The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development

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BACKGROUND: The integrity of sperm DNA is important for the success of natural or assisted fertilization, as well as normal development of the embryo, fetus and child. ICSI, by bypassing sperm selection mechanisms, increases the risk of transmitting damaged DNA and the significance of this requires investigation. METHODS: DNA damage in sperm from an unselected group of 60 men undergoing IVF treatment was measured by single cell gel electrophoresis (Comet assay) and correlated with semen and treatment cycle parameters. RESULTS: Wide spectra of sperm DNA damage were found both within and between men but no specific subgroups were identified. Semen and treatment cycle parameters were not different in men grouped according to high or low sperm DNA damage. However, regression analysis showed that DNA damage was positively associated with age (29–44 years), abnormal sperm and motility and negatively associated with sperm concentration. In ICSI cycles DNA damage was positively associated with impairment of post-fertilization embryo cleavage. CONCLUSIONS: This study contributes to the evidence of DNA damage within sperm. High loads of DNA damage measured by the Comet assay were predictive of failure of embryo development after ICSI. As it is likely that sperm with DNA damage contributed to successful fertilization and in-vitro development, potential adverse effects remain to be clarified.

Key words: Comet analysis/DNA damage/fertility/human/sperm

Introduction

Male factor infertility remains a significant problem contributing to ~50% of the cases attending infertility clinics (Lamb and Lipshultz, 2000; Oehninger, 2000). Clinical evaluation of the contribution of the man towards the infertility of the couple is usually confined to measures of total sperm count and concentration, abnormal forms, motility and seminal factors such as pH and antisperm antibodies. The predictive value of these measurements is limited although some progress has been made in recent years by the introduction of standardized techniques, automation of motility estimations and the organization of quality assurance schemes for semen analysis (Van Voorhis and Sparks, 1999; Oehninger, 2000; Sakkas and Tomlinson, 2000). Although these analyses may describe 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. Abnormalities in the male genome are, however, a clear potential reason for post-fertilization failure (Lopes et al., 1998a; Sakkas et al., 2000; Aitken and Krausz, 2001), and, in rodent studies, embryo toxicity has been associated with chemical- and radiation-induced sperm DNA damage (Hales and Robaire, 1997). The development of ICSI to introduce the male genome directly into the oocyte has been a major advance in the success of assisted reproduction. ICSI overcomes many of the factors related to the production of adequate numbers of sperm as well as impaired fertilization mechanisms. However, since sperm are selected for ICSI based on motility and gross morphology, concern has been expressed that such methods bypass natural selection and could inadvertently introduce a defective paternal genome (Campbell and Irvine, 2000; Hargreave, 2000; Mortimer, 2000; Sakkas et al., 2000). Some progress has been made in addressing this issue and epidemiological analysis of the children arising from ICSI has produced few data of major concern although the risk remains controversial (Palermo et al., 2000; Tarlatzis and Bili, 2000; Ericson and Kallen, 2001; Sutcliffe et al., 2001). As the numbers of children conceived by ICSI is small and they are still young it is possible that deleterious genetic effects which are either subtle, low in frequency or are not expressed until adulthood remain to be discovered.

To assist in the risk assessment of ICSI, it would be appropriate to develop methods to measure DNA damage in the sperm and to correlate this with biological outcomes. DNA abnormalities in sperm are well documented. Cytogenetic
analysis of sperm chromosomes has demonstrated sperm aneu-
plody, which, although low in frequency, is associated with
infertility and adverse pregnancy outcome (Egozcue et al., 2000; Shi and Martin, 2000). Genetic information in the sperm
genome may be mutated or deleted altogether which may be
the cause of some cases of male infertility (Hargreve, 2000;
Foresta et al., 2001). However, it has also become clear that
other subtle genetic changes may be occurring. The nature of
these is not well documented but could give rise to a spectrum
of responses including: failure of fertilization, failure of
preimplantation embryo development, early pregnancy loss or
fetal abnormalities (Hales and Robaire, 1997; Sakkas et al.,
2000; Shen and Ong, 2000).

Several techniques are available to examine the integrity of
sperm DNA. The sperm chromatin structure assay (SCSA)
measures the susceptibility of the DNA to acid denaturization
(Evenson et al., 1999). Abnormal chromatin structure is
measured by flow cytometry that records the ratio of denatured
to native DNA. High ratios correlate with sperm concentra-
tion and sperm head abnormalities. Additionally it has been
shown that if the percentage of cells with abnormal ratios
exceeds 30–40% then fertility is unlikely (Evenson et al.,
1999; Larson et al., 2000; Spano et al., 2000). DNA
strand breaks in sperm have also been directly detected by a
variety of techniques. The terminal deoxynucleotidyl trans-
ferase-mediated dUTP nick-end labelling (TUNEL) assay identifies double and single DNA strand breaks by
enzymatically labelling the free 3’ OH end of the DNA with
a fluorescent substrate. TUNEL-positive cells are identified
either microscopically or by fluorescence-activated cell sorting
(FACS). In the majority of samples only ~5% of sperm have
TUNEL-detectable DNA damage (Manicardi et al., 1995;
Arunvinda et al., 1997; Sun et al., 1997; Donnelly et al., 2000;
Irvine et al., 2000; Oosterhuis et al., 2000; Ramos and Wetzels,
2001). Sperm with DNA strand breaks may persist into the
ejaculate because of a failure in the mechanism of apoptosis,
which normally eliminates them during spermatogenesis
(Sakkas et al., 1999) or alternatively may arise in the re-
productive tract as a result of oxidative damage (Aitken and
Krausz, 2001). Fertilization after ICSI or IVF is reduced if
sperm are retrieved from ejaculates containing high numbers
of TUNEL-positive cells. Additionally, it has been shown that
paternal smoking increases sperm DNA damage measured by
the TUNEL technique and this has been suggested to account
for the increase in childhood cancer (Sun et al., 1997; Potts
et al., 1999).

The Comet assay is extensively used in somatic cells to
measure genotoxic damage, especially single and double strand
breaks, and was originally applied to sperm by Singh (Singh
et al., 1989). This study demonstrated that sperm DNA was
extremely labile in the presence of alkali and it was difficult
to distinguish the level of damage between individual sperm.
However, the Comet assay has been modified and used in
other laboratories to investigate DNA damage in sperm and
its relationship to infertility. Irvine and colleagues showed that
men attending infertility clinics had a higher level of DNA
damage in their sperm, which was also negatively related to
semen concentration (Irvine et al., 2000). Similar DNA changes
could be generated by in-vitro treatments producing oxidative
damage (Aitken et al., 1998; Twigg et al., 1998a; Donnelly
et al., 1999; Shen and Ong, 2000; Ramos and Wetzels, 2001).
However, another study did not detect a difference in DNA
damage between infertile men and normal controls (Hughes
et al., 1996), although subsequent reports suggest that DNA
damage measured by the Comet assay is selectively increased
in the sperm from infertile men after cryopreservation or in-
vitro X-ray radiation (Hughes et al., 1996; Donnelly et al.,
2001). Results from the Comet assay are also correlated with
DNA damage measured by the TUNEL and SCSA methods
(Arunvinda et al., 1997; Donnelly et al., 2000). Close inspection
of these publications reveals significant differences in the
protocols used to treat the cells prior to electrophoresis in an
alkaline buffer. These methodological differences have arisen
from the difficulties encountered in releasing the DNA from
the sperm head due to the unique DNA compaction (Ward
and Coffey, 1991). Combined with the alkaline lability of
sperm DNA, the difficulties encountered in releasing the DNA
electrophoresis have hindered the development of the
Comet assays for comparative studies of male reproduction.
Our laboratory has recently developed a neutral Comet assay
to provide quantitative measures of DNA damage in human
and murine sperm (Haines et al., 1998). This method will
produce a linear increase in DNA damage of sperm irradiated
in vitro at doses that would be expected to produce DNA
strand breakage. We have demonstrated that, in the mouse,
spermatogonial radiation with external X-rays or internal
isotopic contamination results in the appearance of substantial
damage in DNA of the sperm (Haines et al., 2001). These in-
vivo treatments are known to be genotoxic and adversely
effect fertility and reproduction. Additionally we have also
shown that during chemotherapy using fludarabine of a patient
with chronic lymphocytic leukaemia there was a substantial
increase in the sperm DNA damage when measured by the
Comet assay (Chatterjee et al., 2000). DNA damage may be
a good biomarker to relate to fertility problems; moreover, as
the Comet assay is technically straightforward and inexpensive
it may be suitable for routine measurement of DNA damage
once validated by independent laboratories. We have therefore
examined DNA damage in men attending for IVF/ICSI treat-
ment using the Comet assay and correlated the DNA damage
profiles in their semen with treatment cycle outcome.

Materials and methods

Patient selection
Sixty couples requesting IVF treatment at St Mary’s Hospital,
Manchester were recruited into this study after providing written
informed consent for sperm research and with local ethics committee
approval. All women were aged <40 years, men <55 years, and
the couples were childless. Forty of these couples were selected for ICSI
according to guidelines developed at St Mary’s Hospital, based
on semen analysis parameters (World Health Organization, 1999),
fertilization performance in previous cycles, and previous history of
spontaneous conceptions. The other 20 couples received conven-
tional IVF.
**IVF/ICSI treatment cycles**

Ovarian stimulation was achieved using a conventional long protocol down-regulation involving pituitary desensitization with buserelin. Exogenous FSH was administered by a step-down protocol with an initial starting dose between 75 and 450 IU and adjusted throughout the cycle following monitoring of serum estradiol levels. HCG was administered when three or more follicles reached ≥17 mm, and oocytes were recovered 36 h later by ultrasound-guided retrieval. Semen samples were produced by masturbation and analysed for sperm concentration (×10⁶/ml), percentage of sperm which were progressively motile, and percentage abnormal forms, then prepared by density gradient centrifugation using standard protocols. Briefly, sperm were washed free of seminal plasma using sperm culture medium (Medi-Cult UK Ltd, Redhill, Surrey, UK) layered upon a 40: 80% PureSperm (Hunter Scientific, Saffron Walden, Essex, UK) gradient, centrifuged at 600 g for 20 min and resuspended in the sperm culture medium (Horne et al., 1997). For IVF treatment, oocytes were inseminated 4–6 h post recovery with ~1×10⁵ sperm/ml. For ICSI treatment, single motile sperm of normal morphology were microinjected into each oocyte using standard protocols (Van Steirteghem et al., 1993). An aliquot of each sample used for IVF or ICSI was immediately snap-frozen at −20°C until analysed for DNA damage by the Comet assay. Oocytes were cultured overnight at 37°C in 200 µl of Universal IVF medium (Medi-Cult UK).

**Fertilization and embryo development**

Oocytes were assessed for the presence of two pronuclei 16–18 h after insemination, indicative of normal fertilization (day 1). Up to six normally fertilized zygotes were maintained in culture to cleave to the 2- or 4-cell stage (day 2), whilst excess zygotes were immediately cryopreserved. Two embryos, or occasionally one or three, were replaced in the uterus on day 2 following selection based on developmental stage and morphological criteria (Steer et al., 1992). Embryos received a stage score, with one point awarded for each intact blastomere, and a morphological grade, with 4 points awarded for even blastomeres with no fragmentation, 3 points for embryos with up to 10% fragmentation and so on. This scoring system is thought to reflect anucleate fragmentation resulting from cytokinesis and/or loss of blastomeres by apoptosis. The percentage of embryos which failed to cleave (remain at 1-cell) was also scored. Cryopreserved embryos were thawed and replaced in subsequent cycles at patient request. Implantation of fresh or frozen embryos was assessed by measuring serum β-hCG levels 14 days after replacement, clinical pregnancy was confirmed by presence of a fetal heart on scan at 6 weeks, and live birth data were obtained by patient follow-up.

**Comet DNA damage assay**

Single cell gel electrophoresis of sperm DNA (Comet assay) was performed as previously described (Haines et al., 1998). Briefly, sperm cells were thawed rapidly at room temperature, cast into miniature agarose gels on microscope slides and lysed in situ to remove DNA associated proteins and allow the compacted DNA to be relaxed. Lysis buffer (Tris 10 mmol/l, 0.5 mol/l EDTA and 2.5 mol/l NaCl, pH 10) contained 1% Triton X-100, 40 mmol/l dithiothreitol and protease K, 100 µg/ml). Microgels were then electrophoresed (20 min at 25V/0.01A) in neutral buffer (Tris 10 mmol/l containing 0.08 mol/l boric acid and 0.5 mol/l EDTA, pH 8.2), during which the damaged DNA migrated from the nucleus towards the anode. DNA was visualized by staining of the slides with the fluorescent DNA binding dye SYBR Green I (Molecular Probes, Oregon, USA) and sperm identified by size and the presence of a tail. Comet measurements performed were tail length, tail moment and percentage tail DNA using a Nikon Epiphot II epifluorescence microscope and Comet Assay II software (Perceptive Instruments, Haverhill, UK); 100–120 cells were analysed per semen sample (two duplicate sample slides, 50–100 randomly selected cells scored per slide, up to 200 sperm in total). Measurements between the two slides were highly reproducible. For example, when the mean moments were compared for 30 randomly selected samples with moments ranging from 0.7 to 30, the values between the two slides were highly correlated (P < 0.0001, r² = 0.975). Since intra-sample variation in this assay is so low as to be negligible, data are presented throughout as the mean of the sperm analysed on the two slides.

**Statistical analysis**

Data were analysed using SPSS software (SPSS Inc., Chicago, IL, USA). Comet parameters were subjected to further analysis using mean (if normally distributed) or median (if not normal) values. Principle component analysis was used to separate sperm into two populations, which were compared using the non-parametric Mann-Whitney U-test. Multivariate stepwise regression analysis was used to establish relationships between Comet parameters and semen analysis or treatment cycle parameters.

**Results**

DNA damage in the sperm populations collected from 60 unselected men attending for IVF treatment was assessed by the neutral Comet assay. All sperm produced measurable Comet parameters and the summary data are presented in Table I.

**Patient variation in DNA damage profiles**

Upon inspection of the DNA damage moments and tail lengths in the sperm populations, it became clear that there was a wide range of damage distribution profiles. Examples of low, medium and high DNA damage profiles are given in Figures 1–3. Figure 1 shows the DNA moment frequency distribution in sperm with a population profile that is skewed towards zero and the median value is low. Over 50% of sperm in this sample record moments between 5 and 10 and only a small percentage of sperm have large moments of >30. Comet tail lengths were essentially normally distributed although there were a few sperm with short tail lengths. Figure 2 shows an example of a sperm population in which both the tail moment and length were apparently normally distributed, the medians being close to that for all samples in this study. In contrast, some sperm populations contained a substantial number of sperm with high levels of DNA damage (Figure 3). In this particular sample the levels of damage measured by the tail moment, although high, appeared normally distributed. In other samples, however, the moments were skewed towards the

<table>
<thead>
<tr>
<th>Table I. Summary of Comet measurements for the cohort of men (n = 60) investigated in this study</th>
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<tbody>
<tr>
<td>Tail moment</td>
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<tr>
<td>Mean</td>
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<tr>
<td>SD</td>
</tr>
<tr>
<td>Median</td>
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<tr>
<td>Range</td>
</tr>
</tbody>
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higher values with a median ~60, which is about the maximum value that can be achieved using this method as the DNA will not migrate further during the electrophoresis. Tail lengths tend to be longer than in samples with lower levels of DNA damage; in the example shown the distribution is skewed towards the maximum.

Analysis of populations with low and high DNA damage

In view of these observations, the distribution data for tail moments in each sample were analysed by principle components analysis in order to see if the DNA damage profile data could be categorized into one or more populations. For each sample the moments (arbitrary units) calculated for 100 sperm were allocated to size bands of 0–10, 10–20, 20–30, 30–40, 40–50 and >50 and the distribution data converted to the natural log before analysis. The principal component graph (Figure 4) shows that there is a continuum of sperm DNA damage in the different semen samples from low to high values rather than discrete subpopulations.

We have separated these data into groups with either low or high sperm DNA damage profiles (population 1 and 2; Figure 4) for further analysis. The Comet assay parameters (median and range) for population 1 (high DNA damage; n = 31) were: tail moment 33.7 (5.5–39), tail length 113 (74–118), whereas those for population 2 (low DNA damage, n = 22) were tail moment 17.45 (4–49) and tail length 84 (39–145). There was a significant difference between populations 1 and 2 in tail moment ($P < 0.001$) but not in tail length ($P < 0.08$, Mann–Whitney U-test). A comparison of the semen analysis...
and treatment cycle parameters (Age, abstinence, semen volume, sperm concentration, sperm motility, percentage abnormal forms, percentage fertilized oocytes, percentage cleaved embryos, embryo stage, grade and clinical pregnancies) for samples from populations 1 and 2 was also made but none were significantly different. Interestingly, however, percentage fertilization, percent cleavage, embryo cell number and embryo grade all showed a tendency to be lower in the group 1 patients. There were 15 pregnancies (25% per cycle) and 12 live births (20%), evenly distributed between groups 1 and 2. However, of the nine group 1 pregnancies, three ended in early miscarriage, whereas all of the six pregnancies in group 1 yielded live births. Table II gives the summary statistics for all patients.

**Multivariate analysis**

Further investigation of the relationships between the DNA damage tail moment or tail length and clinical measurements were made using multivariate stepwise regression analysis. In addition to the parameters given in Table II, some data modifications were made. Embryo cleavage was subdivided into two groups (50–84 and 100%) and embryo development into three groups: (1) no embryo produced (i.e. 0% cleavage), (2) embryo grade <3.5, and (3) embryo grade ≥3.5.

The summary results from the statistical analysis for all the samples are given in Table III. For many parameters there was no correlation with the measurements of DNA damage, however, only significant correlations are included here.

However, both Comet tail length and moment were significantly correlated with age (\(P = 0.023\) and \(P = 0.032\) respectively) indicating that between the ages of 29–47 years the levels of DNA damage in sperm increased significantly. Additionally, tail length increased with sperm motility (\(P = 0.011\)) and tail moment increased as the proportion of abnormal forms increased (\(P = 0.013\)). Table IV presents the data analysed according to whether the sperm were used for either IVF or ICSI. IVF and ICSI men were not significantly different in age or semen characteristics such as volume or sperm concentration. However, ICSI samples did have a lower motility (\(P = 0.02\)) and percentage morphologically normal forms (\(P = 0.04\)), as expected. IVF and ICSI samples did not differ significantly in their median tail moment or length. In the ICSI group, the associations between DNA damage and age and motility were preserved, whereas the correlation with abnormal forms was lost (although this was not strong in the overall analysis). Interestingly there was a strong inverse correlation between sperm concentration and DNA damage. Furthermore, analysis of embryo cleavage showed that as DNA damage measured by the tail moment increased, embryo cleavage was impaired. Only one significant correlation was obtained for the IVF patients—between tail length and motility (%)—which confirms the association seen in the other analyses. However, as the IVF group size was small it is unlikely that the analysis possessed the power to reveal or confirm the associations documented in Tables III and IV (ICSI).

**Discussion**

The results show that the Comet method we have developed produces an estimate of the DNA damage in every sperm sample (100) and that in sperm from a population of men attending for IVF treatment there is a very wide spectrum of DNA damage both within and between men. This spectrum of DNA damage reflects a continuum, not discrete subpopulations, of DNA-damaged sperm from either the individual men (individual histograms) or the patient population (principal components analysis). This result may have arisen from the deliberate lack of selection of men undergoing IVF/ICSI treatment who have produced a wide spectrum of semen profiles. Others have conducted Comet analysis of DNA damage in human sperm and have recorded the summary statistic of the values for the individual sperm so that the characteristics of the sperm populations have not previously

### Table III. Multivariate stepwise linear regression analysis of Comet tail length and moments versus semen and treatment cycle measurements

<table>
<thead>
<tr>
<th>Variable</th>
<th>Dependent variable</th>
<th>Regression coefficient</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Tail length</td>
<td>1.432</td>
<td>0.023</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>Tail length</td>
<td>0.291</td>
<td>0.011</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Tail moment</td>
<td>0.669</td>
<td>0.032</td>
</tr>
<tr>
<td>Abnormal sperm (%)</td>
<td>Tail moment</td>
<td>0.272</td>
<td>0.013</td>
</tr>
</tbody>
</table>

All the parameters included in Table II were entered into the analysis; however, only significant correlations are included here.
been recognized. Additionally, the method of scoring the Comet data varies between laboratories, which could potentially affect the resolution of the technique. Using some protocols, the presence or absence of a Comet tail is scored, some semen samples producing hardly any ‘Cometed’ sperm (Aravindan et al., 1997). In other protocols, including that reported here, all sperm produced a Comet, so allowing the images to be analysed for % tail DNA, tail length or tail moment. The percentage of DNA which migrates from the sperm head into analysed for % tail DNA, tail length or tail moment. The all sperm produced a Comet, so allowing the images to be discriminated in their analysis (Irvine 1997; Irvine et al., 2000). Although others have previously used the length of the tail to detect the effects of in-vitro irradiation and the effects of chemotherapy upon DNA damage in sperm (Haines et al., 1998; Singh and Stephens, 1998; Chatterjee et al., 2000). Although others have measured tail length, percentage DNA and moment, only results from the percentage tail DNA measurements were documented, presumably because this measure gave better discrimination in their analysis (Irvine et al., 2000). Although all three measurements are interrelated we have chosen to present our data as tail length and tail moment, because in this study these measures exaggerated the differences between patients. The differences in DNA damage between ejaculates were most impressive using the Comet tail moment (see Figures 1–3). This may perhaps be expected as both the length of migration of the DNA and the amounts released from the head are independently increased by DNA damage (Olive, 1999).

It is also important to consider the type of DNA damage the Comet technique is measuring, especially as the methods used for analysis of sperm vary between laboratories. Comet analysis of somatic cells electrophoresed under alkaline conditions measures single DNA strand breaks, whereas neutral conditions reportedly measure double strand breaks. However, many forms of structural damage can be converted to strand breaks during cell preparation and electrophoresis (Collins et al., 1997; Olive, 1999). Early attempts to apply the Comet assay to sperm under alkaline electrophoresis conditions to measure single strand DNA breaks resulted in the majority of DNA migrating into the Comet tail (Singh et al., 1989). This also occurs using the protocol we have used here and has been ascribed to the abundant alkaline-sensitive sites in the sperm DNA and reflects the fragility of the structure in vitro. More recently, several methods have been developed which rely upon harsh biochemical treatments, with or without protease K and reducing agents. These lyse the sperm cell, allowing the DNA to decondense by removing nuclear proteins and their crosslinks. The differences in protocols are increased by the electrophoresis conditions that range from pH 8.2 to 13 (Haines et al., 1998; Singh and Stephens, 1998; Hughes et al., 1999; Irvine et al., 2000; Donnelly et al., 2001). In spite of this, some common results have been reported, especially the observation that radiation of sperm, which is known to induce double and single strand breaks, will also increase the Comet measurements (Haines et al., 1998; Singh and Stephens, 1998; Hughes et al., 1999). It is therefore likely that although the final measurement reflects DNA strand breaks in vitro, it is likely that these have arisen from a variety of in-situ DNA abnormalities ranging from strand breaks per se but also including structural abnormalities. Thus Comet analysis of sperm is most appropriately described generically as DNA damage. Which of the different protocols is the most useful for describing clinically relevant sperm DNA damage remains to be determined.

Whilst this and other studies have established that DNA damage is present in sperm, the biological significance of reproduction with these sperm is not clear. It may be predicted that high loads of DNA damage would be reflected in abnormal fertilization and development, ultimately leading to death of the embryo. In an attempt to address this question, we compared the semen analysis parameters, fertilization, embryo development and pregnancy outcome, from clinical treatment cycles for men grouped according to whether the sperm carried low or high DNA damage loads. Surprisingly, there were no significant differences in any of our measurements. It may be noteworthy that there was a trend for men in the higher DNA damage group to show poorer results in all of the development parameters and that early pregnancy loss occurred only in this group. These associations are potentially interesting and need

### Table IV. Multivariate stepwise linear regression analysis of Comet tail length and moments, and semen analysis for treatment cycles separated into either IVF or ICSI

<table>
<thead>
<tr>
<th>Variable</th>
<th>Dependent variable</th>
<th>Regression coefficient</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICSI (n = 40)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>Tail length</td>
<td>1.611</td>
<td>0.003</td>
</tr>
<tr>
<td>Semen concentration/ml (square root)</td>
<td>Tail length</td>
<td>-2.647</td>
<td>0.002</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>Tail length</td>
<td>0.305</td>
<td>0.036</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Tail moment</td>
<td>-0.666</td>
<td>0.026</td>
</tr>
<tr>
<td>Semen concentration/ml (square root)</td>
<td>Tail moment</td>
<td>-1.144</td>
<td>0.005</td>
</tr>
<tr>
<td>Cleavage (50–84%)</td>
<td>Tail moment</td>
<td>-7.568</td>
<td>0.0271</td>
</tr>
<tr>
<td>Cleavage (100%)</td>
<td>Tail moment</td>
<td>-12.77</td>
<td>0.0003</td>
</tr>
<tr>
<td>IVF (n = 20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility (%)</td>
<td>Tail length</td>
<td>0.52</td>
<td>0.027</td>
</tr>
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</table>

All the parameters included in Table I were entered into the analysis; however, only significant correlations are included here.
to be confirmed by studying a larger, more clearly defined group of men.

However, multivariate analysis of the entire population of samples revealed some interesting correlations. DNA damage increased as a function of age, motility and abnormal forms. When only the men selected for ICSI treatment were analysed, the association with age and motility remained, but additionally semen sperm concentration and embryo cleavage were significantly negatively associated with DNA damage. The association of increased sperm DNA damage with abnormal forms and decreasing sperm count confirms other studies (Sun et al., 1997; Irvine et al., 2000) and is perhaps predictable as inefficiency in spermatogenesis may lead to cells appearing in the ejaculate which either have not completed development or have escaped mechanisms to delete them within the testicular parenchyma (Sakkas et al., 2000). There have been several studies investigating changes in male reproduction with age (Pias et al., 2000; Kidd et al., 2001; Paulson et al., 2001; Rolf and Nieszchal, 2001). Whilst it is clear that endocrine activity in ageing men is less efficient it appears that in many studies sperm production is maintained, although there may be increases in abnormal forms and a decline in motility. We did not detect an age-related decline in sperm count motility or abnormal forms, possibly because the setting of this study resulted in a heterogeneous selection of men with a limited age range of 29–44 years. However, the increase in DNA damage was strongly significant. DNA fragmentation measured by TUNEL is not correlated with age (Sun et al., 1997) although another study has suggested that DNA chromatin condensation is abnormal in sperm from ageing men (Haidl et al., 1996). However, it is fairly clear that conception with sperm from ageing men does not adversely effect fertilization or live birth rate.

Association between sperm DNA damage and impaired fertility has previously been reported. Sperm with high levels of TUNEL labelling are more often found in infertile men, fail to decondense after ICSI and fertilization is often unsuccessful (Sakkas et al., 1996; Lopes et al., 1998b; Host et al., 2000). The SCSA has shown that those men with high levels of chromatin abnormalities in their sperm are likely to have poor fertility, which may be related to impaired fetal development and subsequent miscarriages (Evenson et al., 1999; Larson et al., 2000; Spano et al., 2000). Although it has been shown that there is some correlation between the DNA damage assays, as it is not clear what structural changes these are measuring, it may not be helpful to compare outcomes until further work is done. However, the Comet assay has been used to compare DNA damage in sperm from fertile and infertile men. No differences in sperm Comet parameters between fertile and infertile men could be found in one report (Hughes et al., 1996), however, the study did reveal differences after in-vitro treatment with either X-rays or hydrogen peroxide. This work suggested that sperm DNA from the infertile men was more susceptible to damage arising from oxidative free radical generation (Hughes et al., 1996). This apparent increase in DNA fragility has also been shown by freeze–thawing of samples for fertile and infertile men (Donnelly et al., 2001). The Comet assay has also detected significant correlation between DNA damage and semen parameters in an unselected population of men attending an infertility clinic (Irvine et al., 2000). The latter study also highlighted significant differences in DNA damage between infertility patients and normospermic donors. In the combined analysis of the donors and patients there was considerable overlap of the DNA damage profiles of the two groups. However, there was a highly significant increase in DNA damage as the sperm count and the proportion of morphologically normal forms decreased. The present study confirms the associations between DNA damage and sperm concentration and abnormal forms.

Sperm motility is often used as a predictive measure in semen analyses, high motility being a prerequisite of normal sperm parameters. It was somewhat surprising therefore to find that the higher the motility of sperm in semen, the higher the DNA damage load carried by the sperm populations. In contrast, others (Barroso et al., 2000; Irvine et al., 2000; Zini et al., 2001) report that sperm samples with low motility carried higher loads of DNA damage (TUNEL or Comet) (Irvine et al., 2000). ROS generated in vitro decreases motility as well as inducing sperm DNA damage (Aitken et al., 1998; Twigg et al., 1998a; Donnelly et al., 1999; Shen and Ong, 2000; Ramos and Wetzel, 2001). DNA damage has, however, been both negatively and positively associated with sperm ROS production (Barroso et al., 2000; Irvine et al., 2000). These studies suggest that while nuclear DNA damage may be induced by ROS, at the same time low levels may promote sperm motility (explaining our correlation) whereas higher pathological/pharmacological concentrations impair motility (de Lamirande et al., 1997), explaining the inverse correlation.

The most important observation from this study was that sperm containing high loads of DNA damage detected by the Comet assay gave rise to pronuclei at a normal incidence but were associated with an increase in the percentage of embryos that failed to develop after ICSI. This result is consistent with experiments in which DNA damage in human sperm was created artificially in vitro and after injection into hamster oocytes pronuclear formation was unchanged (Twigg et al., 1998b). DNA damage measured by the TUNEL assay is also negatively related to both fertilization and embryo cleavage rate after IVF, suggesting that DNA damaged sperm will fertilize less efficiently and confirming that early embryonic development is impaired (Sun et al., 1997). The results of our study suggest that selection of the sperm for ICSI in terms of DNA damage was random and that a mechanism for the screening of sperm carrying damaged DNA operates after ICSI to ensure that only those zygotes with a relatively intact genome go on to develop. It would be reassuring to conclude therefore that implantation and pregnancy outcome would not be so adversely affected by using sperm samples carrying high loads of DNA damage. However, the size of this study does not allow us to make this conclusion, and moreover, it remains possible that low, sublethal levels of sperm DNA damage are transmitted through to embryo development. These may be insufficient to trigger a gross response such as cell cycle arrest or apoptosis prior to implantation, or early pregnancy failure, but may nonetheless be expressed in fetal or post-natal development (Hales and Robaire, 1997; Sakkas et al., 2000). It would
be useful if screening semen samples for sperm DNA damage could contribute towards the selection of patients for ICSI. However, we cannot recommend this measurement at this time because significant numbers of sperm in a sample carrying high DNA damage loads may be genetically normal. Further research will be necessary to see if techniques can be devised to identify and select sperm with undamaged DNA for ICSI, or to remove sperm with damaged DNA from sperm samples, to enable the pregnancy outcome after ICSI to be improved. This work may also have implications for the genetic integrity and normal development of children conceived by IVF, especially ICSI.

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References


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