Clinical implementation of sperm DNA fragmentation studies in the assessment of male unexplained infertility

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Background
About 15% of infertile males have normal semen parameters. DNA fragmentation could logically be an additional valuable tool for fertility estimation along with the conventional semen analysis. The connection between infertility and sperm disomy is well reported. The effect of high sperm DNA fragmentation on the results of natural pregnancy and assisted reproductive technology is recorded. There are growing data on the specific characteristics of a man's environment and lifestyle through a variety of physical, chemical, and biological factors in relation to sperm DNA integrity. This work aimed to assess the important role of DNA fragmentation as a diagnostic tool in the diagnosis and explanation of unexplained male infertility and to determine the prevalence of sperm disomy in cases of male infertility.

Patients and methods
This study included twenty infertile men with severe idiopathic oligoasthenoteratozoospermia (group I), 20 infertile men with normal semen parameters (group II), and 10 fertile males as controls. Chromosomal study of peripheral blood, fluorescent in-situ hybridization assessment of sperm using the cocktail X, Y probe, and the Alkaline Comet Assay were performed.

Results
A positive correlation was observed between DNA damage and total disomy. Sperm DNA fragmentation percentage was increased in patients of group II than in patients of group I.

Conclusion
Sperm DNA integrity is essential for the normal production process and its evaluation is recommended in infertile men, especially in infertile men with normal standard semen parameters after repeated analyses. Irrespective of semen parameters, genetic counseling should be provided to infertile men.

Keywords:
chromosomal abnormality, comet assay, disomy, DNA fragmentation, fluorescent in-situ hybridization, male infertility

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Introduction

Infertility is a relatively common problem, affecting between 17 and 25% of all couples, and is defined as the inability of a couple of reproductive age to conceive following 12 months of unprotected intercourse (Agarwal *et al.*, 2015). An increased incidence of chromosomal abnormalities in infertile males has been reported by several studies. Nonetheless, in intracytoplasmic sperm injection (ICSI), any chromosomal abnormality in the male or female partner could influence the outcome and the same also holds true for chromosomal abnormality in a male partner (Harton and Tempest, 2012). Men are identified as having unexplained male infertility (UMI) when they are infertile even with normal semen analysis, normal history and physical examination, and when female factor infertility has been ruled out. The median occurrence of UMI is about 15%, even despite the fact that reports of UMI in study populations have recorded a range from 6 to 37% (Hamada *et al.*, 2012). Potential reasons that might lead to problems in conceiving in UMI include the presence of antisperm antibodies, sperm DNA damage, high levels of reactive oxygen species, and sperm dysfunction (Pandiyan *et al.*, 2017).

Imperfect spermatogenesis of unidentified etiology undetectable by the usual clinical, instrumental, or laboratory methods is defined as idiopathic oligoasthenoteratozoospermia (iOAT). It affects around 30% of infertile men and is usually diagnosed by exclusion. Men with severe OAT have sperm concentration less than 5 millions/ml (Cavallini, 2006; World Health Organization, 2010).

Fluorescent in-situ hybridization (FISH) is considered a good tool and a preliminary step toward understanding the association between male infertility and chromosome segregation shown by the percentage of aneuploid sperm (Ramasamy *et al*., 2014). DNA damage occurs in both developing and mature sperm; however, high levels of DNA damage have been reported in infertile men (Zini and Libman, 2006). The Comet assay is an easy, reliable technique that can detect even low levels of DNA damage (Kawaguchi *et al*., 2010).

This study was designed to determine the chromosomal abnormalities, sperm disomy, and sperm DNA fragmentation (SDF) in infertile men with idiopathic severe OAT and men with unexplained infertility with normal semen parameters.

Patients and methods

Patients

Fifty male participants were enrolled in this study: 20 infertile men with idiopathic severe OAT (group I), 20 men with unexplained infertility with normal semen parameters (group II), and 10 fertile men as controls. Patients were recruited from the Andrology Outpatient Clinic, National Research Center (from September 2014 to July 2016). Informed consent was obtained from the patient or the guardian. The informed consent form was approved by the Medical Research Ethics Committee, NRC. Inclusion criteria comprised age range 20–40 years, primary infertility for more than 1 year, and sperm count less than 5 million sperm/ml for severe OAT and more than or equal to 15 million sperm/ml for men with unexplained infertility. Patients with varicocele, malignancy, and liver or kidney diseases were excluded. Each man was asked to provide a semen sample, and the abstinence period was from 3 to 7 days. For the control group, men with normal semen analysis according to World Health Organization (2010), men free of any systemic and local diseases, fertile men and those who had had children in the last 2 years, and men in age range of 20–40 years were included. Ultrasonography and conventional semen analysis were carried out at least twice.

Methods

Cytogenetic evaluation

Peripheral blood culture of the G-banding technique: peripheral blood lymphocyte microcultures were performed according to standard methods of Hungerford, 1978 (Hungerford and Hungerford, 1978). G-banding on the metaphase chromosome was performed according to Verma and Babu (1995). Twenty metaphases were analyzed for each case. Individual chromosomes were identified, arranged, and karyotyped according to the International System for Human Cytogenomic Nomenclature (ISCN, 2016). Numerical chromosomal abnormalities including aneuploidy (monosomy or trisomy), polyploidy, and structural chromosomal abnormalities, including balanced abnormalities (inversions, translocations, or insertions) were recorded.

Semen processing and FISH analysis

The semen samples were prepared for FISH analysis according to Miharu *et al*. (1994), with minor modifications. The FISH technique was used with a direct labeled cocktail X, Y probe (cytocell) [DXZ1 (spectrum green), DYZI (spectrum red)]. Analysis of FISH was carried out using a Zeiss microscope with an automated stage (Zeiss, Berlin, Germany), coupled with a metasystem image analyzer. One thousand nuclei were analyzed for the number of signals. The number of signals of X and Y was scored per nuclei.

Comet assay for DNA fragmentation

The alkaline Comet assay for SDF was performed according to Ostling and Johanson (1984). Cells were analyzed by examining at least 50 comet images from each slide. Slides were examined using an Olympus BX51 microscope (Olympus, Tokyo, Japan) with a fluorescent attachment and equipped with filter sets. Individual comet images were analyzed for several features including the total intensity (DNA content). The degree of damage was determined by grading the nuclei as undamaged, represented by brilliant intact DNA, and damaged, represented by stretching of attached strands of DNA or migration of individual pieces forming a tail.

Statistical analysis

Statistical analyses of the results were carried out using SPSS version 16 (SPSS Inc., Chicago, IL, USA). Numerical data were expressed as mean ± SD and range. The test of significance was performed using Student's *t*-test with the analysis of variance test. Correlations were tested by regression analysis. Comparisons and correlations were considered statistically significant if $P \leq 0.05$.

Results

The age of the patients in this study ranged from 22 to 40 years, with a mean age of 30.07 years.

Fifty-five percent of the patients were between 30 and 40 years old; 45% were younger than 30 years old. The age of the matched control group of fertile men ranged from 22 to 40 years, with a mean age of 29.1 years. In terms of semen parameters, group I had the lowest sperm count and sperm motility range of 0.1–3 (×10^6/ml) and 5–30%, respectively, compared with those of the controls of 50–83 (×10^6/ml) and 40–73% and of group II of 15–70 (×10^6/ml) and 40–80%. Also, ejaculate volume was low in group I and group II, 0.4–4 and 0.1–4 ml, respectively, compared with the controls: 2.5–5.4 ml. There was a significant directly proportional relationship between age and the total disomic percent and DNA damage percent in group I and group II (Table 1 and Fig. 1).

A significant direct relationship between age and percentage of both total disomy and sperm DNA damage was observed in groups I and II (Table 1 and Fig. 2).

Cytogenetic investigations revealed chromosomal anomalies in 2 out of 40 patients. One patient in group I had a structural chromosomal abnormality (5%) t(9,13)(p; q) (Fig. 3, panel B) and 1 patient in group II had sex chromosome abnormalities: mosaic forms (5%) (46,XY/47, XXY) (Fig. 3, panel C), while the remaining individuals were found to have normal karyotype (46,XY) (Fig. 3, panel A).

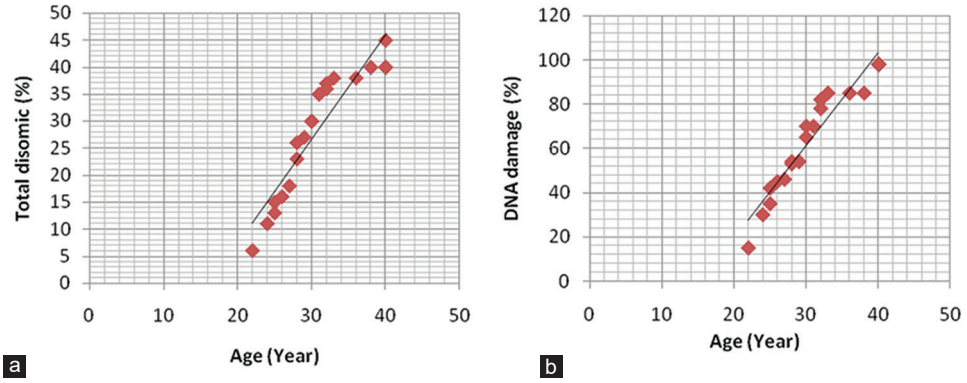
There was a significant inverse correlation between the disomic percent and the semen parameters among the 3 studied groups. The percentage of disomic sperm showed a higher increase in the spermatozoa of men with severe iOAT (group I) than in men with unexplained infertility with high semen quality (group II), compared with the controls (Fig. 4).

Table 1 Descriptive statistics and comparisons of conventional semen parameters, DNA damage, and total disomy in the studied groups

Parameters	Controls (*n*=10)	Group 1 (*n*=20)	Group 2 (*n*=20)	Correlation (*r*)	Significance (*P*)
Age					
Mean±SD	29.1±5.64	30.6±5.9	30.8±5.6		-
Range	22-40	22-40	22-40	-	
Sperm count (×10^6/ml)					
Mean±SD	68.4±10.93	1.79±1.09	35±19.3	–0.41	0.002*
Range	50-83	0.1-3	15-70		
Sperm motility (%)					
Mean±SD	57.4±13.49	15.3±9.02	56.5±12.9	–0.46	0.005*
Range	40-73	5-30	40-80		
Ejaculate volume (ml)					
Mean±SD	3.52±0.87	2.29±0.98	2.26±1.04	–0.87	0.001*
Range	2.5-5.4	0.4-4	0.1-4		
Total disomy (%)					
Mean±SD	8±2.87	43.4±12.7	28.2±11.6	0.63	0.001*
Range	5-12	27-71	6-45		
Sperm DNA (%) damage					
Mean±SD	15.2±5.15	44.9±19.1	64.4±24.3	0.73	0.001*
Range	10-23	20-78	15-98		

Data are represented as mean±SD. *P<0.01, significant.

Figure 1



Correlations: (a) significant positive correlation with total disomy percent (r = 0.63; P = 0.001) and (b) significant positive correlation with DNA damage percent for patients of group II (r = 0.73; P = 0.001).

Moreover, a positive correlation was found between DNA damage and the total disomy percent as shown in Fig. 2. Compared with the control, the SDF percentage increased in men with unexplained infertility (group II) than in patients with severe iOAT (group I) (Fig. 5).

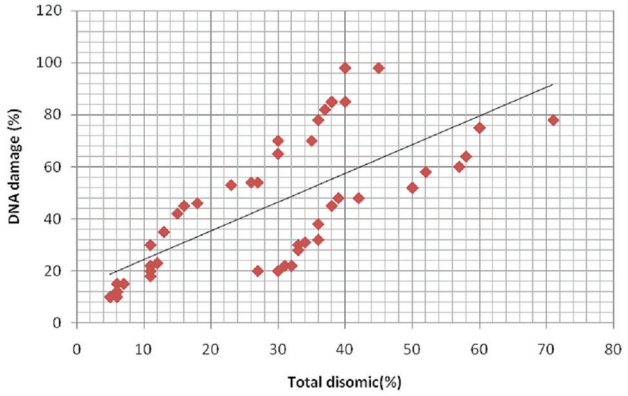
Discussion

A large number of studies have extensively studied and investigated the association between male infertility and elevated proportions of sperm with extra or lost chromosomes in any given ejaculate (Tempest *et al.*, 2004; Hamada *et al.*, 2012; Chatziparasidou *et al.*, 2015; Jungwirth *et al.*, 2017; Esteves *et al.*, 2018), and a highly significant relationship between decreased semen quality parameters and increased sperm disomy was suggested by the majority of studies; this is in agreement with our findings. In humans, levels of sperm disomy can be increased by environmental factors such as alcohol abuse and heavy smoking (Harlev *et al.*, 2015); these findings are in good agreement with ours.

Analyzing the relation between sex chromosome disomy and the decreased semen quality, Mougou-Zerelli *et al.* (2011) found a higher frequency of XY disomy in the spermatozoa of men with abnormal semen than in the control cases. In addition, no statistical difference was found between the XX and YY disomy rate. The results obtained from our study were consistent with their results: the total disomy percentage was considerably increased in group I than in group II. In the spermatozoa of men with idiopathic severe OAT, we reported a higher percentage of XY, XX, and YY disomy than in that in the group of men with unexplained infertility and normal semen quality; XX disomy was the most predominant in the 2 groups (Mougou-Zerelli *et al.*, 2011).

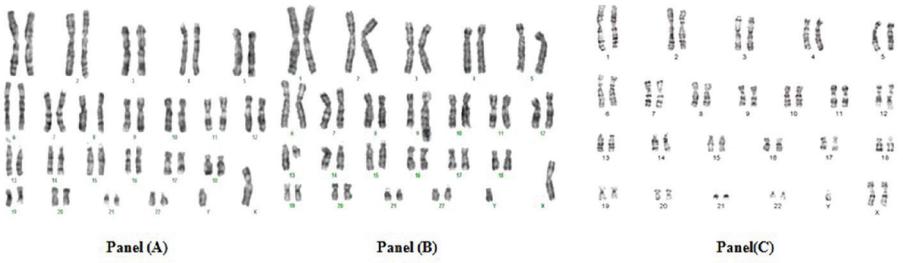
Age is inversely proportional to sperm quality, sperm DNA damage, and sperm disomy. In conventional semen

Figure 2



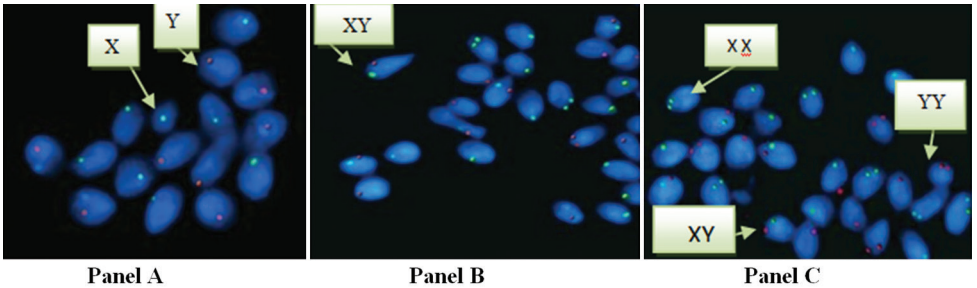
Correlation between DNA damage and total disomy percent (r = 0.51; *P* = 0.0007) for patients of both groups.

Figure 3



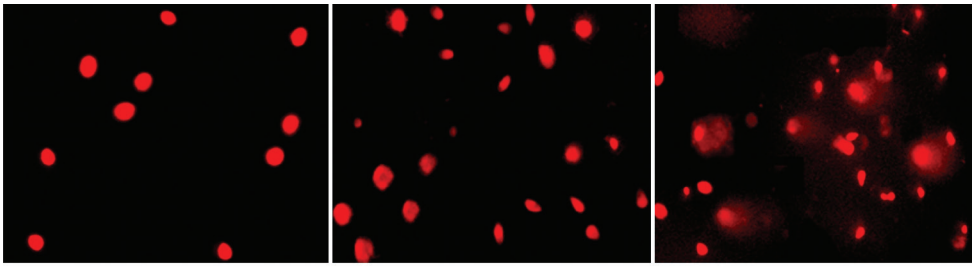
Male karyotype with the GTG-banding technique for the control group (46,XY). Panel A, one patient from group I (translocation 9p, 13q), and panel B, one patient from group II had (47,XXY) 5% cell line, Panel C.

Figure 4



Fluorescent in-situ hybridization technique α-satellite cocktail probe for X and Y chromosomes for the control group C (Panel A), nuclei showing signals for X chromosome (green) and Y chromosome (red). Group I (Panel B) shows low XX,YY and XY disomy and group I (Panel C) shows high XX,YY and XY disomy.

Figure 5



Sperm DNA damage using an alkaline Comet assay for control (Panel A) shows normal cell with no damage, group I (Panel B) shows low DNA damage percent, and group II (Panel C) shows high DNA damage percent.

analysis, ejaculate volume, sperm concentration, motility, and morphology were more consistently reported to decrease with age in the studied infertile patients with an age range of 24–76 years (Brahem *et al*., 2011). Sperm DNA integrity as assessed by the Comet assay was reported by Morris *et al*. (2002). The results of these reports are in agreement with ours, which revealed that age showed a highly significant inverse correlation with semen parameters among the studied groups. There was a highly significant positive correlation between age and percentage of both total disomy and sperm DNA damage among the studied groups. Sivanarayana *et al*. (2014) showed a negative correlation between SDF and semen parameters; the concentration of sperm, motility, and normal morphology were significantly lower in the abnormal DNA group than in the normal DNA group (Sivanarayana *et al*., 2014). In our study, there was a reverse correlation between sperm DNA damage percent and semen parameters in the patients of group I. However, the level of SDF was increased in patients of group II with normal semen parameters than in patients with severe iOAT (group I). This finding could be attributed to the increased SDF as a sole causative factor in unexplained infertility.

Human male infertility is often related to chromosome abnormalities. Ocak *et al*. (2014) recorded structural or numerical chromosomal abnormalities in 12% of patients with azoospermia or severe oligospermia, and 10.9% of Chinese patients with azoospermia or severe oligozoospermia had been discovered to have chromosomal abnormalities (Olewinska *et al*., 2010; Ocak *et al*., 2014; Kaur *et al*., 2015).

Ghorbel *et al*. (2012) reported that Klinefelter syndrome accounts for 66.7% of cytogenetic abnormalities, and Amouri *et al*. (2014) reported that of 52 patients with abnormal cytogenetic defects, Klinefelter syndrome was present in 71% (Ghorbel *et al*., 2012; Amouri *et al*., 2014). Repetitive pregnancy losses are because of structural and rearrangement chromosome abnormalities, which increase the risk of pregnancy loss or transmission of chromosomal abnormalities to offspring because of the production of higher counts of unbalanced spermatozoa (Ocak *et al*., 2014). Our cytogenetic investigations revealed chromosomal abnormalities in 2 out of 40 patients; 1 patient in group I had a structural chromosomal abnormality, t(9,13)(p; q), and 1 patient in group II showed sex chromosome abnormalities in mosaic forms (46, XY/47, XXY). However, the remaining individuals were found to have normal karyotype (46, XY). This is supported by the previous study of Kayed *et al*. (2006).

Conclusions

In conclusion, this study highlights the importance of sperm DNA comet assay analysis for the evaluation of male infertility, especially those with unexplained infertility and normal semen parameters. Therefore, we recommend the inclusion of this test among other routine investigation tests that are carried out in infertile men.

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Conflicts of interest

There are no conflicts of interest.

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