Chapter 4

Poor sperm quality and advancing age is associated with increased sperm DNA damage in infertile men

Introduction

Infertility is a growing problem among couples trying to conceive; and is known to affect nearly 15-20% of couples worldwide. In the past, the female partner was singled out as the primary reason for being unable to bear a child. Research now reveals that male infertility may contribute in up to nearly half of all cases (Sharlip et al., 2002; Marchesi and Feng, 2007). For many years, a conventional semen analysis was considered sufficient to diagnose male infertility. Although fertile men as a group have higher mean sperm parameters (concentration, motility and morphology) than do infertile men, there is significant overlap between these groups (Guzick et al., 2001). Moreover, these factors are generally modest predictors of reproductive outcomes, and their predictive power is highest primarily at the lower ranges of the spectrum (Lefievre et al., 2007; Lewis, 2007). Given the limitations of conventional semen analysis, many investigators have pursued adjunctive technologies for the assessment of male fertility potential and there is now some evidence to suggest that markers of sperm DNA integrity may help in differentiating fertile from infertile men (Evenson et al., 1980; Irvine et al., 2000; Zini et al., 2002) and predict the risk of adverse reproductive events (Lopes et al., 1998b; Morris et al., 2002; Zini et al., 2008).

Clinical evidence derived from assisted reproduction suggests that failure to complete the fertilization process, syngamy, or early cleavage might be the result of an early paternal effect
(Tesarik, 2005). Earlier studies on animal models have clearly shown that embryonic failure to achieve implantation, pregnancy loss, and/or developmental abnormalities may be associated with sperm nuclear/chromatin defects, including sperm DNA damage (Adiga et al., 2007a, 2000b; Fernandez-Gonzalez et al., 2008; Perez-Crespo et al., 2008; Collier et al., 2009; Adiga et al., 2010).

These observations complemented by clinical data, demonstrating higher levels of chromatin damage in men with severe semen defects (Sun et al., 1997) and potential negative impact of high levels of DNA damage on both natural (Evenson et al., 1999; Spano et al., 2000) and ART conception (Larson et al., 2000) has led to considerable attention in the study of the consequences of compromised genetic integrity of the male germ line.

However, the results of recent clinical studies to have examined the relationships between sperm DNA damage and reproductive outcome in the context of natural and ART pregnancies are ambiguous (Barroso et al., 2009; Sakkas and Alvarez, 2010). On the basis of a systematic review and meta-analysis of these studies, sperm DNA damage has been found to be associated with lower pregnancy rates in natural, IUI and IVF, but not with ICSI (Collins et al., 2008; Zini and Sigman, 2009). Interestingly, the literature strongly suggests that sperm DNA damage is associated with an increased risk of pregnancy loss in those couples undergoing IVF or ICSI (Zini et al., 2008). Further, as clinical studies that have contributed to the above observation are generally small, heterogeneous and poorly designed (Collins et al., 2008; Zini and Sigman, 2009), the need for more studies to firmly establish the true clinical utility of sperm DNA damage assessment is clearly warranted (Barratt et al., 2010). The present study has therefore
been planned to analyze the integrity of the male gamete and understand its association as a function of age, standard semen parameters, lifestyle and occupational factors.

**Materials and Methods**

**Subjects:**
Patients visiting the infertility clinic of Kasturba Medical College for fertility evaluation and treatment between 2006 and 2010 participated in this prospective study. This study included a total of 504 patients. After provision of a written, informed consent, a detailed medical history was obtained from the participants and their partners including reproductive history and infertility evaluation. Maximal care was exerted to include patients with normal female partners i.e., normal reproductive history, normal ovulation (by follicular ultrasound study), normal luteal phase progesterone levels and tubal patency (hysterosalpingogram) in the present study. All subjects were asked to provide semen samples after 3-5 days of ejaculatory abstinence. Semen specimens were produced by masturbation directly into a sterile plastic container, in a room specially provided for this purpose and located adjacent to the laboratory.

**Analysis of semen characteristics:**
Routine andrological examination of the semen (volume, count, motility, morphology and vitality) has been performed according to WHO criteria (WHO, 1999). The reference values of semen variables and nomenclature for some semen variables used throughout the thesis has been presented in Chapter 3.

**Terminal deoxy nucleotidyl transferase mediated nick end labeling (TUNEL) assay:**
The assay was carried out according to standard protocol (Piqueras et al., 1996) provided by the manufacturer. One drop of semen sample was placed on a poly-L-lysine coated cover slip and
was allowed to dry at room temperature. The cells were fixed in 4% paraformaldehyde solution for 30 minutes. The sperm membranes were permeabilized by treating with 0.2% triton-X 100 for 30 minutes (on ice) and then washed three times with PBS. The sperm cells were incubated in terminal deoxynucleotide transferase (TdT) and nucleotide mix (Apoalert® DNA Fragmentation assay kit, Cat No. 630108, Clontech, USA) for 1 hour at 37°C in a humidified incubator. The cells were washed three times with PBS and counterstained with Propidium Iodide (PI) solution (10 µg/ml) and mounted on a glass slide. TUNEL positive cells exhibited a strong nuclear green fluorescence which was observed under fluorescence microscope (Imager-A1, Zeiss, Germany) equipped with a 490 nm excitation filter. A total of 2000 spermatozoa were assessed per sample in random fields. DNA damage was expressed as percentage of TUNEL positive spermatozoa.

**Sperm Chromatin Structure Assay (SCSA):**
Sperm DNA damage was measured by SCSA following the procedure as described (Evenson *et al.*, 1999). In the present study, as the SCSA was conducted in a different laboratory, located at a distance of approximately 350 km, the samples were fixed, followed by storage at 4°C. Owing to the same reason, samples collected over the entire study period, were analysed periodically. The sperm density in the semen was adjusted to a concentration of approximately 1-2 millions/ml and then centrifuged at 200 x g for 10 minutes. The supernatant was removed and pellet was resuspended in 200 µl of PBS. The spermatozoa were fixed in 70% chilled ethanol and stored at 4°C in cryovials until analysis. Maximal care was taken while shipping of samples to the SCSA laboratory; the samples were shipped in commercial insulated shipping containers. Small chunks of ice were first placed at the bottom of the shipping container, and then the sample box was placed in the centre of shipping box, followed by placement of ice over and around the box. On the day of analysis, an aliquot of ethanol fixed sperm was washed with TNE buffer (0.01 M Tris,
0.15 M NaCl and 1 mM EDTA) and the pellet was resuspended in 200 µl TNE buffer and treated with 0.4 ml of acid/Triton X-100 solution (0.1% Triton X-100, 0.15 M NaCl and 0.08 M HCl in double distilled water at pH 1.2). After 30 seconds, 1.2 ml of staining solution (0.15 M NaCl, 0.1 M citric acid, 0.2 M Na₂PO₄, pH 6.0 and 6 µg of Acridine Orange) was added. The samples were analyzed on flow cytometer (FACS calibur, Becton Dickinson, San Jose, CA), equipped with an air-cooled argon ion laser. The samples were excited at 488 nm, the green and red emission was collected at 530 nm and 585 nm respectively. The SCSA quantitates metachromatic shift from green (native, double-stranded DNA) to red (acid-denatured, single-stranded DNA) fluorescence. The extent of damage caused by acid denaturation was quantified by calculating alpha t values i.e the mean channel of red fluorescence/ (mean channel of red fluorescence + mean channel of green fluorescence). The alpha t values range between 0 and 1. Higher alpha t values indicate high degree of sperm DNA damage (Evenson et al., 1999).

**Statistical Analysis**

The data obtained was entered and analysed using Statistical Package for Social Sciences (SPSS 16.0). The Kruskal-Wallis test which is the non-parametric alternative to one-way analysis of variance has been used. When the number of independent groups is 2, the Mann-Whitney test has been performed. As non-parametric tests have been applied, the values are as expressed as median, followed by the 25th percentile and 75th percentile (represented within brackets). A P value < 0.05 was considered statistically significant.
**Reagents**

**Sigma Aldrich, St. Louis, MO, USA**

Nigrosin  
Cat No N4754

Poly-L-Lysine  
Cat No P4707

Paraformaldehyde  
Cat No P6148

Triton X-100  
Cat No T8532

Potassium chloride  
Cat No P5405

Tris base  
Cat No T4661

Citric acid  
Cat No C7254

Propidium iodide  
Cat No P4170

**Polysciences, Inc, USA**

Acridine Orange C.I. 46005  
Cat No 04539

**Himedia, India**

Sodium chloride  
Cat No MB023

Disodium hydrogen phosphate  
Cat No RM1154

Potassium dihydrogen phosphate  
Cat No RM249

Disodium Ethylene diamine tetra acetic acid  
Cat No MB011

Tris Hydrochloride  
Cat No RM613

**Merck & Co, USA**

Shorr stain  
Cat No UN1993

Xylene  
Cat No 1330-20-7

Hydrochloric acid  
Cat No 1003172500

Ammonia solution (about 25% pure)  
Cat No 1336-21-6

**Fisher Scientific, UK**

Methanol  
Cat No 32407

**Hayman, UK**

Absolute Alcohol (Ethanol)
<table>
<thead>
<tr>
<th>Company</th>
<th>Product</th>
<th>Cat No</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDH Chemicals, UK</td>
<td>Eosin</td>
<td>3419720</td>
</tr>
<tr>
<td>Dako, California, USA</td>
<td>Dako Fluorescence Mounting Medium</td>
<td>S3023</td>
</tr>
<tr>
<td>Sisco Research Laboratories, India</td>
<td>DPX Mountant for histology</td>
<td>42848</td>
</tr>
</tbody>
</table>
Results

Incidence of sperm DNA damage in patients with different semen characteristics:

The representation of DNA damage assessment by TUNEL assay is given in Figure 4.1. The flow cytometer at National Center of Biological Sciences, Bangalore (FACS calibur) employed for SCSA is represented in Figure 4.2. The median distribution of the standard semen characteristics and sperm DNA damage is presented in Table 4.1. Although those with asthenozoospermia and teratozoospermia had higher median distribution of TUNEL positive sperm compared to normozoospermic group, the difference was statistically insignificant. The median distribution of TUNEL positive sperm was significantly higher (P<0.001) in patients with oligozoospermia, severe oligozoospermia, oligoasthenoteratozoospermia (OAT) and asthenoteratozoospermia compared to normozoospermic ejaculates. In contrast, no difference was observed with respect to sperm chromatin denaturation level (alpha t values) in the various types of ejaculates analyzed (Table 4.1).

With decline in total motility and rapid progressive motility below 40% and 10% respectively, there was a significant increase (P<0.001) in sperm DNA damage as assessed by TUNEL assay. In addition, patients with viability greater than 50% had superior DNA integrity (P<0.001) compared to their counterparts (Table 4.2). Contradicting this observation, the alpha t values assessed by SCSA showed statistically significant increase (P<0.05) with increase in total, rapid progressive motility and viability above the mentioned threshold level.

With increase in morphologically normal sperm above a threshold of 15%, the sperm DNA damage by TUNEL assay showed statistically significant decrease (P<0.001) and is presented in
Table 4.2. In view of the above observation, the percentage of sperm with morphological abnormalities was documented and analysed for possible association with sperm DNA damage. Patients with morphologically abnormal sperm above 60% had significantly higher (P<0.01) median distribution of sperm DNA damage by TUNEL assay. There was however, no significant difference in the alpha t values assessed by SCSA in terms of normal and abnormal sperm morphology (Table 4.2).

*Association between sperm DNA damage, age, life style, occupational factors and pregnancy loss in partner:*

To understand the association between DNA damage and parameters mentioned above, patients were grouped into different categories (Table 4.3) and the incidence of DNA damage observed in their spermatozoa was compared. Patients over 40 years had a significantly higher (P<0.001) incidence of DNA damaged sperm by TUNEL assay. However, there was no difference in the alpha t values assessed by the SCSA with respect to age (Table 4.3).

No significant difference in DNA damage was seen in patients who had developed mumps during their adolescence. The median distribution of DNA damage was not significantly different among smokers/non-smokers and those engaged in high/low risk occupation. Patients who were drivers, courier service men, agriculturists, software personnel, fishermen, mechanical and civil engineers, daily wage workers were considered to be in high risk occupation category while teachers, lecturers, business category, bank employees were segregated into low risk or no risk occupation. Men with varicocele and alcoholics had significantly higher (P<0.01) median distribution of TUNEL positive spermatozoa compared to their counterparts. Alpha t values
assessed by SCSA were not significantly different among the groups studied (Table 4.3). In patients with primary infertility, there was a significant decrease (P<0.05) in chromatin integrity by SCSA compared to their counterparts. Although statistically insignificant, the same effect was seen also in relation to incidence of sperm with DNA damage assessed by TUNEL assay (Table 4.3). A correlation analysis between the TUNEL assay and SCSA yielded an R value of -0.03 (P>0.05, data not shown).
Figure 4.1: TUNEL assay in human spermatozoa, 40X magnification
Red – Intact sperm; Green – sperm with DNA fragmentation
Figure 4.2: Flow cytometer (FACS calibur, Becton Dickinson, San Jose, CA) employed for Sperm Chromatin Structure Assay in human spermatozoa at NCBS Centre of Excellence In Flow Cytometry
Table-4.1: Semen parameters and DNA damage in infertility patients

<table>
<thead>
<tr>
<th>Category of infertility</th>
<th>N</th>
<th>Sperm count (millions/ml)</th>
<th>Total motility (%)</th>
<th>Rapid progressive motility (%)</th>
<th>Sperm with normal morphology</th>
<th>Viability (%)</th>
<th>TUNEL positive sperm (%)</th>
<th>Alpha t (SCSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normozoospermia</td>
<td>123</td>
<td>60 (44,80)</td>
<td>71 (67,75.5)</td>
<td>10 (6,18)</td>
<td>33 (29,38)</td>
<td>72 (66,79)</td>
<td>5 (2,9)</td>
<td>0.24 (0.20,0.26)</td>
</tr>
<tr>
<td>Oligozoospermia</td>
<td>29</td>
<td>13 (9,16.5)</td>
<td>59 (49,67.5)</td>
<td>4 (0, 8.5)</td>
<td>32 (29,36)</td>
<td>62 (50.5,68)</td>
<td>10 (7,14.5) *</td>
<td>0.25 (0.19,0.26)</td>
</tr>
<tr>
<td>Asthenozoospermia</td>
<td>44</td>
<td>42.5 (26, 59.5)</td>
<td>51.5 (43.2,60)</td>
<td>1.5 (0,7.5)</td>
<td>31.5 (28.2,36)</td>
<td>55.5 (67,44.5)</td>
<td>8.5 (3,2,13.7)</td>
<td>0.25 (0.21,0.26)</td>
</tr>
<tr>
<td>Teratozoospermia</td>
<td>60</td>
<td>59 (36,86.5)</td>
<td>68 (61,74)</td>
<td>5 (2,10.7)</td>
<td>20 (17,22)</td>
<td>67 (60,76)</td>
<td>5.5 (3,2,8.7)</td>
<td>0.22 (0.20,0.25)</td>
</tr>
<tr>
<td>OAT $</td>
<td>102</td>
<td>7 (3.5,12)</td>
<td>36 (23,49.5)</td>
<td>0 (0,2)</td>
<td>16 (10,24)</td>
<td>46 (32.5,58)</td>
<td>13 (6,23) *</td>
<td>0.22 (0.19,0.25)</td>
</tr>
<tr>
<td>Severe oligozoospermia</td>
<td>22</td>
<td>0.3 (0.2, 0.5)</td>
<td>29.5 (15.5,40.2)</td>
<td>0 (0,2)</td>
<td>18 (10,30.5)</td>
<td>40 (23.5,50)</td>
<td>14 (7.5,20.7) *</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Asthenoteratozoospermia</td>
<td>124</td>
<td>44 (30,65)</td>
<td>48 (40,56)</td>
<td>1 (0,5)</td>
<td>20 (16,23)</td>
<td>50 (40.2,61.7)</td>
<td>11 (6,17) *</td>
<td>0.24 (0.20,0.26)</td>
</tr>
</tbody>
</table>

* P<0.001 compared to the idiopathic group with normozoospermic semen parameters;

$Oligoasthenoteratozoospermia
Table 4.2: Association between sperm DNA damage, age and standard semen parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>N (Valid Percent)</th>
<th>TUNEL positive sperm (%)</th>
<th>Alpha t (SCSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>21-40 years</td>
<td>399 (79.2)</td>
<td>8 (4.14)</td>
<td>0.24 (0.20,0.26)</td>
</tr>
<tr>
<td></td>
<td>Above 40 years</td>
<td>105 (20.8)</td>
<td>12 (6.19.5) *</td>
<td>0.22 (0.18,0.25)</td>
</tr>
<tr>
<td>Total motility (%)</td>
<td>0-40% total motility</td>
<td>120 (23.8)</td>
<td>16 (7.25) *</td>
<td>0.21 (0.13,0.25) †</td>
</tr>
<tr>
<td></td>
<td>&gt;40% total motility</td>
<td>384 (76.2)</td>
<td>7 (4.12)</td>
<td>0.24 (0.20,0.26)</td>
</tr>
<tr>
<td>Rapid progressive motility %</td>
<td>0-10% G3</td>
<td>405 (80.4)</td>
<td>9 (4.17) *</td>
<td>0.22 (0.19,0.25) ¥</td>
</tr>
<tr>
<td>(G3)</td>
<td>&gt;10% G3</td>
<td>99 (19.6)</td>
<td>6 (3.11)</td>
<td>0.25 (0.20,0.26)</td>
</tr>
<tr>
<td>Sperm viability</td>
<td>0-50% viability</td>
<td>171 (33.9)</td>
<td>13 (8.23) *</td>
<td>0.22 (0.18,0.25) ¥</td>
</tr>
<tr>
<td></td>
<td>&gt;50% viability</td>
<td>333 (66.1)</td>
<td>6 (3.11.5)</td>
<td>0.24 (0.20,0.26)</td>
</tr>
<tr>
<td>Normal sperm morphology</td>
<td>0-15% normal morphology</td>
<td>94 (18.7)</td>
<td>11 (6.22) *</td>
<td>0.21 (0.17,0.25) ¥</td>
</tr>
<tr>
<td></td>
<td>&gt;15% normal morphology</td>
<td>410 (81.3)</td>
<td>8 (4.14)</td>
<td>0.24 (0.20,0.26)</td>
</tr>
<tr>
<td>Sperm with head abnormality</td>
<td>0-60% head defects</td>
<td>432 (85.7)</td>
<td>8 (4.14) †</td>
<td>0.24 (0.20,0.26)</td>
</tr>
<tr>
<td></td>
<td>&gt;60% head defects</td>
<td>72 (14.3)</td>
<td>11 (5.20)</td>
<td>0.22 (0.00, 0.25)</td>
</tr>
</tbody>
</table>

¥P<0.05 compared to other group within the same category;
† P<0.01 compared to other group within the same category;
* P<0.001 compared to other group within the same category
Table 4.3: Association between sperm DNA damage, mumps, varicocele, lifestyle, occupation, duration of infertility and type of infertility

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>N (Valid Percent)</th>
<th>TUNEL positive sperm (%)</th>
<th>Alpha t (SCSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mumps</td>
<td>No Mumps</td>
<td>485 (96.2)</td>
<td>8.5 (4,15)</td>
<td>0.23 (0.20,0.26)</td>
</tr>
<tr>
<td></td>
<td>Mumps in adolescence</td>
<td>19 (3.8)</td>
<td>7 (3,13)</td>
<td>0.25 (0.18, 0.25)</td>
</tr>
<tr>
<td>Varicocele</td>
<td>No varicocele</td>
<td>416 (82.5)</td>
<td>8 (4,14)</td>
<td>0.23 (0.20,0.26)</td>
</tr>
<tr>
<td></td>
<td>Varicocele</td>
<td>88 (17.5)</td>
<td>12 (5,19.7) †</td>
<td>0.24 (0.20,0.26)</td>
</tr>
<tr>
<td>Smoking (Lifestyle)</td>
<td>Non-smoker</td>
<td>459 (91.1)</td>
<td>8 (4,14)</td>
<td>0.24 (0.20,0.26)</td>
</tr>
<tr>
<td></td>
<td>Smoker</td>
<td>45 (8.9)</td>
<td>10.5 (4,21.5)</td>
<td>0.21 (0.16,0.26)</td>
</tr>
<tr>
<td>Drinking (Lifestyle)</td>
<td>Non-alcoholic</td>
<td>445 (88.3)</td>
<td>8 (4,14)</td>
<td>0.24 (0.20,0.26)</td>
</tr>
<tr>
<td></td>
<td>Alcoholic</td>
<td>59 (11.7)</td>
<td>11.5 (4,75,23.5) †</td>
<td>0.21 (0.20,0.26)</td>
</tr>
<tr>
<td>Occupation</td>
<td>Low risk occupation</td>
<td>149 (29.6)</td>
<td>9 (4,14,5)</td>
<td>0.24 (0.20,0.26)</td>
</tr>
<tr>
<td></td>
<td>High risk occupation</td>
<td>355 (70.4)</td>
<td>8 (4,16.5)</td>
<td>0.23 (0.20,0.26)</td>
</tr>
<tr>
<td>Duration of infertility</td>
<td>1-5 years</td>
<td>242 (48)</td>
<td>8 (4,13)</td>
<td>0.24 (0.20,0.26)</td>
</tr>
<tr>
<td></td>
<td>&gt;5 years</td>
<td>262 (52)</td>
<td>9 (4,17)</td>
<td>0.22 (0.19,0.26)</td>
</tr>
<tr>
<td>Type of infertility</td>
<td>Primary infertility</td>
<td>349 (69.2)</td>
<td>9 (4,15)</td>
<td>0.24 (0.20,0.26) ¥</td>
</tr>
<tr>
<td></td>
<td>Secondary infertility</td>
<td>155 (30.8)</td>
<td>7 (4,14)</td>
<td>0.21 (0.18,0.25)</td>
</tr>
</tbody>
</table>

¥P<0.05 compared to other group within the same category, † P<0.01 compared to other group within the same category
Discussion

In the present study, an attempt has been made to investigate the incidence of sperm DNA damage among a total of 504 infertile men, attending the University fertility clinic for evaluation and treatment. An effort has been made to understand the association between sperm DNA damage, sperm characteristics, age of the patients, occupation, lifestyle factors and varicocele.

The average incidence of sperm DNA damage in normozoospermic ejaculates is <10% where as patients with oligozoospermia, severe oligozoospermia, necrozoospermia and multiple sperm abnormalities (oligoasthenoteratospermia) had significantly higher level of DNA damaged sperm by TUNEL assay, suggesting that poor semen quality is associated with increased level of DNA damaged sperm. This is in agreement with previous findings where changes in sperm chromatin conformation were reported to be suggestive of diminished fertility (Evenson et al., 1980). A negative association between semen parameters and sperm DNA fragmentation was reported by earlier studies (Kodama et al., 1997; Sun et al., 1997; Lopes et al., 1998b) which also highlighted that use of sperm with fragmented DNA during ART could result in poor fertilization and/or cleavage rates, correlating with fertilization in vitro. Subsequent studies were targeted at revealing mechanisms of sperm DNA damage, leading to reports of detection of oxidative DNA damage in human sperm, its association with sperm function and male infertility (Irvine et al., 2000; Shen and Ong, 2000). In consequent population-based studies that followed, the above observations were confirmed and optimal sperm chromatin packaging was accepted as being necessary for full expression of the male fertility potential. In addition, tests for chromatin assessment in sperm logically emerged as a predictor of the probability to conceive (Spano et al., 2000). This was followed by attempts to correlate between two markers of sperm DNA integrity-
DNA denaturation and DNA fragmentation, in fertile and infertile men. Additionally, concerns were expressed regarding fertilization with DNA-damaged spermatozoa and increased risk of genetic disease in the offspring (Zini et al., 2001; Zini et al., 2002).

The association between men’s age and sperm DNA damage observed in this study suggests paternal sperm integrity declines with ageing. Since this issue is contradictory (Singh et al., 2003; Wyrobek et al., 2006; Schmid et al., 2007, Vagnini et al., 2007), it needs to be confirmed by multi-centric studies using large number of study subjects.

Owing to lack of correlation between the TUNEL assay and sperm chromatin structure assay (data not shown), it is concluded that these methods are not comparable in estimation of DNA damage. Supporting the statement, these different techniques have been reported to determine different aspects of sperm DNA damage, i.e. ‘real’ DNA damage for the TUNEL assay and 'potential' DNA damage in terms of susceptibility to acid DNA denaturation of the SCSA (Henkel et al., 2010). However, from a practical stand-point, the TUNEL assay assumes greater clinical importance.

Observations from the study also suggests that decline of sperm motility in the ejaculate is associated with increase in sperm DNA damage. These findings are consistent with previous literature (Trisini et al., 2004; Matsuura et al., 2010). The association between morphological normalcy of the sperm and DNA damage is much debated, and has been the focus of many studies over the decade (Avendano et al, 2009; Elshal et al., 2009; Mehdi et al., 2009; Avendano et al, 2010; Oliveira et al., 2010). The association observed between sperm head abnormalities
and increased DNA damage in the present study warrants the need for further studies to explore and establish the possibility of an association between the two variables. In addition, it affirms the need for careful selection of sperm during ICSI as the damage carried by sperm with abnormal morphology can be transmitted to the progeny.

Varicoceles have been associated with sperm DNA damage (Zini et al., 2005a; Sakamoto et al., 2008; Werthman at al., 2008) and the damage has been related to levels of oxidative stress in the semen of these infertile men (Saleh et al., 2003b). In the present study, a similar trend has been observed, with significantly high median distribution of TUNEL positive cells in patients with varicocele. However, it remains yet to be established if sperm DNA damage is specifically increased in men with varicocele. In addition, larger, prospective studies are also indicated to identify if varicocelectomy can reduce the levels of sperm DNA fragmentation.

While a number of studies have been conducted worldwide regarding the association of life style factors and sperm quality (Soares and Melo, 2008; Calogero et al., 2009; Elshal et al., 2009; Viloria et al., 2010), studies on Indian men are lacking. Of the different lifestyle factors studied, the study observed a significantly high incidence of TUNEL positive sperm in men who consumed alcohol. Although this represents only a small proportion of the study population (11.7%), the finding certainly demands further attention. Failure to demonstrate any association between sperm DNA damage and smoking can be attributed to a small fraction of the subjects 8.9% (45/459) who admitted to smoke in the present study. Nevertheless, as strong body of evidence indicates that the negative effect of cigarette smoking on fertility comprises fairly every system involved in the reproductive process (Soares and Melo, 2008), couples in reproductive
age should be strongly discouraged to smoke. While several studies implicate increased sperm DNA damage in couples with recurrent pregnancy loss, (Carrell et al., 2003; Gil-Villa et al., 2010) the observation of a significant increase in sperm chromatin integrity by SCSA in patients with secondary infertility in the present study could possibly suggest the presence of factors apart from sperm DNA damage, leading to recurrent pregnancy loss (3 or more) in infertile couples.

Preliminary reports on association between abnormal semen parameters, particularly teratozoospermia and fertilization disorders in IVF have been reported as early as 1988 (Kruger et al., 1988; Oehninger et al., 1988a, 1988b; 1989). This was followed by studies highlighting an association between abnormal semen parameters and embryo cleavage deficiencies (Ron-el et al., 1991; Parinaud et al., 1993). The results of studies revealing evidence for a low implantation rate when ICSI was performed for patients with severe teratozoospermia (Gorczyca et al., 1993; Grow et al., 1994; Oehninger at al., 1996; Mercan et al., 1998) attracted attention towards the role of the male gamete, leading to more focused studies on the same topic (Hughes et al., 1996; Sun et al, 1997; Lopes et al., 1998b; Duran et al., 2002; Liu et al, 2004). These studies highlighted that spermatozoa from infertile men contain various nuclear alterations such as abnormal chromatin structure, chromosome abnormalities, microdeletions of Y chromosome and DNA strand breaks. Consequently, the reestablishment of an association between poor semen quality, increased sperm aneuploidy and DNA damage specifically in the form of fragmentation, instability and single strand breaks followed (Barroso et al., 2000; Aitken and Krausz, 2001).

The results of the present study agree with above-mentioned studies highlighting an association between abnormal sperm parameters and increased DNA damage, a finding particularly relevant
owing to reports highlighting that Asian ethnicity is associated with decreased pregnancy rates following IUI and (Lamb et al., 2009) and ART (Purcell et al., 2007; Shahine et al., 2009; Langen et al., 2010). Complementing the above observation, a retrospective cohort study which carried out a multivariate analysis of factors affecting probability of pregnancy and live birth with in vitro fertilization revealed that Asians had significantly lower odds of clinical pregnancy compared with whites (Baker et al., 2010). In addition, the same study observed that Asians had a significantly greater risk of pregnancy loss in the second and third trimesters in ART-conceived pregnancies. However, it remains yet to be determined if either sperm DNA damage alone or in combination with other factors is responsible for this trend.

The present study has several limitations. Recently, flow cytometry, coupled with use of TUNEL/PI in semen has indicated the occurrence of two sperm populations, different for the intensity of nuclear staining: one stains more (PI brighter, Pi\text{br} sperm) and the other less (PI dimmer, Pi\text{dim} sperm) with PI (Muratori et al., 2010). These two populations have been reported to differ for: (i) the extent of DNA fragmentation and (ii) the relationship between DNA damage within each population and semen quality. Indeed, whereas in Pi\text{br} populations, a variable fraction of sperm shows fragmented DNA, all sperm in Pi\text{dim} populations show DNA fragmentation (Muratori et al., 2008). In addition, whereas the percentages of DNA fragmented Pi\text{dim} sperm closely associate to poor semen quality, DNA fragmentation in Pi\text{br} population is independent from standard semen parameters (Muratori et al., 2008). The latter finding suggests that DNA fragmented sperm in the Pi\text{dim} population are more likely to exhibit reduced motility and abnormal morphology than those in the Pi\text{br} population. If so, TUNEL/PI, would have the further advantage to quantify, among DNA fragmented sperm, the actual fraction (Pi\text{br} sperm)
having the potential to negatively impact on reproduction outcomes (Muratori et al., 2010). While such precise distinctions as mentioned above may be achievable with flow cytometry, in the present study, fluorescent microscopy has been employed. The ability of the human eye to discriminate sperm populations on the basis of variable PI staining or varying degrees of TUNEL positivity may therefore be limited. Additionally, in view of limited resources and availability, it has not been possible to obtain TUNEL scores from individual investigators and check for comparability.

With regard to SCSA, although frozen samples are preferred and the easiest to work with (Evenson and Jost, 1994), data from fixed samples are essentially similar to that obtained on fresh material (Evenson et al., 1986). It has previously been reported that in samples from individual men, the SCSA parameters were more constant over 8 months than WHO semen analysis parameters (Schrader et al., 1988; Evenson et al., 1991). However, the possibility that SCSA parameters in the present study could be affected by length of storage of samples, cannot be ruled out and requires further validation.

Overall, the results from this study demonstrated the presence of an association between sperm DNA damage, age, standard semen parameters, presence of varicocele and alcohol consumption in infertile men. These findings complement those of a previously published study which analysed human sperm DNA integrity in normal and abnormal semen samples and its correlation with sperm characteristics (Varghese et al., 2009). Although the clinical value of sperm DNA integrity testing remains to be defined (Kodama et al., 1997; Spano et al., 2000; Zini et al., 2001; Zini and Sigman, 2009; Barratt et al., 2010; Sakkas and Alvarez, 2010), estimation of DNA
damage in sperm is clinically relevant given that infertile men (especially those with severe male-factor infertility and with poor sperm DNA integrity) will be seeking treatment with ARTs (Zini et al., 2008).

The findings of an age related increase in sperm DNA damage and association of the same with poor semen quality in the present study, coupled with above-mentioned concerns, adequately stress the need for more studies, with specific reference to the Asian scenario. Supporting the views of the American Society for Reproductive Medicine (ASRM), it is agreed that the need to diagnose sperm at a nuclear level is an area that needs further understanding and refinement.