, Motility and Morphology), and to inspect which of these comet parameters are more reliable when differentiating between fertile and infertile men.

Methods

Study groups

The study was done on 40 infertile men selected from couples attending the Institute of Embryo Reasearch and Infertility Treatment at Al-Kadhimiya City/ Baghdad in the period between Fabruary 2009 and May 2009, with a history of infertility of ≥1 years; and 15 healthy volunteers of proven fertility (initiated a successful pregnancy) served as the control group. Patients age was 35.3 ± 4.2 years (mean ± SD); while control age was (32.2 ± 5.4 years).The study protocol was approved by the local research ethics committee of Al-Nahrain College of medicine. Written informed consent was obtained from each participant.

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 according to the guidelines of the World Health Organization (WHO, 1999)[8]. Sperm parameters were considered normal when sperm concentration was $\geq 20\times10^6$/ml, motility was ≥50% and normal sperm forms were ≥30%. The exclusion criteria were the presence of azoospermia and severe oligospermia. No subjects in either group were on medication, had a history of exposure to chemotherapy or radiation, or a varicocele.

Determination of DNA integrity using a modified alkaline single cell gel electrophoresis (Comet) assay:

The modified alkaline single cell gel electrophoresis (Comet assay) for determination of sperm DNA integrity was carried out according to the procedure of McKelvey-Martin et al., 1997[9], as follows: Fully frosted microscope slides were covered with 1% normal melting point agarose (Sigma–Aldrich, Italy). About 10 µl of human sperm in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS; Sigma–Aldrich) were mixed with 85 µl of 0.5% low melting point agarose (Agarose wide range; Sigma–Aldrich) at 37°C, under yellow light to prevent further induced damage to DNA. This cell suspension was rapidly pipetted on top of the first agarose layer, covered with a coverslip and allowed to solidify at 4°C for 5 min. A final layer of 0.5% low melting point agarose was added to the slide and allowed to solidify at 4°C for 10 min. The cells were then lysed by immersing the slides in a coplin jar containing freshly prepared cold lysis solution (2.5 mol/l NaCl, 100 mmol/l Na_2 EDTA, Tris 10 mmol/l, 10% DMSO with 1% Triton X-100 (pH 10; Sigma–Aldrich) for at least 1 h at 4°C. Then slides were incubated overnight at 37°C with 100 µg/ml proteinase K (Sigma–Aldrich) in order to remove protamines that otherwise impede DNA migration through the agarose.

A horizontal gel electrophoresis tank was filled with alkaline electrophoresis solution (300 mmol/l NaOH, 1 mmol/l EDTA, pH 12.5) at room temperature. The slides were placed into this tank side by side with the agarose end facing the anode and with the electrophoresis buffer at a level of ~0.25 cm above the slide surface. The slides were left in this high pH buffer for 20 min to allow DNA to unwind. The DNA fragments were then separated by electrophoresis for 10 min at 25 V adjusted to 300 mA. After electrophoresis the slides were flooded with two changes of neutralization buffer (0.4 mol/l Tris, pH 7.5) for 5 min each. This removed any remaining alkali and detergents, which could have interfered with staining. The slides were

drained before being stained with 100 µl of 20 µg/ml ethidium bromide (Sigma–Aldrich).

Coded slides were viewed using an Olympus BX41 fluorescence microscope equipped with a filter for ethidium bromide visualization. For each sample, 50 randomly selected sperm nuclei were evaluated by an image analysis system using TriTek comet score TM version 1.5 image analysis software. Scored Comet parameters included comet extent (µm), tail length (µm), Tail DNA %, Tail moment , and Olive tail moment. Comet extent is a measure of the total comet length from the beginning of the head to the last visible pixel in the tail. Percent DNA in tail (% DNA in tail) is a measurement of the proportion of the total DNA that is present in the tail. Tail moment equals (percentage tail DNA multiplied by tail length measured from the edge of the comet head). Finally, olive tail moment is a global comet parameter expressed as [(tail mean × head mean) × (% tail DNA/100)] and used to quantify DNA damage. Comet parameters were expressed as mean from the 50 cells scored per sample.

Statistical analysis:

The results were expressed as (mean ± SD). Results of the standard semen characteristics in addition to the results of the different Comet assay parameters were compared between infertile patients and fertile volunteers using unpaired Student's t- test for two samples of unequal variance. The different types of relationships and correlations accomplished in this work were examined using bivariate Pearson's correlation coefficient (two-tailed) test. A probability (P value) of < 0.05 was considered as statistically significant. Analyses carried out with Microsoft Excel/ Microsoft Office XP 1985-2001 and Statistica/ version 6.0 (USA) statistical package.

Results

Conventional (Standard) Semen Analysis:

The mean of participants' age, semen analysis parameters and comet values in two groups (infertile men and control donors) are summarized in Table 1. As shown in the Table 1, , infertile patients had significantly reduced sperm progressive motility percentage(22.18 ± 11.39% vs. 63.4 ± 11.21%; $P = 0.00001$), poorer sperm morphology percentage(25.27 ± 17.05% vs. 55.82 ± 15.69%;) and lower sperm count(39.5 ± 34.35×10^6/ mL vs. 135.38 ± 109.87×10^6/ mL; $P = 0.005$)as compared to controls (Table 1).

Comet Assay Results:

As compared to healthy volunteers, infertile patients had highly significantly higher values of comet parameters ($P = 0.00001$); these include higher comet extent (94.73 ± 35.62µm vs. 53.29 ± 6.89µm);tail length (44.45 ± 15.54µm vs. 10.62 ± 1.89µm); percentage of DNA in tail (30.12 ± 27.87% vs. 4.72 ± 2.97%); tail moment (10.27 ± 9.98µm vs. 0.53 ± 0.31µm); as well as olive moment (11.07 ± 10.12 vs. 0.86 ± 0.49); (table 1).

Figures 1 and 2demonstrate photographs of single cell gel electrophoresis (Comet assay) of sperm cells stained with ethidium bromide, displaying an intact sperm cell from a fertile volunteer without DNA fragmentation, as revealed from an intact nucleus without a comet tail (figure 1). On the other hand, a photograph of sperm cells from an infertile patient showing variable degrees of nuclear DNA damage as revealed by changes in DNA migration in the comet tail (figure 2).

Correlations Betweenthe Different Studied Parameters:

Table 2 demonstrates different types of correlations between different comet parameters and standard semen characteristics. In all infertile patients, comet extent did not correlate with any of the classical semen parameters. However, there was a significant positive correlation with tail length and olive moment, highly with the former ($P = 0.00001$, $P = 0.02$; respectively). On the other hand, tail length (µm) had significant negative correlations with the three studied standard parameters (higher with sperm morphology, then progressive motility and finally with sperm count) ($r = – 0.5$, $P = 0.001$; $r = – 0.4$, $P = 0.009$, and $r = – 0.39$, $P = 0.013$; respectively). There was highly significant positive correlation between tail length and all other comet parameters ($P = 0.00001$) (table 2).

Concerning percentage of tail DNA, again significant negative correlations was obtained with the three standard semen parameters (highly with sperm count, then sperm morphology and finally with progressive motility) ($r = – 0.6$, $P = 0.00001$; $r = – 0.5$, $P = 0.003$, and $r = – 0.4$, $P = 0.014$; respectively). %DNA in the tail again had highly significant positive correlations with all studied comet parameters, apart from less significant correlation with tail moment and

absence of correlation with comet extent (P = 0.001, P = 0.7; respectively) (table 2).

Significant negative correlations were obtained between both tail moment as well as olive moment with the studied standard semen parameters, again higher for the sperm count (P = 0.001, P = 0.00001; respectively), then sperm morphology (P = 0.017, P = 0.001; respectively) and finally progressive motility (P = 0.027, P = 0.17; respectively) (table 2). Table 2 shows highly significant positive correlation between any of both these parameters with all other tested comet parameters, except with comet extent which was less significant for olive moment and near significance for tail length (P = 0.017, P = 0.06; respectively).

Discussion

Comet assay which was first described by Singh et al.[10]is a sensitive technique that detects the presence of DNA strand breaks and alkali labile damages in the individual cells. The DNA fragments migrate towards the anode pole at the rate inversely proportional to the size of the fragment during electrophoresis[11]. When comparing across the various techniques used to measure DNA integrity, comet assay was found to have the highest resolution for distinguishing between infertile and fertile men[12].In this study we aimed at comparing the levels of sperm DNA damage measured by single cell gel electrophoresis (comet assay) between infertile males and fertile volunteers; and correlating different comet DNA damage parameters with classical semen parameters in infertile males.

Our results demonstratethat infertile patients had a highly significantly more DNA damage than fertile controls, as represented from the higher values of comet parameters: comet extent; tail length; percentage of DNA in tail; tail moment; as well as olive tail moment; (P = 0.00001); which is concordant with previous studies[11, 12, 13, 14].The sperm DFI values for asthenozoospermic, oligoteratozoospermic, asthenoteratozoo-spermic, and oligoasthenoteratozoospermicsemen samples were significantly higher than that observed in normozoospermic semen samples (p<0.05)[14]. In their study, Sheikh et al., indicated that sperm DNA damage 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[11].Wide spectra of sperm DNA damage were found both within and between an unselected group of 60 men undergoing IVF treatment as was measured by single cell gel electrophoresis (Comet assay)[15].

This work also reveals inverse correlations between various comet parameters and standard semen parameters; which were significant correlations for all parameters apart from the comet extent (see results). Different studies have demonstrated different relations between various comet parameters and classical semen profile:Morris et al. studied 60 men participating in IVF treatment and found a highly significant increase in DNA damage as the sperm count and the proportion of morphologically normal forms decreased[15]. Higher degree of DNA damage in oligoasthenoteratozoospermic (OAT) category of infertile patients was attributed to the triple effect of impaired semen parameters (count, motility and morphology) in the OAT category compared to other infertile groups[16]. Tomsu et al. found an inverse relationship between total sperm concentration and the comet length and moment of prepared sperm. Apart from that, there was no correlations between other standard semen profiles and comet assay parameters[3].Trisini et al. found that cells with high DNA damage (CHD) had the most consistent inverse association with semen parameters, followed by comet extent and % DNA in tail[17]. According to Sheikh and his associates, sperm DNA damage assessed by Comet assay demonstrated a very clear negative relationship with sperm motility and morphology[11].Finally, investigators in several other studies did not find consistent relationships between conventional semen parameters and measures of DNA damage, suggesting that measures of sperm DNA damage are independent of semen parameters[18, 19].

In the present study, significant positive correlations were noticed between all the measured comet parameters, except for the comet extent which has an insignificant correlation with tail length and DNA % in tail (table 2). Various studies displayed diverse results concerning comet assay parameters. In some protocols, the presence or absence of a Comet tail is scored, some semen samples producing hardly any `Cometed' sperm[20]; whereas in other protocols, all sperms produced a Comet, so allowing the images to be analysed for % tail DNA, tail length or tail

moment[12, 19]. Irvine and coworkers rely on results from only the percentage tail DNA measurements, presumably as they claim, because this measure gave better discrimination in their analysis[12]. Morris et al. have chosen to present their data as tail length and tail moment only, because these measures exaggerated the differences between patients. They found that differences in DNA damage between ejaculates were most impressive using the Comet tail moment, perhaps, as both the length of migration of the DNA and the amounts released from the head are independently increased by DNA damage[15]. The tail moment is considered to be one of the best indices of comet formation obtained in computerized analysis[21].

To their surprise, Tomsu et al. found no significant correlation between the comet head parameters (mean head densityand integrated head density) and tail parameters (moment and length). They stated that these results indicated that DNA damage in the comet head may not necessarily be reflected in the comet tail, perhaps due to different mechanisms of DNA damage[3]. Duty and coworkers claimed that tail moment is purported to be a more sensitive measure of DNA damage than the comet extent and tail distributed moment (TDM). This increased sensitivity results from observations that with increasing levels of DNA damage, the tail% DNA may continue to increase,but, the tail length may not[22], and this could explain the lack of correlation between the comet extent (that measures total comet length from the beginning of the head to the last visible pixel in the tail) and other measured comet tail parameters found in this study.

To conclude, DNA integrity analysis is a relatively independent measure of semen quality. Sperm DNA damage assessment may be valuable among routine tests for infertility investigations. Comet assay is a very useful technique in assessing DNA damage and is an important requirement in men opting for ART. Comet tail parameters and comet extent, may clarify different aspects of sperm DNA damage, and together give a better insight to the integrity of the male genome.

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Table 1: Comparison of semen characteristics and the values of the different comet assay parameters between infertile patients and healthy fertile volunteers

Variables	Patients (n = 50)	Control donors (n = 27)	*P*value
*Sperm Count** ($\times 10^6$/ml)	39.5 ± 34.35	135.38 ± 109.87	0.005
*Progressive motility** (%)	22.18 ± 11.39	63.4 ± 11.21	0.00001
Normal morphology * (%)	25.27 ± 17.05	55.82 ± 15.69	0.00001
Comet Extent (μm)	94.73 ± 35.62	53.29 ± 6.89	0.00001
TailLength (μm)	44.45 ± 15.54	10.62 ± 1.89	0.00001
% DNA in Tail	30.12 ± 27.87	4.72 ± 2.97	0.00001
Tail Moment (μm)	10.27 ± 9.98	0.53 ± 0.31	0.00001
Olive Moment	11.07 ± 10.12	0.86 ± 0.49	0.00001

* According to WHO criteria1999.

Table 2: Correlations between Different Comet Assay Parameters and Standard Semen Parameters of infertile patients

Correlations r value / ***P* value**	***Comet Extent*** (μm)	***Tail Length*** (μm)	***% DNA in Tail***	***Tail Moment***(μm)	***Olive Moment***
Sperm Count* ($\times 10^6$/ml)	0.0758	-0.3906	-0.5867	-0.5017	-0.5555
	0.642	**0.013**	**0.00001**	**0.001**	**0.00001**
Progressive motility* (%)	-0.1855	-0.4074	-0.3842	-0.3488	-0.375
	0.252	**0.009**	**0.014**	**0.027**	**0.017**
Normal morphology * (%)	-0.2662	-0.5112	-0.4546	-0.3739	-0.5022
	0.097	**0.001**	**0.003**	**0.017**	**0.001**
Comet Extent (μm)	---	0.5823	0.0613	0.306	0.376
	---	**0.00001**	**0.707**	**0.055**	**0.017**
Tail Length (μm)	0.5823	---	0.7884	0.5787	0.8796
	0.00001	---	**0.00001**	**0.00001**	**0.00001**
% DNA in Tail	0.0613	0.7884	---	0.5068	0.8297
	0.707	**0.00001**	---	**0.001**	**0.00001**
Tail Moment(μm)	0.306	0.5787	0.5068	---	0.5887
	0.055	**0.00001**	**0.001**	---	**0.00001**
Olive Moment	0.376	0.8796	0.8297	0.5887	---
	0.017	**0.00001**	**0.00001**	**0.00001**	---

* According to WHO criteria1999.

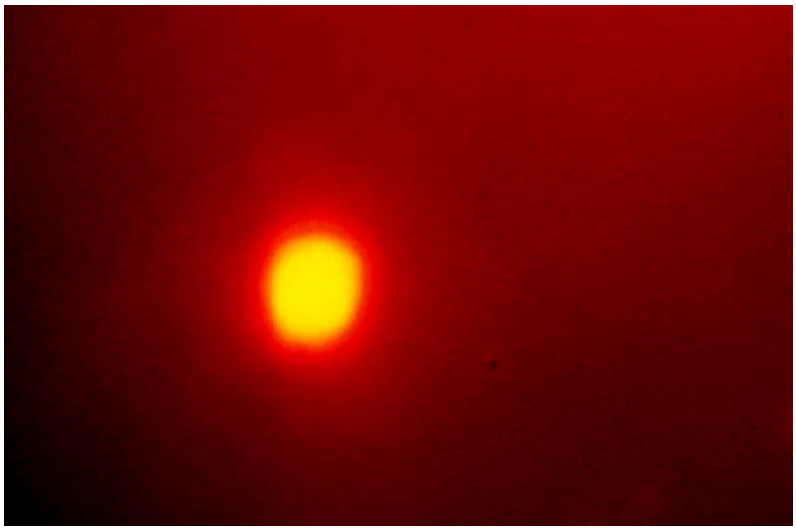

Figure 1: A photograph of single cell gel electrophoresis (Comet assay) of a sperm cell from a fertile volunteer stained with ethidium bromide, showingan intactsperm cellwithout DNAfragmentation.

N.B. Scale bar = 30 µ

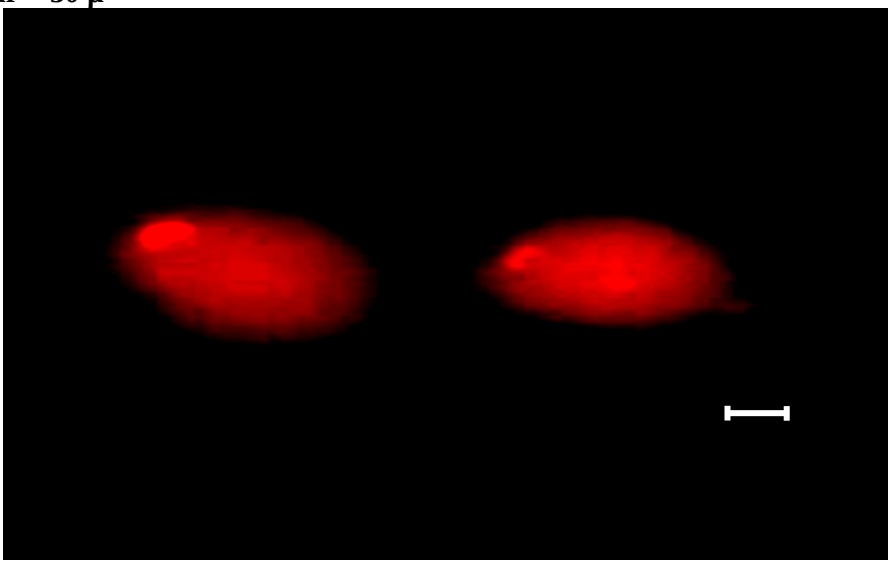

Figure 2: A photograph of single cell gel electrophoresis (Comet assay) of sperm cells from an infertile patient stained with ethidium bromide, showing variable degree of nuclear DNA damage as revealed by changes in DNA migration in the comet tail.
Scale bar = 30 µ

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