Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success

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**Objective:** To evaluate sperm DNA fragmentation and semen parameters to diagnose male factor infertility and predict pregnancy after IVF.

**Design:** Prospective study.

**Setting:** Academic research laboratory.

**Patient(s):** Seventy-five couples undergoing IVF and 28 fertile donors.

**Intervention(s):** Sperm DNA fragmentation was measured by the alkaline Comet assay in semen and sperm after density gradient centrifugation (DGC). Binary logistic regression was used to analyze odds ratios (OR) and relative risks (RR) for IVF outcomes.

**Main Outcome Measure(s):** Sperm parameters and sperm DNA fragmentation in semen and DGC sperm compared with fertilization rates, embryo quality, and pregnancy.

**Result(s):** Men with sperm DNA fragmentation at more than a diagnostic threshold of 25% had a high risk of infertility (OR: 117.33, 95% confidence interval [CI]: 12.72–2,731.84, RR: 8.75). Fertilization rates and embryo quality decreased as sperm DNA fragmentation increased in semen and DGC sperm. The risk of failure to achieve a pregnancy increased when sperm DNA fragmentation exceeded a prognostic threshold value of 52% for semen (OR: 76.00, CI: 8.69–1,714.44, RR: 4.75) and 42% for DGC sperm (OR: 24.18, CI: 2.89–522.34, RR: 2.16).

**Conclusion(s):** Sperm DNA testing by the alkaline Comet assay is useful for both diagnosis of male factor infertility and prediction of IVF outcome. (Fertil Steril 2011;95:652–7. ©2011 by American Society for Reproductive Medicine.)

**Key Words:** Clinical pregnancy, Comet assay, IVF outcome, male factor infertility, sperm DNA fragmentation

Infertility is experienced by 15% of couples attempting to have a family (1) with male factor infertility contributing 40% of these cases (2). Conventional semen analysis continues to be the only routine test to diagnose male factor infertility, although semen parameters have a limited power to predict spontaneous or assisted conception (3). In contrast, a plethora of studies have indicated that sperm DNA damage is closely associated with male factor infertility and may be a useful tool to diagnose male factor infertility and predict assisted reproductive technology (ART) success (4, 5).

Couples undergoing IVF treatment can be divided into those with female, male, and unexplained infertility (6). A large proportion of couples undergoing IVF treatment have female causes and after standard infertility evaluations of semen analysis, and assessment of ovulation and tubal function (7), approximately 15% of couples still have unexplained infertility (8). In this study, to evaluate the effects of sperm DNA damage, couples with female factors or without detectable fertility problems from either partner (idiopathic infertility) were excluded and only couples diagnosed with male factor infertility by presenting abnormal semen parameters according to World Health Organization (WHO) criteria were included.

In the present study the alkaline Comet assay was used. It is highly reproducible (9), with greater sensitivity than other assays (10, 11). At present clinical thresholds have not been reported for sperm DNA testing using the Comet test. The objective of this study is to determine the predictive power of sperm DNA damage to [1] diagnose male factor infertility and [2] predict IVF success.

**MATERIALS AND METHODS**

This project was approved by the Office for Research Ethics Committees in Northern Ireland and the Royal Group of Hospitals Trust Clinical Governance Committee. The study was conducted at the Regional Fertility Centre, Royal Jubilee Maternity Services, Belfast, Northern Ireland, United Kingdom. Sperm samples for research were obtained after written consent was given by each couple.

**Study Population**

Seventy-five infertile men aged 22–46 years, undergoing IVF treatment, were included in the study with the following criteria: [1] a minimum of 1 year unprotected intercourse without pregnancy, [2] no physiological disorders leading to male factor infertility, [3] subnormal semen analysis according to WHO criteria (12), [4] with female partners aged <38 years with no known cause of infertility, and [5] with fresh ET. Sperm from 28 fertile men was obtained from Cryos International, Aarhus, Denmark. Each donor had a seminal profile exceeding minimal characteristics by WHO guidelines. Of the 75 couples initially included, 5 couples had embryos frozen before ET, therefore clinical pregnancy outcomes were obtained for only 70 cases.
TABLE 1

Demographic data on IVF treatment.

<table>
<thead>
<tr>
<th>IVF</th>
<th>Pregnant</th>
<th>Nonpregnant</th>
<th>CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Couples included (n)</td>
<td>20</td>
<td>50</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Female age (y)</td>
<td>33.4 ± 0.9</td>
<td>34.4 ± 0.5</td>
<td>–3.0–1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Oocytes retrieved</td>
<td>9.6 ± 1.4</td>
<td>8.2 ± 0.7</td>
<td>–1.5–4.4</td>
<td>NS</td>
</tr>
<tr>
<td>Oocytes fertilized (with 2 pronuclei)</td>
<td>6.4 ± 0.7</td>
<td>3.9 ± 0.4</td>
<td>0.7–4.3</td>
<td>.007</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>73.9 ± 5.3</td>
<td>51.2 ± 4.6</td>
<td>6.5–38.8</td>
<td>.007</td>
</tr>
<tr>
<td>Total embryo cumulative score</td>
<td>12.0 ± 1.4</td>
<td>7.8 ± 1.1</td>
<td>0.2–8.2</td>
<td>.038</td>
</tr>
<tr>
<td>Male age (y)</td>
<td>35.9 ± 1.1</td>
<td>37.6 ± 0.6</td>
<td>–4.3–0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Semen volume (mL)</td>
<td>2.6 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>–1.2–0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Sperm concentration (10^6/mL)</td>
<td>52.6 ± 7.1</td>
<td>51.4 ± 5.2</td>
<td>–17.8–20.3</td>
<td>NS</td>
</tr>
<tr>
<td>Total sperm output (10^9)</td>
<td>131.6 ± 20.5</td>
<td>150.8 ± 21.2</td>
<td>–91.6–53.3</td>
<td>NS</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>46.8 ± 4.2</td>
<td>44.2 ± 2.1</td>
<td>61.1–11.4</td>
<td>NS</td>
</tr>
<tr>
<td>Normal morphology (%)</td>
<td>32.7 ± 4.3</td>
<td>26.3 ± 1.4</td>
<td>–0.7–13.5</td>
<td>NS</td>
</tr>
<tr>
<td>DNA fragmentation in native semen (%)</td>
<td>33.8 ± 3.6</td>
<td>68.5 ± 2.3</td>
<td>43.3–26.0</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>DNA fragmentation in DGC sperm (%)</td>
<td>23.2 ± 2.8</td>
<td>50.3 ± 2.3</td>
<td>35.5–18.7</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Note: Values expressed as mean ± SD; NS = not significant to P>.05. CI = 95% confidence interval.


Semen Analysis and Sperm Preparation

Semen samples, surplus to clinical requirements, were collected by masturbation from the infertile men on the day of IVF treatment after 2–5 days of recommended abstinence. After liquefaction, routine semen analyses were performed according to WHO guidelines (12) and subsequently, semen was prepared using a two-step discontinuous Puresperm gradient (90%–45%; Hunter Scientific Limited, Saffron Walden, United Kingdom). Two populations of sperm were used to measure DNA damage by Comet assay: the whole population (native semen) and that used for clinical treatment (DGC) for each patient.

IVF Treatment

All IVF cycles (no intracytoplasmic sperm injection [ICSI] cycles were included) were performed according to standard procedures. One or two embryos were transferred into the uterine cavity after an additional 24–48 hours. An intrauterine pregnancy with fetal heart beat was confirmed by ultrasound 5 weeks after ET.

Alkaline Comet Assay

Sperm DNA fragmentation was assessed using single cell gel electrophoresis (Comet) assay, previously optimized for human sperm by our group (13, 14). Our previous study has reported an intra-assay coefficient variation (CV) of 6% for this assay (9).

Statistical Analysis

Data were analyzed using SPSS version 15 for Windows (SPSS Inc., Chicago, IL). Data are presented as mean ± SE. The fertilization rate was calculated as the percentage of all fertilized oocytes. The embryo quality as embryo cumulative score was calculated only in the day 3 embryos (n = 60) by multiplying embryo grade (A = 4, B = 3, C = 2, and D = 1) with the number of blastomeres for each embryo and where a patient had more than one embryo, a mean across embryos was calculated to obtain total quality of all embryos generated.

Semen parameters, male age, and female age were compared for pregnant and nonpregnant couples using the independent sample t-test. Each of the semen parameters was categorized into normal and abnormal categories according to WHO criteria and the odds ratio (OR) to obtain a good fertilization rate (>50%), good embryo quality (embryo cumulative score >16), and pregnancy were determined, if one semen parameter was suboptimal according to the WHO criteria (12). Spearman’s rank correlation coefficient was used to analyze the relationship between semen parameters and sperm DNA fragmentation with IVF outcome.

Logistic regression was used to evaluate the relationship between sperm DNA fragmentation and male infertility, and to compare pregnant and nonpregnant couples. Predicted probability with 95% power was used to calculate the diagnostic and prognostic threshold values. Based on these threshold values OR with 95% confidence intervals (CI), receiver operating characteristics (ROC) curve, sensitivity, specificity, positive and negative predictive power, and relative risk in predicting a successful pregnancy were computed.

RESULTS

There was no difference in any semen parameter or age between groups (Table 1). When the semen parameters were separated into normal and abnormal categories, if one semen parameter was suboptimal according to the WHO criterion, there was no correlation between any semen parameter, sperm DNA fragmentation, and IVF outcomes (Table 2). However, there was a significant positive correlation between sperm motility and fertilization rate (r² = 0.27, P = .03). The OR to obtain a fertilization rate (>70%) with progressive motility (50%) was 5.25 (95% CI: 1.19–24.97).

There was a significant difference in DNA fragmentation of sperm from infertile men (57.92 ± 2.67) and donors (12.47 ± 1.67; P < .001). Ninety-five percent of the fertile population had sperm DNA fragmentation below 25%, whereas only seven men (10%) of the infertile group had sperm DNA fragmentation below 25%. Using this threshold value of 25% DNA fragmentation for the diagnosis of male infertility, the OR (95% CI) was 117.3 (12.73–2,731.83; Table 3). Men with DNA fragmentation more than this threshold value had a relative risk for infertility of 8.75 (95% CI: 4.48–17.08). This diagnostic threshold value showed an area under the ROC of 0.970 cm² with 63.6% sensitivity and 98.5% specificity.

There was a significant decrease in fertilization rate as DNA damage of native semen (r² = −0.243, P = .050) and DGC sperm (r² = −0.276, P = .025) increased. Similarly, an increase in DNA

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damage in native sperm led to a decrease in embryo quality ($r^2 = -0.415, P = .002$) and in DGC sperm ($r^2 = -0.373, P = .007$). The mean percentage of sperm DNA fragmentation was significantly higher in nonpregnant couples ($n = 50$) compared with that of pregnant couples ($n = 20$) in both native semen ($33.8 \pm 3.6$ vs. $68.5 \pm 2.3$) and in DGC sperm ($23.2 \pm 2.8$ vs. $50.3 \pm 2.3$; Table 1). Using a clinically prognostic threshold value (52% for native semen and 42% for the DGC sperm; Fig. 1) to predict successful pregnancies after IVF, the OR (95% CI) was 76.00 (8.69–1,714.44) and 24.18 (2.89–522.34), respectively (Table 3). Measurement of sperm DNA fragmentation in the native semen had a higher specificity (80% vs. 56%) and positive predictive value (66% vs. 46%) to determine IVF pregnancy outcome than the DGC sperm. Men with sperm DNA fragmentation more than the threshold values had an increased relative risk (4.75 for native semen and 2.16 for the DGC sperm) of not achieving a clinical pregnancy. The prognostic ability of these threshold values was supported by the ROC curve, which showed an area of $0.905 \text{ cm}^2$ for the native semen and $0.879 \text{ cm}^2$ for the DGC sperm (Table 3).

Three zones of clinical importance can be marked for successful and unsuccessful couples based on their sperm DNA fragmentation against the threshold values. Zone A is that more than the threshold values, where all couples are unsuccessful after IVF. Zone B is the region less than the threshold value (40%–52%) in the native semen and (28%–42%) in the DGC sperm, where couples unsuccessful after one attempt may be successful by having additional IVF cycles. Three of the four couples in zone B were successful after second attempt. Zone C, less than 40% in the native semen and less than 28% in the DGC sperm, depicts the population of couples likely to be successful after IVF (Fig. 1).

**DISCUSSION**

Semen analysis is currently used both to diagnose the fertility potential of the male partner and to decide the most suitable type of treatment for the couple. Although it is an essential first step in assessing male fertility (15), semen parameters have repeatedly failed to distinguish between fertile and infertile men (16, 17), therefore it is necessary to identify novel biomarkers with greater accuracy. Abnormalities in conventional semen parameters may interfere in the delivery of sperm to the oocyte, thereby resulting in failure to conceive spontaneously. However, postfertilization, these factors become unimportant, whereas sperm DNA quality is crucial from fertilization to delivery. The present study tests the effects of sperm DNA fragmentation, first, on the diagnosis of male factor infertility and second, on the prognosis of IVF outcome, in couples presenting with male factor infertility.

As recommended by Giwercman et al. (17), this study includes only couples presenting with male factor infertility, as defined by WHO criteria. The age of the female partner was standardized, as it has long been recognized as one of the most significant factors in predicting the couples’ fertility. This holds true over a range of
categories from the length of involuntary infertility to strong associations with clinically detected causes of female infertility (18–23). For these reasons we included only women within a young (<38 years) and tightly matched age range (Table 1).

This study shows that there is generally no correlation with conventional semen parameters and IVF success (Table 2). Furthermore, we observed only an inverse correlation between DNA damage and motility, but with no other semen parameter, indicating that sperm DNA damage is important to determine male factor infertility. A reduction in DNA fragmentation was also seen in all samples after DGC. This was probably due to the removal of senescent and defective sperm (24–36). However, it is in conflict with some reports (37–40).

By eliminating known female factors and unexplained causes of infertility, we conclude that sperm DNA fragmentation is an independent factor to diagnose male infertility. Male partners of infertile couples who present with semen quality less than WHO criteria and have native sperm DNA fragmentation >25% by Comet testing, have a high risk of male factor infertility with an OR of 117.25 and relative risk 8.75. Therefore, for accurate diagnosis of male factor infertility, conventional semen analysis should be supplemented with sperm DNA damage testing.

Our second objective was to determine the prognostic value of sperm DNA testing with the alkaline Comet assay. In agreement with previous studies (41–48), we report a significant decrease in fertilization rate as DNA damage of both native semen and DGC sperm increased. Sperm DNA damage is associated with abnormal chromatin packing indicated by alterations in protamine content (49), therefore the reduction in fertilization may be due to a failure of sperm DNA decondensation (50, 51). In contrast, some studies show no correlation between sperm DNA damage and fertilization rate (24, 32, 52–55).

A good fertilization rate is necessary to maximize the number of embryos available for transfer (56). In IVF, strong progressive motility is essential for the sperm to penetrate the extracellular matrix and surrounding membranes of the oocyte in vitro for fertilization to occur (57, 58). In support of this, we noticed that the influence of progressive motility on the fertilization rate (>70%) in vitro had an OR >5.0. Our study also showed a strong negative relationship between sperm DNA fragmentation and embryo quality in both native and DGC sperm. Again, this is in contrast to studies that show no association between embryo quality and sperm DNA damage measured by sperm chromatin structure assay (SCSA) and terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay (10, 24, 43–45, 47, 52–55, 59, 60). Such contradictions could result from the different sensitivity of tests or specific types of DNA damage measured by each tests.

Most important, this study is in agreement with previous reports showing that sperm DNA fragmentation is associated with IVF pregnancy (17, 24, 52, 59, 61–64). In contrast, other studies have also showed no relationship between sperm DNA damage and clinical pregnancies (32, 37, 42–48, 53, 54, 65–68). One explanation for the conflict lies in the patient inclusion factor. These studies have included couples with female factors, therefore the effect of sperm DNA damage on pregnancy is compromised by female infertility factors. For example, in the study by Payne et al. (45), more than half of the couples had been diagnosed with female infertility. Such inclusions confound any data generated. In our study we...
have carefully excluded female factors. We obtained high ORs in both native semen and DGC sperm, (76 and 24, respectively) and the elimination of both female and unexplained infertility contributions may well have contributed to this powerful finding.

We found all, but one couple, who achieved a clinical pregnancy had sperm DNA fragmentation <52% in native semen and <42% after DGC. Of the 40 samples with >52% DNA damage, 26 samples also had damage >42% after DGC, thus remaining in zone A (Fig. 1). None of these couples achieved a clinical pregnancy. Seventy-five percent of the couples having their second attempt in (42, 44, 46, 53, 59, 70, 71), and for SCSA, 20% (68), 27% (24, 33). Adequate, the threshold levels for TUNEL assay vary from 4%–35%, and OR must be calculated. These vary enormously (69) depending again the prognostic value of sperm DNA damage in both populations of sperm.

To quantify the clinical usefulness of a test, its threshold value and OR must be calculated. These vary enormously (69) depending on the assay, preparation and, not least on scientific choice. For example, the threshold levels for TUNEL assay vary from 4%–35% (42, 44, 46, 53, 59, 70, 71), and for SCSA, 20% (68), 27% (24, 32), 30% (45, 61, 72, 73). This wide variation in threshold values reflects the absence of standardized laboratory protocols for TUNEL assay, its use in native and DGC populations, patient groups, and the type of ART treatment performed. Of all the DNA tests commonly used, a robust threshold value (30%) has only been established for the SCSA (55, 74, 75). In our study, we calculated, for the first time, prognostic thresholds for native and DGC sperm using the Comet assay. Our thresholds of 52% for native sperm and 42% for DGC sperm are higher than the 30% threshold for native semen using SCSA. This is due to the sensitivity of the Comet assay in that all double and single strand breaks, throughout the entirety of relaxed chromatin are revealed, in contrast to other assays where perhaps more peripheral DNA damage is determined. The sensitivity and accuracy of the Comet assay was also confirmed by the wide range of sperm DNA damage among the men analyzed in this study, 4%–98% in the native semen and 3%–82% in the DGC sperm (Fig. 1).

When all the studies using TUNEL and SCSA were drawn together by meta-analyses, a weak impact (OR: 1.44) of sperm DNA damage was observed on IVF outcomes (69, 76) compared with our study showing 76.00 and 24.18 in native semen and DGC sperm, respectively. Again, such conflict in the literature is probably due to differences in methodology (between and within DNA tests) and patient groups. In conclusion, our study provides robust evidence to show that the sperm DNA fragmentation is a useful biomarker of both male infertility and predicted IVF outcome. Measurement of sperm DNA fragmentation in native semen and also in DGC sperm using the alkaline Comet assay offers the sensitivity and specificity needed to determine IVF success.

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