

## ORIGINAL ARTICLE

**Human sperm DNA integrity in normal and abnormal semen samples and its correlation with sperm characteristics**A. C. Varghese<sup>1</sup>, F. M. Bragais<sup>2</sup>, D. Mukhopadhyay<sup>3</sup>, S. Kundu<sup>3</sup>, M. Pal<sup>4</sup>, A. K. Bhattacharyya<sup>3</sup> & A. Agarwal<sup>2</sup>

1 Fertility Clinic and IVF Department, AMRI Medical Centre, Kolkata, India;

2 Center for Reproductive Medicine, Glickman Urological and Kidney Institute, Cleveland Clinic, Cleveland, OH, USA;

3 Department of Biochemistry, University of Calcutta, Kolkata, India;

4 Department of Statistics, University of Calcutta, Kolkata, India

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**Correspondence**

Prof. Ashok Agarwal, Director, Center for Reproductive Medicine, Glickman Urological and Kidney Institute and Department of Obstetrics-Gynecology, Cleveland Clinic, 9500 Euclid Avenue, Desk A19.1, Cleveland, OH 44195, USA.

Tel.: +1 216 444 9485;

Fax: +1 216 445 6049;

E-mail: agarwaa@ccf.org

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**Summary**

Reports indicate an increase in the incidence of DNA fragmentation in male factor infertility and its role in the outcome of assisted reproductive techniques (ART). However, reports are conflicting between the relationships of sperm DNA integrity with conventional semen parameters. We examined the relationship between conventional sperm parameters and DNA integrity using acridine orange (AO) test. The study included 373 patients and 28 fertile volunteers. DNA normality was compared with semen parameters between the patient and donor populations. Significant correlations were noted between DNA normality and sperm concentration ( $r = 0.18$ ,  $P = 0.000$ ), motility ( $r = 0.21$ ,  $P = 0.0001$ ), rapid motility ( $r = 0.19$ ,  $P = 0.000$ ), normal morphology by World Health Organization ( $r = 0.15$ ,  $P = 0.019$ ) and head defects ( $r = -0.15$ ,  $P = 0.023$ ). A significant difference was noted in AO levels between donors and patients with asthenozoospermia ( $P = 0.002$ ) and oligoasthenozoospermia ( $P = 0.001$ ). A significant difference in DNA integrity was noted in samples having <30% and >30% normal morphology. A wide range of % DNA normality was observed in the patient group. Sperm assessment for DNA status using AO is reliable and shows good correlation with sperm count, motility and morphology. Assessment of sperm DNA status with AO staining may be helpful prior to ART.

**Introduction**

A broad spectrum of assisted reproductive technologies (ART), which include *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI), currently have a major role in infertility management. Success rates depend on a variety of factors, but the structural and functional integrity of the gametes have a decisive role. Human sperm quality is usually defined by standard World Health Organization (WHO) semen analysis parameters: count, motility and morphology. These factors are generally modest predictors of reproductive outcomes (Evenson *et al.*, 1999). Current methodologies and techniques employed in the field of andrology can be

enhanced by the addition of new tests for assessing semen quality (Giwerzman *et al.*, 2003).

It is evident from current researches that oxidative stress, sperm DNA damage and apoptosis are the possible independent or interlinked molecular events, in infertile males, that are associated with various clinical and laboratory manifestations. Sperm DNA contributes one half of the genomic material to offspring and the integrity of sperm DNA is of crucial importance for balanced transmission of genetic information to future generations. The incidence of DNA-fragmented sperm in human ejaculate is documented, particularly in men with poor semen quality (Sun *et al.*, 1997; Lopes *et al.*, 1998; Høst *et al.*, 1999a; Irvine *et al.*, 2000). Poor chromatin packaging has been

shown to correlate with numerous reproductive outcomes: decreased fertility of couples after intercourse (Evenson *et al.*, 1999; Spanò *et al.*, 2000), poor fertilisation after IVF and ICSI (Lopes *et al.*, 1998; Esterhuizen *et al.*, 2000) and a higher incidence of pregnancy loss (Evenson *et al.*, 1999). Conflicting reports on the pre-fertility necessity of sperm nuclear DNA screening have emerged over the last decade. Normal and fertile sperm donors were found to have lower levels of nuclear DNA defects when compared to men undergoing fertility work-ups (Irvine *et al.*, 2000).

The possibility of fertilisation with DNA-damaged sperm during ICSI has aroused wide concern over the hidden consequences that it may have on the normality of the embryos and the resultant fetuses (Fatehi *et al.*, 2006; Zini & Libman, 2006). The mounting evidence that sperm DNA damage is common in infertile men, together with the increasing concern over genetic and epigenetic abnormalities in children conceived through assisted conception, urge us to explore the subject of human sperm genomic integrity further (De Baun *et al.*, 2003; Neri *et al.*, 2004; Varghese *et al.*, 2007).

Sperm DNA integrity can be assayed in direct methods such as single cell electrophoresis or terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL). These methods require expensive equipment and they are often inaccessible to most infertility laboratories. Indirect techniques such as sperm chromatin structural assay (SCSA) and DNA intercalating dyes such as AO are practical alternatives to these tests (AO; Darzynkiewicz *et al.*, 1975; Darzynkiewicz, 1994). These indirect methods are based on the principle that damaged DNA denatures much faster than undamaged DNA when subjected to stresses such as heat and pH changes (SCSA; Evenson *et al.*, 1980). AO binds to denatured (single-stranded) DNA and emits red fluorescence. DNA that is associated with disulphide-rich protamines is resistant to denaturation procedures; hence AO binds to double-stranded DNA and emits green fluorescence (Kosower *et al.*, 1992). The human sperm DNA integrity as assessed by AO stain (microscopic), although still controversial (Duran *et al.*, 1998; Evenson *et al.*, 2002; Chohan *et al.*, 2006), has been widely used for evaluation of male infertility (Tejada *et al.*, 1984; Shibahara *et al.*, 2003; Henkel *et al.*, 2004) and pregnancy outcome in ART (Virant-Klun *et al.*, 2002; Shibahara *et al.*, 2003; Henkel *et al.*, 2004).

The reports on correlation of nuclear integrity with semen parameters have been somewhat inconsistent. The objective of the present investigation was to evaluate semen samples for the status of sperm DNA integrity by AO method and to find the interrelationship of the DNA normality with conventional sperm quality parameters in a larger study population.

## Materials and methods

The study was carried out according to the guidelines and approval of the ethical committee of the University of Calcutta, India. The study's participants were the male partners of couples who attended the Andrology Laboratory at University of Calcutta between January 2003 and May 2005 for diagnostic andrology ( $n = 373$ ; patient group) and volunteers who donated semen samples ( $n = 28$ ; donor group). Post-vasectomy semen samples and those of men receiving treatments for infertility with hormonal and antioxidant supplements were excluded from the study group.

### Semen analysis

Each man produced semen sample by masturbation into a sterile wide-mouthed plastic specimen container. The men were instructed to abstain from ejaculation for 2–3 days before producing the semen. The sample was allowed to liquefy at 37 °C for 20 min before analysis. Measurement of both sperm concentration and motility [reference values: concentration  $\geq 20 \text{M ml}^{-1}$ , motility  $\geq 50\%$  (a + b category)] was carried out in a pre-warmed (37 °C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel).

Normal saline was used for sperm washing and two slides were prepared with the washed specimen using the feathering method (WHO, 1999). Sperm washing removed seminal plasma. It decreased background staining and provided clear images of sperm in high magnification. The resulting thin smear was allowed to air-dry for 1 h before it was stained with Papanicolaou staining method. A minimum of 200 sperm cells were scored from two slides for each specimen. Spermatozoa were considered as normal when the head had a smooth oval configuration with well-defined acrosome involving about 40–70% of the sperm head, and no defects of neck, mid-piece or tail. Semen samples showing  $< 30\%$  spermatozoa with normal morphology (WHO, 1992) were considered as abnormal. All slides were scored by one technician to preclude inter-technician variability in scoring.

### Sperm chromatin integrity by AO fluorescence

For this study, a smear was drawn from each sample and placed on a clean glass slide on the same day of the analysis. This was fixed overnight in a freshly prepared Carnoy's solution (3 parts of methanol and 1 part of glacial acetic acid). The next day it was air-dried and stained with AO solution (10 ml of 1% AO in distilled water added to a mixture of 40 ml of 0.1 M citric acid and 2.5 ml of 0.3 M  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) for 5 min. The slides

were then gently rinsed in distilled water and kept in a cool and dark place until the evaluation was carried out. Sperm chromatin status was evaluated, using the method described by Acosta & Kruger (1996). The percentage of spermatozoa with normal DNA was determined by counting at least 300 spermatozoa under a fluorescent microscope (BX41; Olympus, Tokyo, Japan) in 400 × magnification, with excitation at 450–490 nm. Spermatozoa with normal, intact double-stranded DNA stained green and those with denatured ones showed red or orange fluorescence. The pictures were taken with the help of a camera attached to the microscope and directly connected to a computer that used an RS IMAGE™ software (Roper Scientific, Inc., Tucson, AZ, USA) to capture the image from the camera. A single observer interpreted the fluorescence colour shades to rule out inter-technician variability.

### Statistical analysis

Statistical analysis of the data was performed with MINITAB™ Statistical Software (version: 13.31; Minitab Inc., PA, USA). The distributions of variables were examined, and where necessary, they were normalised by log transformation. Differences between groups on DNA integrity were examined by means of Students *t*-test or by Mann–Whitney *U*-test. A *P*-value of <0.05 was considered as statistically significant. Pearson-product moment correlation coefficient was used to measure the degree of linear relationship between two variables.

### Results

The study population consisted of patients attending infertility clinics for initial evaluation and from a group of donors who volunteered to provide semen sample. The mean age of the patient population was 33 (24.0–48.0) and that of donors 23 (22.0–25.0). Normal semen parameters were noted in the entire donor group. Among the patients, 123 (33%) had asthenozoospermia, 53 (14.2%)

had oligoasthenozoospermia and 197 (52.8%) had normal semen parameters. The standard semen parameters of donors and patients are described in Table 1. For patients, significant correlations were noted between DNA normality and the semen parameters: sperm concentration ( $r = 0.18$ ,  $P = 0.000$ ), motility ( $r = 0.21$ ,  $P = 0.000$ ), rapid forward progressive motility ( $r = 0.19$ ,  $P = 0.000$ ), normal morphology ( $r = 0.15$ ,  $P = 0.019$ ) and head defects ( $r = -0.15$ ,  $P = 0.023$ ), which implied that there was significant linear relationship between them (Fig. 1). For donors, the sperm parameters showed insignificant correlation with the DNA normality. The Pearson correlation coefficients and associated *P*-values for both patients and donors are given in Table 2.

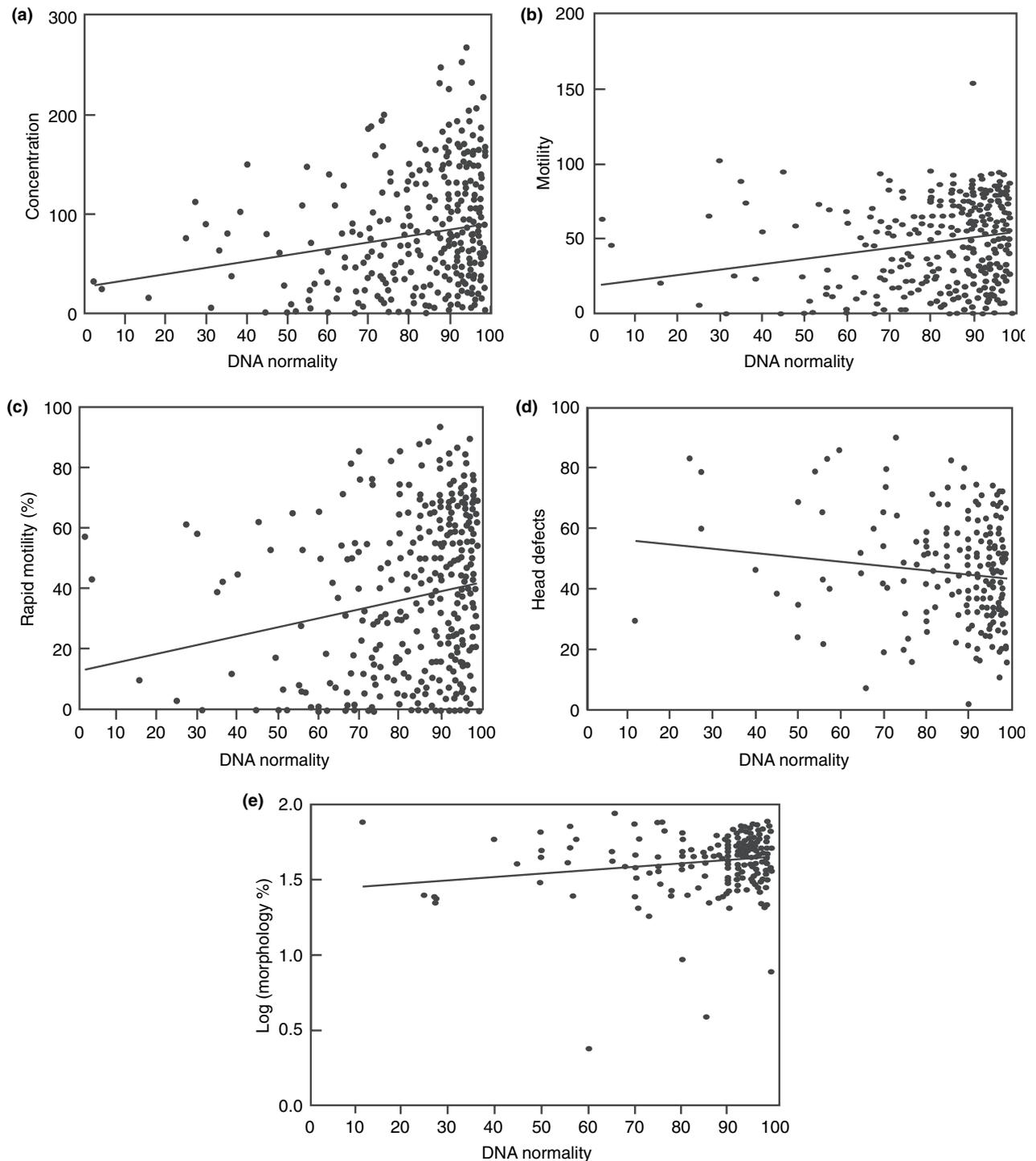
Table 3 gives the mean values and standard deviations of DNA normality in donors and the above three categories of patients. A significant difference was noted in AO normality between donor group and patients with asthenozoospermia ( $P = 0.002$ ) and oligoasthenozoospermia ( $P = 0.001$ ) (Table 3). Similar significant differences in DNA integrity were seen when normozoospermic samples from patients were compared to asthenozoospermic ( $P = 0.002$ ) or oligoasthenozoospermic samples ( $P = 0.001$ ). The mean percentage DNA normality level as determined by AO was the highest in donor group. Patients with normal sperm parameters had better quality spermatozoa compared with patients having asthenozoospermia or oligospermia. But there was no significant difference in AO levels when compared between donors and patients with normal semen parameters, even though a wide range of % DNA normality was observed in the latter group (Fig. 2). Figure 2 in the histogram showing frequency distribution of DNA normality in (a) normozoospermic patients and (b) donors.

Among the patient samples, we compared the DNA integrity status based on percentage of normal morphology (<30%,  $n = 53$ ; >30%  $n = 180$ , Table 4). Both parametric and nonparametric tests (Students *t*-test and Mann–Whitney *U*-test) showed that the mean DNA normality for patients with morphology <30% was significantly less than that for patients with morphology ≥30%

**Table 1** Standard semen parameters in patients and donors

Parameters	Patients ( $n = 373$ )		Donors ( $n = 28$ )	
	Median	IQR	Median	IQR
Sperm concentration ( $\times 10^6 \text{ ml}^{-1}$ )	76	37.0–119.0	86	64.3–120.2
Motility (%)	52.1	24.0–71.5	76	58.7–83.1
Rapid forward progressive motility (%)	40	14.2–58.0	67.5	53.8–74.2
Normal morphology (%)	45 ( $n = 230$ )	37.0–55.0	52	40.0–58.7
Head defects (%)	42 ( $n = 230$ )	32.0–51.2	25.7	13.7–36.1

IQR (interquartile range): Q1 and Q3 are the 25 and 75 percentile points.



**Fig. 1** Linear regression of (a) concentration ( $r = 0.18$ ,  $P = 0.000$ ), (b) motility ( $r = 0.21$ ,  $P = 0.000$ ), (c) rapid forward progressive motility ( $r = 0.19$ ,  $P = 0.000$ ), (d) normal morphology (log transformed,  $r = 0.15$ ,  $P = 0.019$ ), (e) head defects ( $r = -0.15$ ,  $P = 0.023$ ) on percentage of sperm with DNA normality by AOT. A negative correlation between head defects and DNA normality was found, while the other sperm parameters showed an increase with the increase in DNA normality.

( $P = 0.020$  for parametric test and  $P = 0.011$  for nonparametric test). We also compared the per cent DNA normality of oligozoospermic samples having  $>25\%$  ( $n = 20$ )

and  $<25\%$  ( $n = 33$ ) motility (Table 4). It was seen that the % DNA normality is significantly more ( $P = 0.0013$ ) in oligozoospermic samples having  $>25\%$  motility.

**Table 2** Correlation between sperm parameters and DNA normality in patients and donors

Parameters	Patients			Donors		
	<i>n</i>	Correlation with DNA normality	<i>P</i> -value	<i>n</i>	Correlation with DNA normality	<i>P</i> -value
Concentration ( $10^6 \text{ ml}^{-1}$ )	373	0.180	0.000	28	0.076	NS
Motility (%)	373	0.205	0.000	28	-0.157	NS
Rapid forward progressive motility (%)	373	0.187	0.000	28	-0.149	NS
Normal morphology (%)	230	0.154 <sup>a</sup>	0.019	28	-0.075 <sup>a</sup>	NS
Head defects (%)	230	-0.150	0.023	28	-0.054	NS

<sup>a</sup>Log transformation was used for normal morphology (%).  
NS, not significant.

**Table 3** DNA normality levels in study population based on sperm parameters

Variables	<i>n</i>	Mean $\pm$ SD	Range	<i>P</i> -value compared with donors	<i>P</i> -value compared with normozoospermic patients
Donors	28	88.8 $\pm$ 7.6	71.10–99.50		
Normozoospermic	197	86.9 $\pm$ 14.7	2.00–99.20	NS	NS
Asthenozoospermic	123	82.7 $\pm$ 16.0	4.00–99.00	0.002	0.002
Oligoasthenozoospermic	53	79.6 $\pm$ 18.5	15.90–99.50	0.001	0.001

NS, not significant.

## Discussion

In general, sperm quality has mainly been judged by parameters such as number, motility and morphology. The influence of seminal parameters as a whole or individually on fertility has been a matter of investigation and discussion since the early days of infertility treatment. Several new tests have been added lately and are gaining access to ART centres for the precise diagnosis of male factor infertility. Among these, test of sperm chromatin integrity has gained considerable interest due to its influence on ART outcome and in view of increased use of ICSI with subnormal semen samples in recent times.

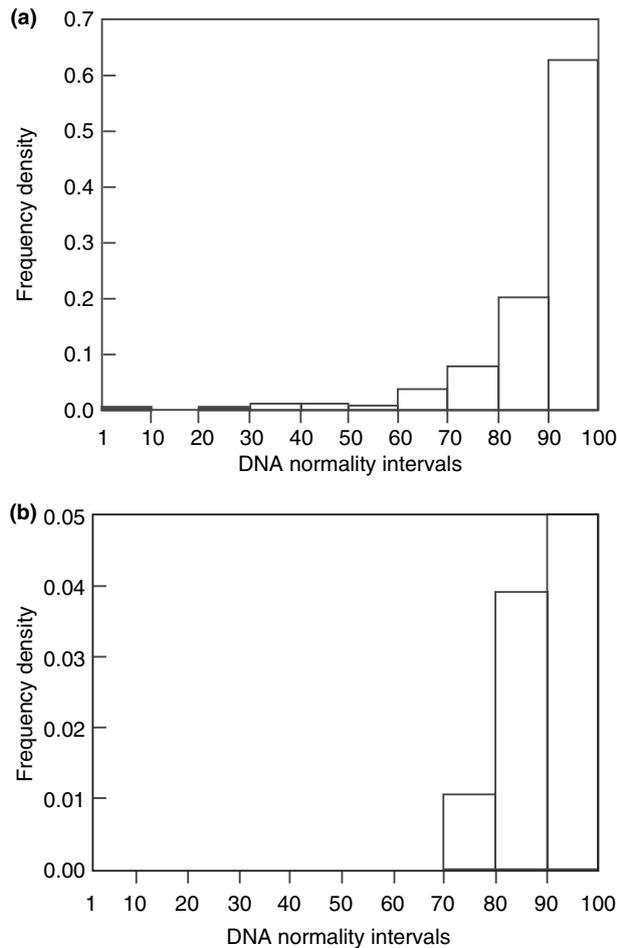
There are numerous reports published regarding the relationship between DNA integrity and conventional semen parameters (Sun *et al.*, 1997; Irvine *et al.*, 2000; Erenpreiss *et al.*, 2002; Trisini *et al.*, 2004). Our results are in accordance with other reports of increased percentage of DNA abnormalities with a rise in semen abnormalities. Furthermore, the same reports support our findings that the fertile or 'normal' population exhibits the least amount of DNA abnormalities versus the subfertile population.

Sun *et al.* (1997) demonstrated a significant negative correlation between semen quality (motility, morphology and concentration in descending order of significance) and the incidence of DNA strand breaks in spermatozoa

from 285 men attending an infertility clinic. Incidentally, our report also showed a similar correlation coefficient ( $r = 0.18$ ) for concentration and DNA integrity as observed by their group. However, in our study, the correlation of sperm parameters in relation to DNA integrity was: motility, rapid forward progressive motility, concentration, normal morphology and head defects in descending order of significance.

In this study, the normal sperm morphology and head defects were correlated with incidence of sperm DNA normality (sperm head showing green fluorescence in AO) in the patient population. This is in agreement with other investigations that found similar correlation (Tomlinson *et al.*, 2001; Liu & Baker, 2007). We observed samples with >30% normal morphology with less DNA defects compared to the samples with <30% normal morphology ( $P = 0.020$ ). Recent reports have found higher aneuploidy rates in teratozoospermic and globozoospermic patients versus sperm from fertile population. (Strassburger *et al.*, 2007).

It was also found that, as in Fig. 2, approx. 30% of the patients with normal sperm parameters had DNA normality varying from 1% to 70%. Similar observations were reported by earlier workers too. (Høst *et al.*, 1999a,b; Evenson *et al.*, 1999). These high variations in DNA normality among normozoospermic patients may account for the incidence of unexplained infertility (Høst



**Fig. 2** Showing frequency distribution of DNA normality in (a) normozoospermic patients, and (b) donors.

*et al.*, 1999a,b) and low fertility after regular unprotected intercourse in couples having normal range of classical semen parameters (Evenson *et al.*, 1999). Another study by Fatehi *et al.* (2006) showed that the DNA damage (75% as assessed by TUNEL assay) induced by high irradiation (upto 10 Gy) did not impair progressive motility or fertilisation by conventional IVF compared to controls.

Morris *et al.* (2002) also showed that (although contrary to expectations) DNA damage was positively associated with motility, suggesting that the higher the motility the higher the DNA damage in sperm. In a very recent study to assess the chromatin status of zona bound human sperm, it was found that human sperm bound to the zona pellucida have normal nuclear chromatin as assessed by AO. However, around 8% DNA damaged sperm (showing red fluorescence) were also bound to the zona (Liu & Baker, 2007). It has also been proved using zona-free hamster oocyte penetration assay that sperm with high DNA damage can bind to the oocyte and undergo nuclear decondensation (Lo *et al.*, 2004). This means that in conventional IVF or natural conception, the damaged sperm with good motility can reach the vicinity of fertilisation and can initiate the fertilisation process. So, the concern is not only ICSI, but also normal fertilisation process with patients having high loads of sperm DNA damage. In fact, several studies have linked positive correlations in increased childhood cancers from offspring of men, with high oxidative stress in the ejaculate and DNA damage to spermatozoa (Ji *et al.*, 1997; Sorahan *et al.*, 1997; Aitken *et al.*, 1998).

The mounting evidence that sperm DNA damage is common in infertile men together with the unknown consequences of iatrogenic transmission of abnormal genetic material during assisted conception urge us to explore therapies (corrective microsurgies, empirical therapies with neutraceuticals etc.) that may potentially reduce sperm DNA damage. Zini *et al.* (2005) have reported the beneficial effect of microsurgical varicocele-tomy on human sperm DNA integrity. Although the literature shows contradictory reports on *in vivo* and *in vitro* antioxidant therapy on sperm DNA integrity, some patients may benefit from it (Kodama *et al.*, 1997; Comhaire *et al.*, 2000; Agarwal *et al.*, 2004; Agarwal & Said, 2005; Greco *et al.*, 2005). In view of current evidence correlating increased smoking with sperm DNA damage (Zenzes, 2000), life style changes may prove beneficial.

The clinical value of the AO test in semen for predicting ART outcome is still contradictory in current

**Table 4** Sperm DNA normality (%) and its relation with % motility of oligoasthenozoospermic samples and relation with % morphology of whole patient group

Variable	<i>n</i>	Mean $\pm$ SD	Median	IQR	Range	<i>P</i> -value
Oligoasthenozoospermia						
% motility >25%	20	89.72 $\pm$ 9.4	92	89.40–94.78	55.7–99.0	0.0013
% motility <25%	33	73.82 $\pm$ 20.0	78	62.05–91.00	15.9–99.5	(nonparametric test)
Normal morphology (%)—whole patient group						
>30%	180	89.18 $\pm$ 12.6	93	88.00–96.00	11.90–99.20	0.011
<30%	52	82.46 $\pm$ 18.2	89	75.08–95.65	25.00–99.00	(nonparametric test)

literature as some reports show that AO gives higher DFI value (Chohan *et al.*, 2004). According to Duran *et al.* (1998) and Evenson *et al.* (1999), the major pitfalls of AO based slide test are the subjectivity in reading and interpretation of the results, problems with rapidly fading fluorescence and heterogeneous slide staining. These obstacles have limited the application of AO in andrology clinics (Evenson *et al.*, 2002; Perreault *et al.*, 2003). However, the AO test, particularly with the microscopy method, is simple, quick and can provide valuable information about overall sperm chromatin status. It can act as a screening test which can be routinely incorporated in standard semen analysis.

Modifications in sperm selection (Spanò *et al.*, 1999; Gil-Guzman *et al.*, 2001; Varghese *et al.*, 2004; Damayandi *et al.*, 2006) and cryopreservation procedures (Bhattacharyya *et al.*, 2006; Grunewald *et al.*, 2006) to select sperm with normal DNA integrity, short co-incubation of OCC with sperm (Kattera & Chen, 2003), intracytoplasmic morphologically selected sperm injection (Berkovitz *et al.*, 2005), the novel method of hyaluronic acid bound sperm selection (Jakab *et al.*, 2005) etc. are currently being tried by several workers to circumvent the problems associated with sperm nuclear damage. Further research on causes and impact of sperm genomic integrity and translation of this knowledge from research bench to clinical applications with more co-operations between andrology and gynaecology disciplines is needed.

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