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Comet assay: A prognostic tool for DNA integrity assessment in infertile men opting for assisted reproduction

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***Background & objectives*: The growing concern on transmission of genetic diseases in assisted reproduction technique (ART) and the lacunae in the conventional semen analysis to accurately predict the semen quality has led to the need for new techniques to identify the best quality sperm that can be used in assisted procreation techniques. This study analyzes the sperm parameters in the context of DNA damage in cytogenetically normal, AZF non deleted infertile men for DNA damage by comet assay.**

***Methods*: Seventy infertile men and 40 fertile controls were evaluated for the semen quality by conventional semen parameters and the sperms were also analyzed for DNA integrity by comet assay. The patients were classified into oligozoospermic (O), asthenozoospermic (A), teratozoospermic (T), oligoasthenoteratozoospermic (OAT) categories and infertile men with normal semen profile. The extent of DNA damage was assessed by visual scoring method of comets.**

***Results*: Idiopathic infertile men with normal semen profile (n=18) according to conventional method and patients with history of spontaneous abortions and normal semen profile (n=10) had high degree of DNA damage (29 and 47% respectively) as compared to fertile controls (7%). The O, A, T and OAT categories of patients had a variably higher DNA damage load as compared to fertile controls.**

***Interpretation & conclusions*: The normal range and threshold for DNA damage as a predictor of male fertility potential and technique which could assess the sperm DNA damage are necessary to lower the trauma of couples experiencing recurrent spontaneous abortion or failure in ART.**

Key words Assisted reproduction - comet assay - DNA damage - DNA fragmentation index - DNA integrity - infertility - recurrent spontaneous abortion

DNA damage in the male germ line is associated with fertilization failure, impaired pre- and post-implantation development and poor pregnancy outcome[1]. During spermatogenesis nucleoproteins exchange occurs involving replacement of somatic histones by transition proteins and subsequently by protamines[2]. Only 15 per cent histones are retained in the ejaculated sperms. The developing sperms are also succeptible to a number of endogenous and exogenous stress factors, which affect DNA integrity[3]. Recent

studies indicate that DNA integrity of male gamete is one of the most discriminating parameters for fertility assessment[3].

Evaluation of DNA integrity is an important parameter, having more diagnostic and prognostic significance as compared to routine semen analysis for assisted reproduction[4,5]. Till recently, it was believed that motility and morphology of sperm are most important predictors of fertility potential in man, however presence of genetic abnormality (as large aberrations detected cytogenetically, small aberrations detected by molecular techniques like PCR, RFLP, sequencing and DNA breaks assessed by comet) have more profound implications on fertilization, cleavage, embryogenesis and pre- and post-natal growth[6-10].

Sperms have evolved multiple repair mechanisms to prevent DNA damage. Prior to fertilization either the aberrant sperm genome is rectified or if the damage is beyond repair, the sperm is apoptized by a stringently regulated cascade. Post-fertilization, the oocyte actively repairs the sperm DNA damage, however, errors in this repair process would generate mutations that are linked to the increased incidence of dominant genetic diseases and childhood cancer[11].

With increase in number of infertile couples opting for assisted reproduction techniques (ART), the decline in fertility potential (decrease in sperm count at the rate of 2% annually), and growing concern about the transmission of genetic diseases, highlight the need for diagnostic techniques which could asses paternal germ cell DNA damage. The traditional semen analysis focuses on analysis of sperm concentration, motility, and morphology but does not asses quality of DNA. It is also important to analyse the percentage of cells with damaged DNA because if the DNA fragmentation index (DFI) is ≥27-30 per cent, it results in fertilization failure or early foetal loss. In this study we planned to analyze DNA integrity in men with idiopathic infertility who were both cytogenetically normal and did not harbour Yq microdeletions, mitochondrial alterations. Alkaline single cell gel electrophoresis (SCGE) assay or Comet assay was used to evaluate sperm DNA damage in infertile patients.

Material & Methods

Patients: Seventy men with idiopathic infertility attending the infertility clinic Department of Urology, AIIMS, ART Centre of Department of Obstetrics and Gynaecology, All India Institute of Medical Sciences (AIIMS), and ART Centre, Army Research and Referral Hospital, New Delhi from April 2007 to June 2008 and 40, age matched, fertile controls (who had fathered a child in last one year and had normal semen profile) were included in the study after informed consent and permission of institute ethical clearance board. The infertile men had normal endocrinal profile. Patients with abnormal sperm parameters had either one or a combination of sperm parameter defects for motality, concentration or morphology. On this basis these patients were classified into oligozoospermic (O) (sperm concentration < 20 million per ml), asthenozoospermic (A) (progressive motility <50), teratozoospermic (T) (normal sperm morphology < 30) or oligoasthenoteratozoospermic (OAT). Exclusion criterion included infertility attributed to obstructive pathology, infection, medication or trauma. Samples with azoospermia, leukocyte concentration greater then 10^6 / ml and hyper-viscosity were excluded. Infertile men who had a history of smoking, alcohol and any other drug abuse or men with occupational exposure to high temperature, fertilizers or other toxins were excluded from the study.

Cytogenetic analysis by G-banding[12]: Heparinized blood (5 ml) was drawn and the syringe was kept in an upright position at 37°C for 30-45 min. Then, the plasma and the settled lymphocyte (PLS, plasma lymphocyte suspension) in buffy coat (within syringe) is tapped and mixed together gently. The needle was bent and 0.5 ml of PLS was transferred into a sterile culture vial containing 5 ml of media RPMI 1640 and 0.2 ml Phytohaemagglutinin A (PHA). The culture was incubated for 72 h at 37°C. After 70 h of incubation, 0.1 ml (0.2%) of colcemid was added to the cultures. At 72 h (*i.e.,* 2 h after colcimid is added), the samples were transfered to 15 ml centrifuge tube and then centrifuged at 80 *g* for 10 min, the supernatant was discarded and freshly prepared prewarmed hypotonic solution (0.56% KCI) was added and incubated for 20-25 min at 37°C. The cell suspension was centrifuged again and after discarding the supernatant, freshly prepared chilled carnoys fixative (methanol + acetic acid 3:1 v/v) was added to the cell pellet slowly. The cell pellet in carnoys fixative was vigourously mixed and then kept at 4^0 C for overnight. The next day the cell pellet was washed thrice atleast at 80 *g* for 10 min with carnoys fixative, until the pellet becomes pale white. The supernatant was discarded and two drops of cell suspension were dropped from a height on a clean wet slide then slides are kept at 37 ^{0}C for 72 h.

G-banding[13]: Giemsa staining of chromosome preparation after proteolytic enzyme treatment reveal G-banding. The 3 days old matured unstained chromosome preparations (slides prepared and incubated at 37 ^{0} C for about 72 h) were stirred in 0.25 per cent trypsin for 10-15 seconds, and then rinsed in phosphate buffer saline.

The slides were stained in 2 per cent Gimesa stain for 5-7 min, thereafter, washed in, Germany distilled water. Metaphases were observed in (ZEISS microscope) (Germany) using cytovision software supplied by Applied Imaging Corp (USA) and analyzed according to ISCN 1995.

Polymerase chain reaction (PCR) analysis: PCR screening was done for Yq microdeletion. Genomic DNA was isolated from peripheral blood by phenol-chloroform (Organic) method. Each of the patients and fertile controls were examined for 3 AZF (azoospermic factor) loci, mapped to the interval 5 and 6 of the long arm of Y chromosome. The primers used were for AZF a- sY 84; AZF b – sY 127; AZF c- sY 254. The internal control used was SRY (Sex Determining Region)- sY 14, on the short arm of Y chromosome[14]. Fertile male and female samples were used as positive and negative controls and water was used as blank. Samples were subjected to PCR amplification using 40 cycles of 94^0 C for 1 min, 59^0 C for for 45 sec and 72^0 C for 45 sec. Initial denaturation was done at 94^0 C for 3 min and final extension at 72^0 C for 5 min. The PCR products were analyzed on 1.8 per cent agarose gel containing ethidium bromide (0.5 µg/ml).

Semen analysis: Semen samples were obtained by masturbation and collected into sterile containers after a period of 72-96 h of sexual abstinence. Specimens were allowed to liquefy for 30-40 min at room temperature and conventional semen analysis was performed (WHO criterion, 1999)[15] within 1 h of collection. Sperm motility was expressed as the percentage of spermatozoa that showed forward progression. The viability was expressed as percentage of live cells after analysis of minimum of 100 spermatozoa. The morphology was expressed as percentage abnormal sperms, including the head, midpiece and tail defects.

Comet assay: Comet assay was done to assess the DNA integrity of sperms[16]. All chemicals were purchased from Sigma-Aldrich Chemical Co. (USA) unless otherwise stated and the entire process was performed in low light conditions to prevent induced DNA damage.

Frosted microscopic slides (Hi Media, Mumbai 1.2 mm) were covered with 100 µl of 0.7 per cent high resolution agarose in Ca^{2+} and Mg^{2+} free phosphate buffered saline 1X, *p*H 7.4 and immediately covered with a large coverslip (24mm×50mm; Corning Glass Works, Corning, NY). When the agarose had solidified, the coverslips were removed. These base slides were stored in dark, cold and moist conditions till the next step of embedding of sperms in agarose gel.

Embedding of sperm in agarose gel (microgel preparation): 200 µl of 1 per cent agarose was layered over the first base layer then the cover slip was placed till gel solidified. 60 µl of sperm agarose mixture prepared by adding 10 µl PBS diluted semen containing 1 to 1.5 lacs sperm per slide to 50 µl 0.7 per cent agarose, was pipetted on the slide as the second layer over the base slide. Cover slip was then removed; 200 µl of 0.7 per cent agarose was then pippeted to sandwich the cell agarose layer, and therefore prevent the handling induced abrasions. The slides were then processed for lysis.

Positive controls by H_2O_2 treatment: As a positive control, 3 microgel slides of semen sample from a fertile control were treated with H_2O_2 (8.8 and 70.4 µM) and 3 slides of the same fertile control were treated with only 1× PBS (*p*H 7.4) at 37 ^{0}C for 30 min. The H_2O_2 treated slides were gently washed with PBS. These sperm were then processed for the lysis and electrophoresis along with other microgel slides.

Lysis and electrophoresis: Slides with microgels were incubated in a prewarmed (37 ^{0}C) lysing solution (NaCl, sodium *N*-lauroyl sarcosinate, tetrasodium ethylene diaminetetraacetic acid, tris, reduced glutathione, and of DNAse free proteinase K) for 2 h at 37 ^{0} C. After lysis, the slides were placed on a horizontal electrophoretic unit. The unit was filled with chilled electrophoresis buffer (*p*H 9). Slides with microgels were allowed to equilibrate for 20 min and electrophoresed for 20 min at 24V and 300mA while the electrophoresis tank was packed with ice packs to maintain cold conditions.

Neutralization, DNA precipitation, and staining of microgels: Slides for neutralization and DNA precipitation were immersed in freshly prepared tris, *p*H 7.4 in 50 per cent ethanol with 1 mg/ml of spermine for 15 min. This step was repeated thrice with fresh solution. Slides were air-dried. The staining was done by propium iodide dye.

Slide analysis: Images were observed on a Olympus microscope (BX 51, Germany) at 400× magnification.

The scoring was done by visualizing a minimal 100 sperms per sample and tail length was determined to assess the level of DNA damage using an eye piece micrometer.

The comets were classified into 4 groups (A-D) according to the tail length. Comets which have tail length less than 20 µm were placed in group A. Comets with tail length between 20.01 and 30.00 and 30.01 and 40.00 µm were placed in groups B and C respectively. Group D comets had tail length greater than 40.00 µm.

Statistical analysis: To minimize run to run and person to person variability, visual scoring step was performed by one person only. To reassure, 12 slides were analyzed blindly by the same person, thrice in random order. The difference in results of analysis was found to be non-significant. The consistency of the measurements for cell to cell variability was estimated by scoring three samples two times and by scoring five slides of one sample and analyzing 411 comets in this particular sample.

To minimize run to run variability 4 semen samples (of different men) were selected and were aliquoted as such that these samples can be processed along with other samples in each batch (run) of comet assay. The comet analysis of these 4 semen samples did not reveal any significant difference in the result, for all the batches run for the DNA damage study by comet assay. The mean of number of comet in each group was approximated to nearest whole value in each patient subcategory (O, A, T or OAT). The scoring was done by visualizing minimum 100 sperms per slides. Comparison of percentage comet grade sperm cells between the infertile groups and controls was done by one way ANOVA followed by bonferroni test. $P<0.05$ was considered as significant between the groups.

Results

Based on semen analysis, the mean sperm count in infertile group was 8 million / ml and in control group was 35 million/ml. In infertile men 21 per cent of the sperms were morphologically normal as compared to 43 per cent in the control group. Progressive motility (sum of rapid linear progression and slow progression *i.e.,* grades A and B) was 42 and 67 in patients and controls respectively (Table I). In the patient group, oligozoospermic (n=17), asthenozoospermic (n=13), teratozoospermic (n=9), oligoasthenoteratozoospermic (n=3), primary infertile with normal semen profile (n=18) and men with normal sperm parameters whose spouse had experienced abortions (n=10) were observed as per semen profile. The female partners were normal on gynaecological examination and had normal 46, XX chromosomal complement. All the healthy controls had a normal semen profile of progressive motility greater than 50 per cent, minimum sperm concentration of 20 million per ml and minimum 30 per cent normal morphology.

Table I. Semen analysis in patient and control group (progressive motility expressed as sum of per cent of grades A and B motile sperms)

	Patient group (expressed as mean± SD) (n = 70)	Control group (expressed as mean± SD) (n = 40)
Sperm count (million/ml)	8.01 ± 2.03	35.24 ± 4.67
% Sperm motility (PM)	42.34 ± 5.67	67.51 ± 6.96
% Normal sperm morphology	21.23 ± 2.97	43.14 ± 5.72

Comet preparations represent native semen and had a variable range of head to tail ratios. The comets were classified into four groups (A-D) (Figs 1 and 2). The circular halos of the nuclei represented the sperms with high degree of DNA integrity (A) and were predominant in the controls (69%) as compared to the patients (26%) averaged for the different subcategories. Stretched DNA continuous with the halo (B) and a halo with stretched DNA along with separated fragments (C), indicative for DNA breaks represented the intermediate degree of fragmentation and were present in 20 and 25 per cent respectively in the patients and 13 and 11 per cent respectively in the healthy fertile controls. Nuclei where DNA was no longer visible as threads and a halo, but appeared as a collection of fluorescent spots largely migrated away from the remnant of the sperm nucleus (D) represented the sperm with the least DNA integrity and was present in 29 per cent in the patient group and 7 per cent in the controls (Table II).

Discussion

The results of this pilot study suggest that conventional tests intended to detect abnormalities in sperm are not a reliable indicator of semen quality, although infertile patient group had normal semen profile with a high percentage of sperms with DNA damage as compared to healthy controls.

The OAT category had the expected very high DNA damage load which affected all the conventional semen parameters. Greater number of cells with most

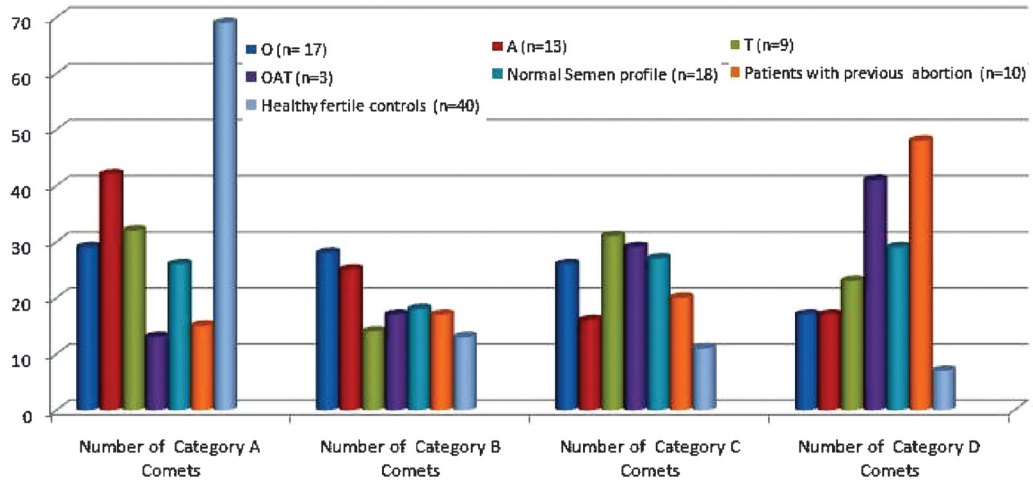

Fig. 1. Results of comets classified according to tail migration in different patient subcategories. (Expressed as per cent, approximated to the nearest whole figure value). Group A, Comets tail length less than 20 µm; Group B, Comets length between 20.01 and 30.00 µm; Group C, Comet tail length between 30.01 and 40.00 µm, Group D, Comets tail length greater than 40.00 µm. O, oligozoospermic; A, asthenozoospermic; T, teratozoospermic; OAT, oligoasthenoteratozoospermic men.

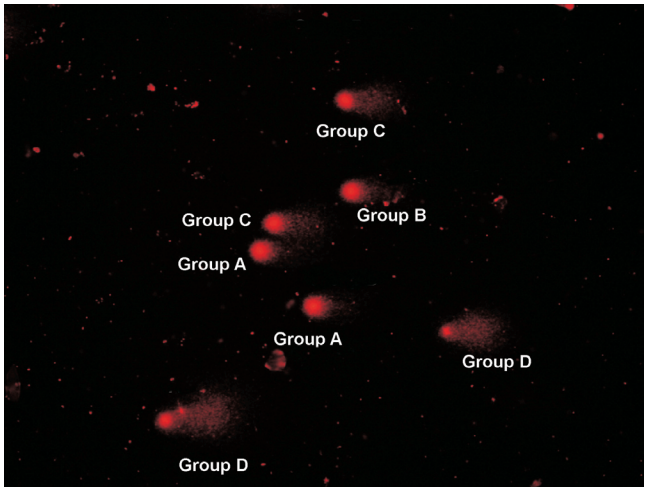

Fig. 2. Classification of comets into different categories (Group A to D) based on their tail length. Group A, Comets with tail length less than 20 µm; Group B, Comets with tail length between 20.01 and 30.00 µm; Groups C, Comets with tail length between 30.01 and 40.00 µm; Group D, Comets with tail length greater than 40.00 µm.

damaged DNA was found in OAT category (40%) as compared to 17 per cent in the oligozoospermic (O), 17 per cent in the asthenozoospermic (A), 23 per cent in the teratozoospermic (T) group. The higher degree of DNA damage can be postulated as a reason for this triple effect of impaired semen parameters in the OAT category.

Several factors cause DNA damage in sperm, including nuclear remodelling[17,18], apoptosis[19] and seminal oxidative stress in the ejaculate[20-23]. Seminal oxidative stress is mainly caused by activated leucocytes, sperms with abnormal morphology, redox cycling of metabolites or xenobiotics or exposure to radio frequency radiation[24-26].

Of single stranded (S5) and ds DNA breaks, in the sperms the latter have greater mutagenic potential, because of the absence of an error free copy as a template for DNA repair in the G1 phase male pronucleus. The indices of DNA damage increase with decreasing sperm counts[27]. It may also explain for poor ART outcome despite use of professional expertise and state of art technology. Compared with distribution of comet number in the different categories in healthy fertile males and oligozoospermic group, our study also indicates that the sperm number is in direct correlation with the DNA damage.

Different studies[28] on infertile patients and in patients experiencing recurrent spontaneous abortion (RSA) have shown varying results concerning the extent of sperm DNA damage. The inconsistency in the results in different studies could be largely attributed to the different sensitivities of the techniques used and inclusion of different patient population. It is still believed that RSA are due to underlying female factor but there may be sperm factors which are actually involved in aetiopathogenesis of abortions and ART failure[28].

Table II. Results of comets classified according to tail migration in different patient subcategories

Groups	Percentage comet categories			
	A	B	C	D
Oligozoospermic (n=17)	29.11 ± 5.40	28.41 ± 5.83	25.70 ± 5.35	16.76 ± 5.97
Asthenozoospermic (n=13)	41.84 ± 3.95	25.38 ± 3.04[a]	16.15 ± 4.09	16.61 ± 6.65[a]
Teratozoospermic (n=9)	31.77 ± 2.53[a]	14.33 ± 2.59	31.11 ± 2.66	22.77 ± 2.63
Infertile men with normal semen profile (n=18)	26.38 ± 5.32[a,b]	17.61 ± 5.33[b]	26.66 ± 3.51[a]	29.33 ± 3.46
Infertile men with previous abortion (n=10)	15.30 ± 3.40	16.60 ± 3.16[b,c]	20.40 ± 3.20[e]	47.70 ± 4.80
Oligoasthenoteratozoospermic (n=3)	13.23 ± 3.12	17.51 ± 4.01	29.20 ± 5.98	40.06 ± 6.35
Controls (n=40)	69.10 ± 7.26	12.85 ± 3.51[b,d]	11.50 ± 4.54	6.55 ± 3.49
P value	<0.0001	<0.0001	<0.0001	<0.0001

Value are mean ± SD

[a]Non-significant compared to Oligozoospemic men; [b]Non-significant compared to Teratozoospemic men; [c]Non-significant compared to infertile men with normal sperm profile; [d]Non-significant compared to infertile men with previous abortions; [e]Non-significant compared to Asthenozoospermic men. Values without superscript are significant when compared by one way ANOVA followed by bonferroni test.

Group A- Comets tail length 20 µm, Group B- Comets length between 20.01 and 30.00 µm, Group, C-Comet tail length between 30.01 and 40.00 µm, Group D- Comets tail length 40.00 µm.

In our study, the male partners of females who had abortions had high degree of DNA damage as compared with fertile controls. Sperm DNA damage adversely affects embryogenesis and implantation and also highlight the role of sperm factor in etiology of recurrent spontaneous abortion/early pregnancy loss.

Comet assay is a very useful technique in assessing DNA damage and is an important prerequisite in men opting for ART, in couples experiencing recurrent ART failure and in cases of bad obstetric history. However for technical and biological reasons, the comet assay underestimates the true frequency of DNA breaks. This may be due to several possible causes: *(i)* masking, overlapping and entangling of migrating fragments[29,30], *(ii)* incomplete chromatin decondensation may not allow all breaks to be revealed, *(iii)* due to loss of small pieces of DNA from agarose during various steps involved in the comet assay there may be fragments which are too small to be visualized. Thus the DNA damage observed is less than the actual DNA damage providing an approximate assessment for level of DNA damage. However, since significant effect of DNA damage is only observed when DFI is greater than 27 to 30 per cent, this technique proves to be ideal in diagnostic workup of idiopathic infertile men and couples with bad obstetric history.

Apart from halo formation, two types of nuclear modification are distinguished after electrophoresis of lysed and proteinase K digested sperm: extension of DNA fibres (stretched DNA) and separation of DNA fragments.

To conclude, normal structure of sperm chromatin is not only essential for the fertilizing ability of spermatozoa but also affects implantation, embryogenesis, placentation and has lifelong implications on health. DNA integrity analysis is a relatively independent measure of semen quality that yields diagnostic and prognostic information complementary to, but distinct and more significant than standard sperm parameters. Sperm DNA damage assessment may be valuable among routine tests for infertility investigations.

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