Human semen quality and sperm DNA damage assessed by comet assay in clinical groups

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1. Introduction
Infertility is the inability of a couple to conceive naturally after one year of regular unprotected sexual intercourse. Infertility is perceived as a social and public problem across virtually all cultures and societies. It affects 13%–15% of the reproductive-aged couples worldwide. The prevalence of infertility varies widely from region to region, being higher in developing countries, where there is a lack of resources for investigation and treatment, than in developed countries (1). Infertility is one of the most important and underappreciated reproductive health problems in developing countries (2,3). The inability to procreate is considered a personal tragedy and a curse for couples, impacting the entire family and even the local community (4).

Male infertility is the inability of a male partner to achieve a pregnancy in a fertile female partner. Male infertility is directly or indirectly responsible for 60% of the cases involving the reproductive-aged couples with fertility related issues (5–7). During ejaculation, semen is produced from a concentrated suspension of spermatozoa stored in the epididymis and mixed with fluid secretions of the accessory sex glands. Semen has two major quantifiable attributes. Firstly, the total number of spermatozoa, which reflects sperm production by the testes and secondly, the patency of the posttesticular duct system and total fluid volume contributed by the various accessory glands, which reveal the secretory activity of the glands. The nature of the spermatozoa (vitality, motility, and morphology) and the composition of the seminal fluid are important parameters for proper sperm function (8). A deficiency in semen, either quantitative or qualitative, is the most common cause of male infertility. Semen analysis is the single most important and fundamental initial laboratory investigation for the assessment of male infertility (9).
Clinical evaluation of the contribution of a male towards the infertility of a couple is usually confined to measures of total sperm count, sperm concentration, normal and abnormal sperm forms, and motility. The predictive value of these measurements is limited only to describing some aspects of the function of the testis and sperm. They do not address the integrity of the male genome contained in the head of the sperm (6,10,11). The DNA integrity in sperm is essential for the success of natural or assisted fertilization as well as the normal development of the embryo, fetus, and child. Moreover, DNA damage in sperm may carry mutations into the next generation or result in male infertility (12,13).

This cross sectional study was designed to determine the semen quality of fertile and infertile Pakistani men, to identify the relationship of sperm DNA integrity to seminal parameters and reproductive hormonal levels in the infertile men, and to make a comparison with those of the fertile ones.

2. Materials and methods
The study included 180 male subjects aged 18–50 years. Among the 180 male subjects, 26 (14.45%) were fertile (proven fathers) and 154 (85.55%) were infertile (those whose marital duration was more than one year and had failed to procreate during the last one year of regular unprotected sexual intercourse). The infertile subjects were further subdivided into 22 (12.22%) with asthenozoospermia, 9 (5%) with asthenoteratozoospermia, 20 (11.12%) with azoospermia, 58 (32.22%) with normozoospermia, 7 (03.88%) with oligozoospermia, 12 (06.66%) with oligoasthenozoospermia, and 26 (14.45%) with oligoasthenoteratozoospermia. This division of infertile subjects into different groups was based strictly on the semen analysis according to the nomenclature of the WHO Laboratory Manual for the Examination and Processing of Human Semen (9). All subjects were subjected to thorough clinical examination to exclude those suffering from chronic health problems.

The study was approved by the Institutional Review Board (IRB) and Advanced Studies and Research Board (ASRB) of Khyber Medical University, Peshawar, Pakistan. Written informed consent was obtained from the subjects and participation in the study was voluntary. Fertile and infertile subjects were recruited from the two private clinics in Dera Ismail Khan, Pakistan.

2.1. Semen samples
The semen samples were collected by masturbation into preweighed labeled containers at Bilal Clinical Laboratory after a sexual abstinence of 3 days, were kept at 37 °C during liquefaction, and were analyzed soon after liquefaction according to WHO guidelines. The viscosity of each sample was evaluated by introducing a glass rod into the sample and observing the length of the thread that formed upon the withdrawal of the rod. Semen volume was assessed by weighing the sample in the vessel in which it was collected and subtracting the weight of the empty container, assuming the semen density to be 1 g/mL. Semen pH was measured with the help of pH paper in the range of 6 to 10. Sperm motility was assessed using 10 μL of well-mixed semen placed on a clean glass slide, covered with a 22 × 22 mm coverslip, and then examined at a total magnification of 400×. The sperm were classified as progressively motile, nonprogressively motile, and immotile in order to record the proportion of motile spermatozoa. For the assessment of sperm concentration, the samples were diluted in a solution of 0.6 mol/L NaHCO₃ and 0.4% (v/v) formaldehyde in distilled water, and subsequently assessed using an improved Neubauer hemocytometer. Only complete sperms (sperms with tails) were counted. Smears were prepared for morphological evaluation, sperm vitality, and sperm DNA fragmentation. Sperm morphology and sperm vitality smears were stained with Spermac stain and VitalScreen, both manufactured by FertiPro N.V. (Belgium). Approximately 200 spermatozoa in each replicate were evaluated. The comet assay protocol was performed on all semen samples according to the Enciso (14) method. Sperm cells were diluted to a concentration of 10 × 10⁶ spermatozoa/mL in phosphate-buffered saline (PBS). Next, 25 μL of the cell dilution was mixed at 37 °C with 50 μL of freshly prepared 1% low-melting point agarose (Sigma Aldrich, St Louis, MO, USA) in distilled water. An aliquot of 15 μL of the mixture was placed on a pretreated slide for gel adhesion (1% low-melting point agarose), covered with cover slips, and allowed to gel on a cold plate at 4 °C for 5 min. As soon as the gel solidified, the cover slips were smoothly removed and the slides were submerged sequentially in two lysing solutions: lysing solution 1 [0.4 mol/L Tris–HCl, 0.8 mol/L dithiothreitol (DTT), 1% sodium dodecyl sulfate (SDS), pH 7.5] for 30 min, followed by lysing solution 2 [0.4 mol/L Tris–HCl, 2 mol/L NaCl, 1% SDS, 0.05 mol/L EDTA, pH 7.5] for 30 min. Then slides were rinsed in TBE buffer (0.09 mol/L Tris–borate, 0.002 mol/L EDTA, pH 7.5) for 10 min, transferred to an electrophoresis tank, and immersed in fresh TBE electrophoresis buffer. Electrophoresis was performed at 20 V (1 V/cm), 12 mA for 12.5 min. After washing in 0.9% NaCl, the nucleoids were unwound in an alkaline solution (0.03 mol/L NaOH, NaCl 1 mol/L) for 2.5 min, transferred to an electrophoresis chamber, and oriented 90° to the first electrophoresis. The second electrophoresis was performed at 20 V (1 V/cm), 12 mA for 4 min in 0.03 mol/L NaOH. Then the slides were rinsed once in a neutralization buffer (0.4 mol/L Tris–HCl, pH 7.5) for 5 min, briefly washed in TBE buffer, dehydrated in increasing concentrations of ethanol, and air dried. All
comet assay samples were stained with DAPI (Invitrogen, Eugene, OR, USA) and were evaluated using a fluorescence microscope, counting at least 200 spermatozoa per sample. The sperm cells were classified according to fragmented and nonfragmented sperm (15).

2.2. Blood samples
Nonfasting venous blood samples were collected from the subjects using disposable sterile syringes. The serum was separated through centrifugation at 1600 × g and stored at −80 °C for subsequent hormonal analysis. Follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were quantitatively determined using chemiluminescence assay (CLIA) kits manufactured by Monobind Inc. (USA). Assays were done according to the manufacturer's instructions.

2.3. Statistical analysis
Results were expressed as mean ± SD. The obtained results were analyzed and compared by one-way analysis of variance followed by a post-hoc Tukey test using SPSS version 16 (SPSS Inc., Chicago, IL, USA). P < 0.05 was considered to be statistically significant.

3. Results
Mean age (years) and body mass index (BMI) (kg/m²) of the fertile and their age-matched infertile subjects are summarized in Table 1, which clearly shows that mean age and BMI did not differ significantly between the fertile and all of the infertile groups.

Subjects included in the study had normal seminal viscosity.

The seminal parameters of both groups are presented in Table 2. No significant difference (P > 0.05) was found between the semen pH, liquefaction time (minutes), and semen volume (mL) of the fertile and all of the infertile groups. Sperm concentration was observed to be significantly lower (P < 0.001) in all of the infertile groups except the normozoospermic group (P > 0.05) as compared to the fertile group (Table 2). Sperm vitality was observed to be significantly lower (P < 0.001) in the asthenozoospermic, asthenoteratozoospermic, oligozoospermic, and oligoasthenoteratozoospermic groups as compared to the fertile group, while no significant difference (P > 0.05) was observed between the oligoasthenozoospermic, normozoospermic, and fertile groups (Table 2). Similarly, total sperm motility and progressive sperm motility were observed to be significantly lower (P < 0.05) in all of the infertile groups except the oligoasthenozoospermic group (P > 0.05) as compared to the fertile group, as shown in Table 2.

Further morphological examinations revealed that normal sperm forms were significantly lower (P < 0.001) in all of the infertile groups relative to those of the fertile group as depicted in Table 2. Malformations of the head and midpiece were significantly higher in all of the infertile groups except the oligoasthenozoospermic group relative to the fertile males. Tail defects showed no significant difference between the fertile and all of the infertile groups (Table 2).

We assessed sperm DNA fragmentation between infertile and fertile subjects by using a comet assay. Sperm DNA fragmentation (SDF) is graphed in the Figure, which clearly shows that SDF (%) was significantly higher (P < 0.001) in all of the infertile males as compared to the fertile males.

Reproductive hormone levels are summarized in Table 3. FSH levels (mIU/mL) were found to be significantly lower (P < 0.001) in the infertile normozoospermic and asthenozoospermic groups as compared to the fertile group, while no significant difference (P > 0.05) was observed between the other infertile groups as compared to the fertile group. On the other hand, significantly lower serum LH (mIU/mL) and testosterone (ng/mL) levels were observed in all of the infertile (P < 0.05) groups compared to the fertile group.

4. Discussion
Semen analysis is the most useful and basic investigation in the search for the cause of male infertility. It provides insight into not only sperm production (count) but also...

### Table 1. Mean age (years) and BMI (kg/m²) of the fertile and infertile normozoospermic (NZ), azoospermic (AZO), asthenozoospermic (AZ), asthenoteratozoospermic (ATZ), oligozoospermic (OZ), oligoasthenozoospermic (OAZ), and oligoasthenoteratozoospermic (OATZ) males.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fertile (n = 26)</th>
<th>Infertile (n = 154)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NZ (n = 58)</td>
<td>AZO (n = 20)</td>
</tr>
<tr>
<td></td>
<td>AZ (n = 22)</td>
<td>ATZ (n = 9)</td>
</tr>
<tr>
<td></td>
<td>OZ (n = 7)</td>
<td>OAZ (n = 12)</td>
</tr>
<tr>
<td></td>
<td>OATZ (n = 26)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>33.23 ± 5.81</td>
<td>30.24 ± 5.74 NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.98 ± 3.31</td>
<td>23.36 ± 2.72 NS</td>
</tr>
</tbody>
</table>

Values = mean ± SD and NS = statistically nonsignificant as compared to the fertile group.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fertile ( (n = 26) )</th>
<th>Infertile ( (n = 154) )</th>
<th>NZ ( (n = 58) )</th>
<th>AZO ( (n = 20) )</th>
<th>AZ ( (n = 22) )</th>
<th>ATZ ( (n = 9) )</th>
<th>OZ ( (n = 7) )</th>
<th>OAZ ( (n = 12) )</th>
<th>OATZ ( (n = 26) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen pH</td>
<td>7.6 ± 0.32</td>
<td>7.78 ± 0.41(^{NS})</td>
<td>7.7 ± 0.39(^{NS})</td>
<td>7.74 ± 0.26(^{NS})</td>
<td>7.67 ± 0.21(^{NS})</td>
<td>7.65 ± 0.19(^{NS})</td>
<td>7.65 ± 0.27(^{NS})</td>
<td>7.62 ± 0.32(^{NS})</td>
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<tr>
<td>Semen liq. time (min)</td>
<td>24.4 ± 5.48</td>
<td>23.78 ± 6.32(^{NS})</td>
<td>24 ± 6.12(^{NS})</td>
<td>24.68 ± 5.25(^{NS})</td>
<td>25.5 ± 5.16(^{NS})</td>
<td>25.67 ± 4.13(^{NS})</td>
<td>25.42 ± 5.88(^{NS})</td>
<td>24.27 ± 6.58(^{NS})</td>
<td></td>
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<tr>
<td>Semen vol. (mL)</td>
<td>3.16 ± 1.11</td>
<td>3.89 ± 2.42(^{NS})</td>
<td>2.83 ± 1.33(^{NS})</td>
<td>4.54 ± 1.99(^{NS})</td>
<td>2 ± 0.76(^{NS})</td>
<td>1.66 ± 0.52(^{NS})</td>
<td>2.41 ± 0.93(^{NS})</td>
<td>3.22 ± 2.66(^{NS})</td>
<td></td>
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<tr>
<td>Sperm conc. ((\times 10^6/\text{mL}))</td>
<td>71 ± 22.73</td>
<td>63.96 ± 20.85(^{NS})</td>
<td>0.00</td>
<td>47.27 ± 17.59(^{***})</td>
<td>32 ± 10.58(^{***})</td>
<td>10.33 ± 2.25(^{***})</td>
<td>11.92 ± 2.05(^{***})</td>
<td>10.38 ± 8.83(^{***})</td>
<td></td>
</tr>
<tr>
<td>Sperm vitality (%)</td>
<td>81.1 ± 7.39</td>
<td>73.41 ± 9.36(^{NS})</td>
<td>0.00</td>
<td>32.64 ± 15.59(^{***})</td>
<td>36.75 ± 20.12(^{**})</td>
<td>76.67 ± 6.83(^{NS})</td>
<td>45.5 ± 13.33(^{**})</td>
<td>23.85 ± 15.89(^{**})</td>
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<tr>
<td>T. sperm mot. (%)</td>
<td>73.9 ± 8.06</td>
<td>64.83 ± 13.92(^{***})</td>
<td>0.00</td>
<td>28.27 ± 10.96(^{***})</td>
<td>32.5 ± 19.09(^{***})</td>
<td>70 ± 8.94</td>
<td>38.33 ± 12.67(^{**})</td>
<td>14.38 ± 13.83(^{**})</td>
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</tr>
<tr>
<td>Prog. mot. (%)</td>
<td>64.8 ± 6.72</td>
<td>50.86 ± 12.07(^{***})</td>
<td>0.00</td>
<td>14.18 ± 10.03(^{***})</td>
<td>17.5 ± 11.02(^{***})</td>
<td>53.33 ± 6.83(^{NS})</td>
<td>15.83 ± 7.02(^{**})</td>
<td>0.54 ± 1.42(^{**})</td>
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<tr>
<td>Normal form (%)</td>
<td>77.5 ± 9.84</td>
<td>60.97 ± 15.42(^{***})</td>
<td>0.00</td>
<td>28.64 ± 11.77(^{***})</td>
<td>27.5 ± 0.46(^{**})</td>
<td>55 ± 11.83(^{***})</td>
<td>19.17 ± 7.64(^{**})</td>
<td>1.31 ± 1.35(^{**})</td>
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<tr>
<td>Abnormal head (%)</td>
<td>11.1 ± 5.58</td>
<td>18.38 ± 6.97(^{**})</td>
<td>0.00</td>
<td>29.55 ± 7.71(^{***})</td>
<td>38.75 ± 7.91(^{**})</td>
<td>20 ± 0.01(^{NS})</td>
<td>36.67 ± 11.55(^{**})</td>
<td>47.85 ± 12.34(^{**})</td>
<td></td>
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<tr>
<td>Abnorm. midpiece (%)</td>
<td>7.31 ± 3.78</td>
<td>14.72 ± 8.94(^{***})</td>
<td>0.00</td>
<td>30.91 ± 6.84(^{**})</td>
<td>35 ± 12.54(^{**})</td>
<td>16.67 ± 9.31(^{NS})</td>
<td>25.83 ± 8.75(^{NS})</td>
<td>33.23 ± 13.44(^{**})</td>
<td></td>
</tr>
<tr>
<td>Abnormal tail (%)</td>
<td>4.15 ± 3.73</td>
<td>6.45 ± 3.73(^{NS})</td>
<td>0.00</td>
<td>9.55 ± 4.06(^{NS})</td>
<td>11.25 ± 5.83(^{NS})</td>
<td>10 ± 4.47(^{NS})</td>
<td>21.67 ± 15.28(^{NS})</td>
<td>16.77 ± 19.67(^{NS})</td>
<td></td>
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</tbody>
</table>

Values = mean ± SD, NS = statistically nonsignificant as compared to the fertile group, * = \(P < 0.05\), ** = \(P < 0.01\), and *** = \(P < 0.001\) compared to the fertile group.
Liq = liquefaction, vol = volume, conc. = concentration, T. sperm mot. = total sperm motility, Prog. mot. = progressive motility, and Abnorm. midpiece = abnormal midpiece.
sperm quality (motility and morphology). More than 90% of cases of male infertility are due to low sperm count or poor semen quality or both.

The prevalence of normozoospermia in our study was 32.95%, azoospermia was 10.22%, asthenozoospermia was 12.51%, asthenoteratozoospermia was 4.54%, oligozoospermia was 3.41%, oligoasthenozoospermia was 6.81%, and oligoasthenoteratozoospermia was 14.78%. The prevalence of oligoasthenoteratozoospermia observed in our study is comparable to that of Jahan et al. (2011), who observed the prevalence as 11.69% (16). The findings of the present study are also in agreement with those of Butt and Akram, who noted the prevalence of oligozoospermia as 11.11%, oligoasthenoteratozoospermia as 9.09%, and azoospermia as 14.89% (17). Similarly, the results of our work are also in agreement with the work done by Khan et al. They observed the prevalence of azoospermia as 14.28% and that of oligozoospermia as 21.43% (18). However, another study revealed the prevalence of azoospermia as 13.3%, oligozoospermia as 23.3%, normozoospermia as 14.5%, and asthenozoospermia as 35.2% in the Pakistani population (19). The incidence of azoospermia in Pakistan was reported as 12.32% and 16% in two separate studies, which is comparable to our study (20,21). In connection with that, the incidence of azoospermia in the Pakistani population is comparable to the USA and Kenya, with reported rates of 10% and 11.35% respectively (22,23).

Age and BMI are other parameters that affect the fertility of patients. In this study, age and BMI of the fertile and infertile subjects revealed no significant difference. This can be compared with similar studies of both Pakistani subjects in different cities (16) and other studies from around the world (24,25).

Alterations in seminal pH may reflect an abnormality in the functioning of the accessory sex glands or ejaculatory duct obstruction. The present study revealed no significant difference in the mean seminal pH of the fertile and infertile subjects (Table 2). Studies conducted on seminal pH levels in the USA (26), Norway (27), and in the Pakistani population (19) also expressed similar results. Similarly, in the present study, the mean semen liquefaction time of the fertile and infertile subjects

<table>
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<th>Fertile (n = 26)</th>
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<tbody>
<tr>
<td></td>
<td>NZ (n = 58)</td>
<td>AZO (n = 20)</td>
</tr>
<tr>
<td>FSH (mIU/mL)</td>
<td>8.17 ± 3.76</td>
<td>3.51 ± 3.59***</td>
</tr>
<tr>
<td>LH (mIU/mL)</td>
<td>4.22 ± 1.59</td>
<td>1.52 ± 1.91***</td>
</tr>
<tr>
<td>Testosterone (ng/mL)</td>
<td>8.57 ± 0.82</td>
<td>2.34 ± 1.50***</td>
</tr>
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</table>

Values = mean ± SD, NS = statistically nonsignificant as compared to the fertile group, * = P < 0.05, ** = P < 0.01, and *** = P < 0.001 compared to the fertile group.
showed no significant difference. Semen liquefaction time was noted as normal in all subjects (Table 2) and is comparable to the results of other studies conducted in China (28) and Pakistan (18,19).

Low semen volumes may reflect an abnormality in the functioning of the accessory sex glands. The mean semen volumes of the infertile and fertile subjects showed no significant difference (Table 2). The majority of the subjects participating in this study had a normal semen volume and this result is in agreement with the studies conducted in different parts of Pakistan (17–19) and the world (24,25,29–31). The adequate volume of ejaculated semen obtained in our study may be due to the 3–7 days of sexual abstinence prior to sample collection.

Sperm concentration in the ejaculate reflects the activity and functioning of the gonads in males and the patency of the posttesticular duct system. Studies have revealed that the pregnancy rates in couples decline as sperm concentrations decrease (32,33). Sperm concentration in the present study is outlined in Table 2 and was observed to be significantly lower in all of the infertile groups except the normozoospermic group as compared to the fertile group. Other studies in Pakistan have revealed similar results of sperm concentration in infertile men (17–19). The results are also comparable to those of a similar study by Mortimer et al., with a mean sperm density of 84.3 ± 78.3 (34).

Vitality assessment is essential to differentiate dead sperm from immotile spermatozoa. Sperm vitality is summarized in Table 2 and it was significantly lower in all of the infertile groups except the normozoospermic and normozoospermic groups as compared to the fertile group. The results of a similar study conducted on Chinese men revealed sperm viability to be 73.8% in healthy Chinese men, which is comparable to the present study (35).

Sperm motility is essential for the sperm to pass through the cervical mucus plug in order to fertilize the ovum. Total sperm motility and progressive sperm motility are summarized in Table 2 and were observed to be significantly lower in all of the infertile groups except the oligozoospermic group as compared to the fertile group. Similar results for sperm motility in fertile and infertile groups were also shown in other studies of the Pakistani population (17–19,21). A similar study conducted on the Pakistani population showed a prevalence of asthenozoospermia as 25% (17); however, a study conducted at the NIH in Islamabad, Pakistan expressed the prevalence as 21.42% (19). Yet another study observed the prevalence of asthenozoospermia as 18% (18). The mean sperm motility of the fertile, normozoospermic, and oligozoospermic subjects in our study is similar to those of other studies conducted on fertile men (24,25,29,35,36).

Normal sperm morphology, i.e. the differential development of the head, midpiece, and tail of a mature spermatozoon from the spermatid and spermatocytes, is a function of the testes as well as the epididymis. Normal sperm forms are outlined in Table 2 and were significantly lower in all of the infertile groups as compared to the fertile group. The results of our study are comparable to those of another study that showed mean normal morphology in normozoosperma samples as 65 ± 14% compared to 45 ± 15.65% in oligozoospermic samples (17). Furthermore, abnormal sperm morphology has been observed in 53% of oligozoospermic males and abnormal motility has been observed in 60% of oligozoospermic males (18). A similar pattern of abnormal sperm forms in different infertile and fertile groups was also shown by a study conducted at the NIH in Islamabad, Pakistan (19).

Sperm DNA damage is not currently included in routine clinical investigations of infertility. Investigation of sperm DNA damage has been demonstrated as a promising tool in determining a male patient’s fertility status as well as the outcomes following assisted reproduction treatment. Many techniques have been described to detect the status of sperm DNA damage, such as the sperm chromatin structure assay (SCSA), sperm chromatin dispersion test (SCD), DNA breakage detection–fluorescent in situ hybridization (DBD–FISH) assay, deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) test, in situ nick translation, comet assay, and the measurement of 8–hydroxy–2–deoxyguanosine (8–OHdG) by high performance liquid chromatography (HPLC) (37). However, little data are available to make a comparison of these methods. According to researchers (37–41), amongst the methods currently available to evaluate sperm DNA damage status, the comet assay is considered to be the most sensitive and reliable (38,41). It is the only technique that allows quantitative assessment of DNA damage in individual cells and so it is particularly useful in heterogeneous cell populations like spermatozoa. The comet assay measures single- and double-strand breaks as well as abasic sites (41). In addition, it requires only a small number of cells, making it suitable to evaluate DNA damage in semen samples (42). It has also been demonstrated to provide a stronger prognostic ability to predict fertilization after IVF than progressive motility (43).

The DNA integrity of sperm is essential for the accurate transmission of genetic information, and therefore the maintenance of good health of future generations. DNA damage in sperm may carry mutations into the next generation or may result in male infertility (44). Moreover, a compromised DNA integrity of sperm has been linked to failed fertilization (45). Sperm DNA fragmentation is depicted in the Figure and was observed to be significantly elevated in all of the infertile groups as compared to the
fertile group, indicating that subjects that had low semen quality also had higher levels of DNA damage. Sperm DNA damage was significantly increased in infertile patients suffering from varicocele (n = 32) as compared to the fertile ones and could have been due to increased exposure to reactive oxygen species (46–49). The sperm DNA damage in the infertile normozoospermic group with normal sperm parameters and the oligozoospermic group was significantly higher than that for the fertile subjects but not significantly different from that of the other infertile subgroups. It is now clear that many men with normal seminal parameters have elevated levels of sperm DNA damage. Increased sperm DNA damage may be responsible for the low fertility in men who otherwise have normal standard seminal parameters on repeated analysis and are diagnosed as unexplained or idiopathic. The mechanism(s) underlying the increased sperm DNA damage in normozoospermic males could be an inherent defect in the sperm chromatin packaging or the damage could occur after spermeation (46,47). Similar results were noted in studies conducted in Scotland (38), Spain (15), and Canada (50) that revealed the impaired semen quality observed in the infertile group was associated with a significantly increased rate of DNA damage as compared to the fertile group. Similar results were also shared in the studies conducted by Benchab (51) and Morris (10).

The reproductive hormonal measurements of the present study revealed that serum LH and testosterone levels were significantly lower in all of the infertile groups than in the fertile group, while FSH was significantly lower in all of the infertile groups as compared to the fertile group but did not differ significantly between the other infertile groups and the fertile males, as shown in Table 3. It has been previously reported that the serum levels of both LH and FSH are higher (52–54), lower (55), or unmodified (56) in infertile and azoospermic males as compared to normal males. On the other hand, no such change was observed in the FSH serum level in oligozoospermic males relative to that of fertile ones (57,58). It has been shown that high levels of intratesticular testosterone secreted by the Leydig cells are necessary for spermatogenesis. Inside the Sertoli cells, testosterone selectively binds to the androgen receptor and leads to the activation and maintenance of spermatogenesis while the action of FSH minimally serves to promote spermatogonial output by increasing the number of Sertoli cells (59,60).

The study revealed that the seminal parameters in all of the infertile groups were lower than those for the fertile group. The sperm DNA fragmentation was higher in all of the infertile subjects as compared to the fertile ones and showed a negative correlation with the seminal parameters, i.e. the sperm DNA damage increases with lower levels of semen quality. The levels of reproductive hormones were also reduced in infertile males.

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References


