Reliability of the comet assay in cryopreserved human sperm

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BACKGROUND: Although the comet assay has potential value for measuring DNA damage in large epidemiological human sperm studies, it is impractical to perform the assay daily on fresh semen samples. Therefore, before its use in epidemiological studies, the reliability of the comet assay in measuring DNA damage in cryopreserved sperm should be compared with that in fresh human sperm. METHODS: Semen samples from 16 men were cryopreserved in liquid nitrogen (LN) using four methods: flash freezing with and without cryopreservative, and programmable freezing with and without cryopreservative. Neutral microgel electrophoresis was performed and comets were stained with YOYO-1. Comet length was measured using an eyepiece micrometer at ×400 magnification. RESULTS: The highest correlation was between comet assay results obtained from fresh human semen compared with semen flash frozen without cryopreservative (R = 0.88). However, the method of cryopreservation, as compared with other sources of variability, accounted for only 6% of the variability. Inter-individual variability accounted for 20%, and individual sperm-to-sperm variability within an ejaculate accounted for 65%. CONCLUSIONS: Flash-freezing in LN without cryopreservative most closely reproduced the results obtained using fresh human semen samples, and thereby represents the most appropriate cryopreservation method for human semen in epidemiological studies utilizing the neutral comet assay.

Keywords: comet assay/cryopreservation/human sperm/intra-class correlation/reliability

Introduction

The single-cell gel electrophoresis (SCGE) or ‘comet assay’ is used to measure the amount of DNA fragmentation in individual cells. In the assay, a cell with fragmented DNA has the appearance of a ‘comet’ with a brightly fluorescent head and a fluorescent tail, the intensity of which represents the relative amount of DNA strand breaks present (Singh et al., 1988; Hughes et al., 1997; Singh and Stephens, 1998). Although the comet assay has potential value for measuring DNA damage in human sperm in epidemiological studies, it is impractical to perform the assay daily on fresh semen samples. The use of frozen semen will enhance the feasibility of conducting the assay on a large number of archived samples, thereby increasing the power of the study. In several types of genetic integrity semen studies, including hamster oocyte/human sperm fusion techniques, sperm chromatin structure assay (SCSA), and fluorescence in-situ hybridization (FISH) (Evenson et al., 1991; Martin, 1993; Lahdetie et al., 1996), freezing of human semen samples has not been associated with adverse results compared with fresh semen. However, more recent studies utilizing the alkaline comet assay and tests of chromatin stability have detected DNA damage from cryopreservation in semen from infertile men (Hammadeh et al., 1999; Donnelly et al., 2001). The effects of freezing on human semen samples has not been fully examined in the neutral comet assay. Therefore, before using the comet assay in epidemiological studies, it is important to ascertain if frozen semen samples can be used instead of fresh samples.

Freezing can be detrimental to the cell because of osmotic imbalances and ice crystal formation. Ice crystals form at different rates during the cooling process, and cooling that is either too fast or too slow can disrupt cellular membranes and organelles (Morris et al., 1999; Gilmore et al., 2000). Damage from freezing can be minimized by optimizing freezing rates and using cryopreservatives. To optimize post-freezing motility, several different cryopreservation methods have been tested to determine optimal freezing rates and cryopreservative (Verheyen et al., 1993; Morris et al., 1999; Gilmore et al., 2000; Hallak et al., 2000).

The present study examined the effect of rate of freezing
Comet assay reliability in cryopreserved human sperm

and use of cryopreservative on the reliability of the comet assay. The comet assay for human sperm was adapted from methods used on somatic cells, and can be conducted under either alkaline or neutral conditions. Neutral conditions were used for human sperm because of the abundance of alkali-sensitive sites in sperm. Alkaline test conditions can induce damage at alkali-labile sites and produce single-strand DNA breaks (Singh et al., 1989). It was necessary to modify the assay as packaging of sperm DNA is much more compact than that in somatic cells (Singh and Stephens, 1998). The six-fold increase in compactness is accomplished by disulphide bonding between DNA and protamines; this causes sperm DNA to fold upon itself, thereby protecting it from damage caused either by exogenous agents or by hydrogen peroxide generated intracellularly (Adler, 1996). Hence, the modified protocol utilized an extensive lysis step to ensure effective removal of DNA-associated proteins.

In the present study, a decision was made to use Medi-Cult (Medi-Cult Inc., Hopkinton, MA, USA) sperm freezing medium because the protein particles were smaller than those in commonly used egg-yolk buffer, and might cause less interference with fluorescence signalling during the analysis. Medi-Cult medium also has a longer shelf life and enhanced ease of use. The results of the comet assay comparing fresh, unfrozen samples with flash-frozen and controlled-rate frozen samples in the presence or absence of Medi-Cult cryopreservative are reported.

Materials and methods

Patients
The study was approved by the Harvard School of Public Health and Massachusetts General Hospital (MGH) Human Subjects Committee, and all subjects provided their informed consent to participate. Subjects were male partners of subfertile couples who presented to the andrology laboratory at MGH, Boston, MA, USA for semen analysis as part of an infertility investigation. Eligible men were those aged between 20 and 51 years. Men presenting for postvasectomy semen analysis were excluded, as were those who were not English speaking.

Sample collection
Semen was produced on site at MGH by masturbation into a sterile plastic specimen cup after a recommended 48 h of abstinence. After liquefaction at 37°C for 30 min, ~1 ml of semen was archived for the main study (not described here) and the remaining semen was divided for use in the comet assay. Logistical constraints prohibited conducting the comet assay at the recruitment site on freshly produced semen samples. Therefore, the semen sample was placed on ice within 1 h of ejaculation and kept on ice for 18–24 h before processing for the comet assay. To validate the use of the ‘24-h ice’ semen as the reference category, the comet assay was conducted on a subgroup of samples processed for the comet assay within the 2.5 h of production. The latter samples were referred to as ‘fresh’, and were compared with the ‘24-h ice’ result.

Preparation of sample
In order to minimize the production of reactive oxygen species (ROS), the semen sample was split into two portions without separation of sperm from semen by Percoll gradients and centrifugation. One portion was kept undiluted and divided into eight 50 µl volumes, in 0.25 ml cryogenic straws (CryoBiosystem, I.M.V. Division, San Diego, CA, USA). The other portion was diluted 1:1 with Medi-Cult cryopreservative, ensuring that both semen and cryopreservative were at the same temperature. Medi-Cult cryopreservative is composed of modified Tyrodes with HEPES buffer containing sucrose, glucose and sodium lactate; synthetic serum replacement; glycerol; human serum albumin; penicillin 50 000 IU/l and streptomycin 50 mg/l. The medium was stored at 2–8°C, with protection from light, and discarded 28 days after opening. Medi-Cult was added dropwise to semen over 1 min. The mixture was allowed to equilibrate for 10 min before freezing to allow time for diffusion of freezing medium into the intracellular compartment. This mixture was then divided into eight cryogenic straws in 50 µl aliquots. Cryostaws were used rather than cryovials in order to minimize the air-to-surface ratio and reduce oxidative DNA damage (Bwanga et al., 1990). The larger volume-to-surface ratio also promotes more uniform freezing and thawing throughout the sample. Straws were sealed with glass beads or labelling rods.

Freezing methods
Freezing methods included slow freezing using a controlled-rate freezer with custom computer software to program the desired rate of freezing, as well as rapid (or flash) freezing which entailed immersing samples directly into LN. The four freezing method categories were a combination of freezing rate and cryopreservative use:

1. Flash-freezing without cryopreservative semen without cryopreservative was frozen in 0.25 ml cryostraws by direct immersion in LN (−196°C).
2. Flash-freezing with Medi-Cult cryopreservative; semen was diluted 1:1 with Medi-Cult freezing medium and frozen in 0.25 ml cryostraws by direct immersion in LN (−196°C).
3. Slow or controlled-rate freezing without cryopreservative; semen-filled 0.25 ml cryostraws were loaded into a programmable freezer (Custom Biogenic CL3000) and frozen at a rate of 1°C/min from room temperature to 4°C, then held for 5 min at this temperature before decreasing the temperature again at 8°C/min from 4°C to −80°C. Straws were then immersed into LN (−196°C).
4. Controlled-rate freezing with cryopreservative; this procedure followed the same protocol as (3), but used straws filled with semen, diluted 1:1 with Medi-Cult cryopreservative.

Thawing of samples
Before processing for the comet assay, the straws were thawed by gently shaking in a 37°C water bath for 10 s and then immediately processed for the comet assay.

Experiment design
The study was designed to compare DNA damage levels between fresh and frozen samples. Each electrophoresis experiment consisted of 16 slides. All slides were run under identical experimental conditions. Two electrophoresis experiments were needed to compare ‘24-h ice’ samples with ‘fresh’ samples. This was accomplished in a 2-day process; on the day of semen production the fresh samples and their corresponding flash-frozen samples, with and without cryopreservative, were processed for the comet assay. The slowly frozen samples could not be frozen and transported to the laboratory within the 2.5-h window required for processing ‘fresh’ samples. Hence, on the following morning the semen which had been kept on ice (‘24-h ice’) was processed along with duplicate flash-frozen samples as well as with the slowly frozen samples, with and without cryopreservative. This design allowed for a comparison of ‘24-h ice’
and ‘fresh’ samples. The replicate flash-frozen samples allowed for assessment of slide-to-slide variability.

Comet assay

The entire procedure was conducted under low indirect incandescent light situations (60 W) in order to minimize light-induced damage to sperm DNA. All reagents were purchased from VWR Scientific (West Chester, PA, USA) unless otherwise specified. After thawing, ~0.25 µl semen was mixed with 50 µl of 0.7% agarose (3:1 high-resolution; Amresco, Solon, OH, USA) embedded between two additional 200 µl layers of 0.7% agarose on specially designed, partially frosted, microgel electrophoresis glass slides with a clear central window (Erie Scientific, Portsmouth, NH, USA). Cover glasses were removed before submersion of the slides in cold (4°C) lysing solution of 2.5 mol/l NaCl, 100 mmol/l EDTA tetrasodium salt, 10 mmol/l Tris-base (pH 10), 1% sodium lauroyl sarcosine and 1% Triton-X (Boehringer Mannheim, Germany). This step mainly allowed for dissolution of the cell membrane such that the sperm chromatin could be accessed by the next two enzyme digestion steps. The slides were then transferred to enzyme treatment (2.5 mol/l NaCl, 5 mmol/l Tris, 0.05% sodium lauroyl sarcosine with pH adjusted to 7.4) and 350 µg RNase (Amresco). After 4 h at 37°C the slides were transferred into enzyme treatment plus 33 mg of DNase-free proteinase K (Amresco) for a minimum of 18 h at 37°C. These two steps were crucial to decondense sperm chromatin and allow migration of DNA from the nucleus. Slides were then equilibrated in neutral electrophoresis solution (300 mmol/l sodium acetate, 100 mmol/l Tris, pH 9) for 20 min before being electrophoresed under neutral conditions at 12 V and 90–100 mA for 1 h at room temperature. This was followed by precipitation and fixation of cells: first, in absolute alcohol mixed with 10 mol/l ammonium acetate; and second in 70% ethanol with 100 mg spermine, each for 15 min. The resulting slides were air-dried and subsequently stained with YOYO dye (Molecular Probe), which is an intensely fluorescent DNA dye. Fluorescent comet patterns were examined with a Leica fluorescence microscope (model DMLB) under ×400 magnification and fluororesothiocyanate filter combination.

Measurement of DNA damage

The comet length was measured in µm, using an eyepiece micrometer calibrated with a stage micrometer. A single technician (S.M.D.) who was blinded to the freezing method performed all measurements. Comet length measurements were taken starting from the far left border of fluorescence associated with the head of the comet, to the furthest visible pixel in the tail. Some comets were too long to measure as the maximum length of the eyepiece micrometer was 250 µm. In such cases the comet was assigned a value of 300 µm. A total of 200 cells was analysed on each slide.

X-ray dose–response

To determine the sensitivity of the comet assay, control semen and semen with damage induced by X-irradiation were compared. One semen sample was divided into five 50 µl volumes in 0.25 ml cryostraws. Samples were irradiated with 25, 50, 100 or 200 rad (cG) X-irradiation using a Philips MG165 constant potential X-ray system (Phillips Electronic Instruments Co., Alpaharetta, GA, USA) operated at 160 kV and 18 mA at room temperature. The dose rate was 0.74 Gy/min. Samples were kept at room temperature for 1 h after irradiation, and then flash-frozen in LN at −196°C. The straws were thawed at 37°C in a water bath for 10 s before processing for the comet assay. A total of 100 cells was analysed for each dose, using VisComet image analysis software (Impuls Bildanalyse GmbH, Germany).

Image analysis

To explore DNA damage induced by X-irradiation, ‘comet extent’ and ‘distributed moment’ were measured with VisComet software. The VisComet software was unavailable for the main portion of the reliability study and only used for the X-irradiation image analysis. Comet extent is a measure of total comet length from the beginning of the head to the last visible pixel in the tail. This was the same measure obtained by manual analysis using an eyepiece micrometer. The distributed moment (which is also known as ‘DNA migration’) was an integrated value that took into account both the distance and intensity of comet fragments. The formula used was: \( M_{\text{dist}} = \Sigma (1 - X)/\Sigma I \), where \( I \) is the sum of all intensity values belonging to the head, body or tail, and X is the x-position of intensity value. A total of 100 cells per X-irradiation dose was analysed.

Statistical analysis

A central goal of this analysis was to assess the reliability of the four different freezing methods relative to that of fresh sperm. In an ideal setting, this might be carried out by subjecting each sperm cell to the various methods being considered and then comparing results. In practice, clearly each cell can be assessed only once. Hence, the study approach was to take different aliquots of the same semen sample and then to apply the different methods. Consequently, it was important for the analysis to consider effects related to between-person and between-method differences as well as cell-to-cell differences. The inherent variability of comet lengths has not been well described in human sperm. To determine the effect of freezing method on comet length, it is important to determine the proportion of variance due to between-person variability, slide-to-slide variability, cell-to-cell variability, and intra-technician reliability. In order to estimate intra-technician reliability, 15 slides were analysed blindly by one technician, twice in random order. These were referred to as duplicates. To determine slide-to-slide variability, two slides of the same method per person were run together in the same experiment and analysed; these were referred to as replicates. With the duplicate and replicate readings it was possible to apportion variability into: (i) between-person variance; (ii) between-method variance; (iii) slide-to-slide variance; (iv) intra-technician variance; and (v) cell-to-cell variance. There are therefore several layers of repeated measures, or ‘nesting’, all within the individual semen sample. A simple linear regression was not appropriate as it would not take into account the repeated measures, and would constrain variances between methods to be equal—which may not be appropriate (Littell et al., 1996). To study the variation between freezing methods, the variance components method was utilized (Box et al., 1978). Two different methods of analysis were used in linear mixed models. In the analysis of the mean-level data, the person was allowed to have random effects; in the analysis of cell-level data, nesting of random effects within the person was allowed. In this way it was possible to obtain variance component estimates and then to determine intra-class correlations.

All statistical analyses were carried out using in SAS-PC version 7.0 (SAS Institute Inc., 2000) unless otherwise noted. Linear regression with mixed effects and nesting was used to analyse between freezing method differences, to apportion inter-individual, inter-method and intra-individual variances, and to determine intra-technician reliability. The intra-class correlation coefficient (ICC) was the appropriate correlation measure for determining reproducibility of replicate measures from the same subject where the multiple measurements should ideally be measuring the same quantity. Unlike the Pearson correlation coefficient, which allows variance to change with the mean, the ICC does not (Rosner, 2000). The ICC is a ratio of between-person variance to total variance, and was based on the mean of 100–200 cells per freezing method for each person. According to a previous
report (Fleiss, 1999), an ICC <0.4 indicates poor reproducibility, while levels between 0.4 and 0.75 represent good reproducibility. Excellent reproducibility is measured by an ICC >0.75. Paired t-tests were used for pairwise comparisons of the effects of each radiation dose to the control sample. Sensitivity of the comet assay to detect irradiation damage was also performed with a non-parametric trend test, which is an extension of the Wilcoxon rank-sum test (nptrend: Intercooled Stata 6.0 software for Windows, Stata Corp., College Station, TX, USA). A P-value < 0.05 was considered statistically significant.

Results

Semen samples

Semen samples were obtained from 16 men seeking fertility evaluation between June 2000 and November 2000. Demographically, the group was diverse, with 44% born outside the USA, and with ages ranging from 25 to 49 years (Table I). Three subjects were current smokers. All 16 men had ‘24-h ice’ aliquots, and eight also had ‘fresh’ aliquots. A univariate analysis of comet length revealed a non-normal, skewed distribution due to the assignment of 300 µm to those comets too long to measure. Log transformation only moderately normalized the distribution, but minimized the effect of outliers and stabilized residuals. All subsequent analyses were based on log-transformed data.

Comparison of fresh and ‘24-h ice’ semen

In order to validate the ‘24-h ice’ aliquot as the fresh reference group, the comet length measurements in eight ‘fresh’ aliquots were compared with the corresponding ‘24-h ice’ aliquot. The data showed no significant difference in mean comet length between ‘fresh’ and ‘24-h ice’ aliquots (118.6 and 127 µm respectively; P > 0.2), based on 3510 sperm from eight men. The ICC was calculated in order to explore further the relationship between ‘fresh’ and ‘24-h ice’ aliquots. Comparison of ICC values for ‘24-h ice’ and fresh samples was only good, but not excellent (R = 0.63), indicating that ‘24-h ice’ aliquot should not be used as the ‘gold standard’; subsequently, the four freezing methods were compared with the fresh aliquot.

Effect of different freezing methods on DNA damage

Cell-level data

Cell-level descriptive statistics for the two fresh and four freezing methods are presented in Table II. A total of 200 was analysed for each sample as cell count permitted. Standard deviations were slightly higher in frozen aliquots (57–64 µm) compared with fresh aliquots (53–56 µm), and all methods appeared to have a right-skewed distribution with means 15–20 µm longer than the median. The medians were similar across all methods, ranging from 100 to 115 µm. Over 70% of all cells in each freezing method were between 50 and 150 µm in length. In general, flash-freezing produced comet lengths similar to the fresh aliquot and 6–14 µm shorter than comets from slowly frozen aliquots. The use of cryopreservative did not affect comet lengths as much as the rate of freezing did. The aliquot kept on ice overnight had comet lengths more comparable with the slowly frozen aliquots. None of these differences was statistically significant on mixed effects linear regression. However, it was found not sufficient simply to examine the difference in means between methods to determine the optimum freezing method, as both the means and variances changed.

Mean-level data

The distribution of mean comet length of 200 cells per person is shown in Figure 1. Although median values were similar, the method-to-method variability of data was quite different. The distribution of mean comet length for flash-frozen samples was more similar to the fresh semen sample than for slowly frozen samples, though some data points obtained for the flash-frozen method were more than 1.5 times the interquartile range. Compared with fresh samples, those with cryopreservative had the greatest variance, with some data points more than 1.5 times the interquartile range. Slowly frozen semen exhibited higher median values and more variability than did fresh samples.

ICC

Each cryopreservation method was compared with both the ‘24-h ice’ and ‘fresh’ aliquots by use of the ICC. The ICC values for flash-freezing methods were always higher than for slow-freezing methods when the fresh aliquot was used as the ‘gold standard’ (Table III). Additionally, lower correlations were seen with cryopreservative use, regardless of the rate of freezing. When using the 24-h ice aliquot as the reference, the correlations were all only modest, and none was greater than flash-freezing without cryopreservative (R = 0.66). Interestingly, samples that were slow frozen with cryopreservative has a larger pairwise ICC with the 24-h ice sample than with the fresh sample; the ICC was low, however (R = 0.51). The freezing method which was consistently better than all others, and which was most comparable with the fresh aliquot, was flash-freezing without cryopreservative (R = 0.88). The ICC, although good, was less than expected. Even though the ICC was based on mean values, it was likely that the reported ICC was an underestimation of the true reliability as it may have reflected some residual cell-to-cell variability.

Intra-technician reliability and apportionment of sources of variability

By using cell-level data with mixed effects linear regression and appropriate nesting, the different sources of variability in the data were apportioned. The vast majority of the total

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**Table I. Demographic characteristics of subjects (n = 16)**

| Age (years) | 35.4 (25.4–48.7) |
| Body weight (kg) | 83.5 (69.2–118.0) |
| Height (cm) | 175.7 (161.2–187.5) |
| Race (n) |  
| White | 13 (81) |
| Black/African American | 2 (13) |
| Other | 1 (6) |
| Ever smoker (n) | 4 (25) |
| Current smoker (n) | 3 (19) |
| Born outside USA (n) | 7 (44) |

Values in parentheses are percentages.

Values are mean (range)
Table II. Mean (± SD) comet length by method

<table>
<thead>
<tr>
<th>Cryopreservation method</th>
<th>No. of subjects</th>
<th>No. of sperm measured</th>
<th>Length (µm)</th>
<th>Mean ± SD</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>’Fresh’</td>
<td>8</td>
<td>1596</td>
<td></td>
<td>118.6 ± 53.3</td>
<td>105.0</td>
</tr>
<tr>
<td>24-h ice</td>
<td>16</td>
<td>1914</td>
<td></td>
<td>127.0 ± 56.2</td>
<td>107.5</td>
</tr>
<tr>
<td>Flash-freeze without cryopreservative</td>
<td>16</td>
<td>2100</td>
<td></td>
<td>120.2 ± 57.5</td>
<td>100.5</td>
</tr>
<tr>
<td>Flash-freeze with cryopreservative</td>
<td>16</td>
<td>2100</td>
<td></td>
<td>118.5 ± 59.1</td>
<td>100.0</td>
</tr>
<tr>
<td>Slow-freeze without cryopreservative</td>
<td>16</td>
<td>1760</td>
<td></td>
<td>132.2 ± 63.6</td>
<td>115.0</td>
</tr>
<tr>
<td>Slow-freeze with cryopreservative</td>
<td>16</td>
<td>1554</td>
<td></td>
<td>126.7 ± 64.0</td>
<td>105.0</td>
</tr>
</tbody>
</table>

24-h ice = semen stored on ice for 24 h before processing for the comet assay; ’Fresh’ = fresh semen sample processed for the comet assay within 2.5 h of production.

Figure 1. Box and whisker plot showing distribution of mean comet length (200 cells per person) by method. Medians are indicated by open boxes; interquartile ranges (25–75%; IQRs) are indicated by solid boxes. The whiskers are 1.5 times the IQR distribution, with individual outliers depicted as solid bars. Fresh = sperm processed for comet assay within 2.5 h of production; Flash frozen = direct immersion in liquid nitrogen; Slowly frozen = frozen by programmable freezer over 35 min before immersion in liquid nitrogen. NP = no cryopreservative added to semen; P = cryopreservative added to semen; 24-h ice = sperm kept on ice overnight and processed for the assay on the following morning.

Figure 2. Relative apportionment of several sources of variability in comet length measurements. Cell = cell-to-cell variability; Duplicate = intra-technician variability (same slide read twice); Method = between-method variability (flash or programmable freezing with and without cryopreservative); Person = between-subject variability; Replicate = slide-to-slide variability/both intra- and inter-experiment variability.

Table III. Pairwise intra-class correlation coefficient (ICC)a

<table>
<thead>
<tr>
<th>Cryopreservation method</th>
<th>Fresh</th>
<th>24-h ice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>Flash-freeze without cryopreservative</td>
<td>0.88</td>
<td>0.66</td>
</tr>
<tr>
<td>Flash-freeze with cryopreservative</td>
<td>0.74</td>
<td>0.46</td>
</tr>
<tr>
<td>Slow-freeze without cryopreservative</td>
<td>0.66</td>
<td>0.51</td>
</tr>
<tr>
<td>Slow-freeze with cryopreservative</td>
<td>0.28</td>
<td>0.51</td>
</tr>
</tbody>
</table>

aICC calculated on a mean of 200 sperm measured per person. 24-h ice refers to semen stored on ice for 18–24 h before processing for the comet assay; Fresh refers to semen processed for the comet assay within 2.5 h of production.

Discussion

The present study showed that cryopreservation of human semen does not reduce the reproducibility of the neutral comet
assay results. These results were consistent with other genetic integrity studies of sperm where freezing did not adversely affect the outcome of specific genetic integrity tests, such as FISH, TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL), SCSA and the hamster-oocyte/human sperm fusion techniques (Evenson et al., 1991; Martin, 1993; Lahdette et al., 1996). A recent study explored the effect of two different freezing methods on DNA damage in testicular sperm as compared with fresh testicular sperm, and found no difference in the percentage of undamaged DNA in the comet head between fresh or frozen human sperm (Steele et al., 2000). Interestingly, these authors also found that the percentage of undamaged DNA in ejaculated sperm was much lower than in testicular sperm. Others (Evenson et al., 1991) found no difference in SCSA results whether sperm were cryopreserved or not, nor if sperm were flash-frozen or slowly frozen; rather, the cryoprotectant was found to interfere with the fluorescence signal. Validating the reliability of the neutral comet assay in cryopreserved human semen may enhance the widespread application of the assay to large-scale epidemiological studies where it is not feasible to analyse fresh specimens.

In the present study it was shown that the majority of variability in comet lengths is due to the inherent heterogeneity of the population of sperm cells within the individual, rather than to either freezing methods or intra-technician measurement error. Although the selection of a more homogeneous population of sperm by the use of Percoll gradients or swim-up techniques might reduce this variability, there is evidence that the processing of sperm increases DNA damage, most likely from the resultant production of ROS (Zini et al., 1999).

Few published human studies exist which have examined the effect of environmental chemicals on DNA integrity in sperm as measured by the comet assay. Using the alkaline version of the comet assay, it was found that frozen sperm from both fertile and infertile samples produced similar responses upon exposure to a metabolite of 1,3-butadiene, as well as dibromochloropropane and two different estrogens when compared with fresh sperm (Anderson et al., 1997). Unfortunately, sperm from the same person was not tested before and after freezing. This would ensure that any difference in response to the chemical was not attributed to the freezing process. However, the parameter used in this study was percentage DNA in the head (% head DNA), and median baseline levels were quite consistent whether fresh or frozen, or from fertile or infertile donors.

The comet assay has proven to be a very sensitive test for detecting low levels of DNA damage in human sperm (Hughes et al., 1996; Sun et al., 1997; Singh and Stephens, 1998; Irvine et al., 2000). The radiation dose–response data obtained in the present study provided additional evidence of the sensitivity of the comet assay in detecting low levels of DNA damage, and were comparable with those reported elsewhere (Olive et al., 1991; Hughes et al., 1996; Singh and Stephens, 1998). It was found that an integrated measure of tail moment (distribution moment) was a more sensitive parameter for detecting low-dose irradiation-induced DNA damage in human sperm as compared with simple comet length. This was consistent with findings by others (Olive et al., 1991), who concluded that ‘tail moment’—an integrated measure of the product of tail length and the amount of DNA in the tail—was a more sensitive measure of irradiation-induced DNA damage in Chinese hamster V79 cells than was simple tail length.

In conclusion, the results obtained with the neutral comet assay using cryopreserved sperm were reproducible and comparable with those obtained from fresh human sperm. The lack of DNA damage might be the result of the unique packaging of sperm DNA which protects it from intracellular fluid shifts and ice crystal formation during the freeze–thaw cycle. The neutral comet assay is also sensitive to the detection of in vitro-induced radiation damage as low as 50 rads. It was possible to apportion the different sources of variability in DNA measurements and show that cell-to-cell variability accounted for the vast majority of the variance, and that the four freezing methods tested each had minimal impact on DNA damage. In addition, the use of cryopreserved sperm in the neutral comet assay was validated, and this should enhance the applicability of this assay in large-scale epidemiological studies. Moreover, the data also showed that the use of semen that has been kept overnight on ice before processing for the comet assay is less reliable than freezing semen immediately in liquid nitrogen. However, because the method used was seen to account for very little of the overall variability of the data, it remains feasible—when liquid nitrogen is not available—to keep semen samples on ice overnight before subsequent analysis. The use of ‘24 h ice’ semen samples may however slightly reduce the ability of the comet assay to detect very small differences in DNA damage.

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