Human sperm DNA integrity assessed by the Comet and ELISA assays

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DNA integrity in sperm is essential for the accurate transmission of genetic information and therefore the maintenance of good health in future generations. The ELISA and Comet assays, two techniques that detect DNA damage in cells, are compared in this study of DNA integrity in human sperm. Both techniques rely on alkaline unwinding for the release of single strands of DNA from the nucleus. The Comet assay measures single strands drawn out by electrophoresis, stained with ethidium bromide and quantified by image analysis. The two techniques, both modified for use with sperm, detect similar levels of baseline DNA damage along with similar dose-dependent patterns of induced damage by X-ray irradiation at 10 and 30 Gy (P < 0.05). The assays are also comparable in the detection of a significant protective effect by ascorbic acid (300 and 600 µM) and α-tocopherol (30 and 60 µM) on DNA integrity, both at baseline levels and following X-ray irradiation (P < 0.01). The advantages and disadvantages of each technique are discussed.

Introduction

The function and replication of every cell is dependent on its DNA. DNA damage may result in cell death or induction of mutations. In sperm, DNA damage may carry mutations into the next generation or result in male infertility. DNA integrity may become even more important as an indicator of sperm health with the newest treatment for male infertility; intracytoplasmic sperm injection (ICSI) (Palmero et al., 1993). Using ICSI, the classical parameters of sperm motility and the ability to recognize and penetrate the oocyte are no longer necessary for fertilization, so a more pertinent test is urgently needed. Many assays have been used to measure DNA damage in mature and immature sperm cells, such as acridine orange staining (Tejada et al., 1984; Peluso et al., 1992), flow cytometry (Evenson et al., 1991), alkaline elution (Joshi et al., 1990; Van Loon et al., 1993), enzyme-linked immunosorbant assay (ELISA) (Van Loon et al., 1991), the single cell gel electrophoresis (Comet) assay (Hughes et al., 1996). Two of these assays, ELISA and Comet, are used in this study to compare DNA damage in mature sperm cells. The Comet assay was chosen as it gives detailed information on the quantitation of DNA integrity of individual sperm. The ELISA assay was chosen over the more detailed information on the quantitation of DNA integrity of mature sperm cells. The Comet assay was chosen as it gives

Materials and methods

Semen analysis

Semen samples were obtained from 50 patients, after recommended abstinence from sexual activity for 3 days. Routine semen analysis was carried out by light microscopy. Semen samples from eight normozoospermic (concentration 600 × 10⁶/ml, motility >50%, normal morphology >30%) men of recently proven fertility and 42 asthenozoospermic (concentration 20 × 10⁶/ml, motility <50%, normal morphology >30%) men were used for the study.

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To select the subpopulation of sperm with the best motility and morphology routinely used in assisted conception techniques, the samples were prepared by two-step discontinuous Percoll gradient centrifugation. Freshly liquefied semen was treated as follows: (i) two layer Percoll (95.0–47.5%) centrifugation at 500 g for 20 min; (ii) concentration by centrifugation at 250 g for 10 min; (iii) dilution with Biggers–Whitten–Whittingham medium (BWW; Biggers et al., 1971) to a concentration of 1×10^7/10 µL.

**Antioxidant incubation**

Semen from 30 of the asthenozoospermic infertile subjects was divided into three prior to Percoll preparation. One control aliquot was then prepared with Percoll as described, while the other two aliquots were prepared in the presence of one of the antioxidants ascorbic acid (300 or 600 µM) or α-tocopherol (30 or 60 µM).

**Induced oxidative damage to DNA and antioxidant protection**

Sperm from each of the three aliquots from the antioxidant-treated group were divided into two so that sperm from the same sample could be compared with and without subsequent induced damage. To induce oxidative DNA damage sperm from each sample were irradiated with a dose of 30 Gy at room temperature (Hughes et al., 1996).

Sperm from 20 subjects which had not been treated with antioxidants were divided into three aliquots; one control, one subjected to 10 Gy and the last aliquot to 30 Gy irradiation. X-irradiation was performed using a 300-kV Siemens Stabilipan X-ray source at a dose rate of 2.6 GY/min.

**Comet assay**

The modified alkaline Comet assay for sperm (Hughes et al., 1996, 1997) was carried out on the prepared samples. A slide was prepared for each control, antioxidant-treated and irradiated aliquot.

Fully frosted slides (Richards Supply Company Ltd, London, UK) were covered with 100 µl 0.5% normal melting point agaroose (Sigma Chemical Co., Poole, UK), a coverslip added and the agarose allowed to solidify. The slides were covered with 1×10^5 cells in 10 µl BWW and mixed with 90 µl 0.5% low melting point agarose (Sigma) used to form the second layer. The slides with coverslips removed were then placed in lysis buffer for 1 h [2.5 M NaCl, 100 mM NaEDTA, 10 mM Tris, 1% Triton X-100 and 10% dimethyl sulphoxide (Sigma), pH 10]. The slides were then incubated overnight at 37°C in 100 µl/ml proteinase K (Sigma) added to the lysis buffer. Antioxidants were present in the lysis buffer throughout the incubation.

After draining the proteinase K solution from the slides, they were placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution containing 300 mM NaOH and 1 mM EDTA (Sigma), pH 12.5, for 20 min to allow the DNA from the cells to unwind. Electrophoresis, for 10 min was performed at room temperature, at 2 V/0.714 V/cm and 300 mA, obtained by adjusting the buffer level. The slides were then washed with a neutralizing solution of 0.4 M Tris (Sigma), pH 7, to remove alkali and detergents. After neutralization, the slides were stained with 50 µl 20 µg/ml ethidium bromide (Sigma) and covered with a coverslip. All steps were carried out under yellow light to prevent further DNA damage.

Fifty cells from each slide were selected randomly and analysed by image analysis using Hewlett and Packard Super VGA and Feretena Comet Software (v.2.2). Observations were made at magnification ×400 using an epifluorescent microscope (Olympus BH2). Statistical analysis was carried out on the values obtained for the percentage head DNA of each cell. Percentage head DNA is obtained by taking the mean sample values by the corresponding 100% unwound controls and the mean background value was subtracted from these figures. Percentage single-strandedness for each dilution of the sample was calculated by dividing the mean sample values by the corresponding 100% unwound control sample values and multiplying by a dilution factor of 10.

All of the chemicals used for the ELISA were obtained from Sigma Chemical Co. The antibody and conjugated antibody were obtained from Dr G.P.Van der Schans (Rijswijk, The Netherlands).

**Statistical analysis**

Statistical analysis was carried out using non-parametric statistics with the Statistics (Statsoft Inc.) package on the values obtained from both assays. Analysis was carried out to determine if the damage induced by X-irradiation was significantly different from the control values within each sample and if the antioxidants had any protective effect against the induced oxidative damage using Student’s t-test for dependent samples.

**Results**

**DNA integrity in fertile and infertile subjects**

DNA integrity determined by the ELISA and Comet assays was similar for both fertile and infertile men (Figure 1), with 81.3% undamaged DNA (Comet) compared with 80.8% (ELISA) for the infertile group and 81.2% (Comet) compared with 78.8% (ELISA) for the fertile group (Figure 2). Following X-irradiation there was good correlation (P < 0.01, r = 0.67) in the pattern of DNA damage detected by the two techniques, each exhibiting dose-dependent increases in damage (Figure 2). Significant amounts of damage were detected by ELISA...
DNA integrity assessed by the Comet and ELISA assays

![Fig. 2](image-url) The effect of X-irradiation on sperm from (a) fertile and (b) infertile men as determined by the Comet and ELISA assays. The mean values ± SD for percentage intact DNA for control □, 10 Gy ■ and 30 Gy □ are shown. *Significantly different from control (P < 0.05); **significantly different from control (P < 0.01); ***significantly different from control (P < 0.001)

![Fig. 3](image-url) The effect of ascorbic acid present during sperm Percoll preparation as determined by (a) the Comet and (b) the ELISA assays. The mean values ± SD for percentage undamaged DNA for control □, 300 µM ascorbic acid ■ and 600 µM ascorbic acid □ compared with those exposed to 30 Gy radiation. **Significantly different from the control (P < 0.01); ***significantly different from the control (P < 0.001)

at 10 Gy irradiation (P < 0.001) and the Comet assay (P < 0.05) in sperm from both fertile and infertile subjects (Figure 2).

The effect of antioxidant supplementation in vitro on DNA integrity

The effects of antioxidant supplementation on sperm DNA show a similar pattern of results for the two techniques, with the presence of antioxidants in the preparation medium significantly improving DNA integrity. There was an increase in intact DNA with ascorbic acid (300 µM) (Figure 3) and α-tocopherol (30 µM) (Figure 4) detected by ELISA (P < 0.001) and the Comet assay (P < 0.01). In addition, in both assays 30 Gy X-irradiation significantly damaged the DNA compared with that of control sperm (P < 0.001). The presence of antioxidants provided such protection from X-ray damage that DNA integrity remained at baseline levels (P > 0.05). Again, this effect was observed with both assays (Figures 3 and 4).

Discussion

The Comet assay is relatively new in its application to determine DNA damage in sperm, so in this study we compared it with the more established ELISA assay to see how comparable the assays were. Our results indicate that similar baseline DNA integrity is detected for sperm of fertile and infertile subjects using both techniques. Similar dose-dependent patterns of damage induced by X-irradiation were also detected. High doses of X-irradiation were again used in this study as these doses were previously shown to be required to induce significant damage to sperm DNA (Hughes et al., 1996, 1997). This is probably due to the unique tight packaging arrangement of DNA within the sperm nucleus (Barone et al., 1994).

The DNA integrity of sperm by both techniques is low compared with that of somatic cells. This has previously been shown in sperm using the Comet assay (Hughes et al., 1996).
and to a lesser extent in spermatids, shown by alkaline elution (Van Loon et al., 1993) and ELISA (Van Loon et al., 1991). As sperm and spermatids no longer possess significant cytoplasm (Sega and Generoso, 1990), this higher level of DNA damage may be a result of oxidative attack in the absence of repair (Van Loon et al., 1993). It has, however, been shown that some repair of sperm DNA may take place in the oocyte after fertilization (Ashwood-Smith and Edwards, 1996), which may explain why a population of cells which appear to be damaged are still capable of creating a genetically healthy embryo.

The addition of antioxidants to the preparation medium during Percoll centrifugation resulted in a higher percentage of intact DNA as measured by both assays, suggesting that some of this baseline DNA damage is due to oxidative attack in the absence of any cellular antioxidant protection. In human semen, defective sperm and neutrophils are potential sources of the oxidant hydrogen peroxide (Aitken et al., 1992), therefore centrifugation in the presence of such oxidants during Percoll preparation leaves sperm susceptible to oxidative insult. Both ascorbic acid and α-tocopherol significantly prevented DNA damage from occurring at the baseline level and following X-irradiation, which was evident from each assay.

Ascorbic acid has previously been shown to reduce DNA damage in studies measuring 8-hydroxy-2-deoxyguanosine, one of the major products of DNA damage (Fraga et al., 1991; Jacob et al., 1992). The reason for the positive effects of this antioxidant is its role as a scavenger of all major reactive oxygen species, including the hydroxyl radical, the major product of X-irradiation. Ascorbic acid appears to be the most important chain breaking antioxidant for sperm DNA protection, as it is the most abundant one found in seminal plasma (Lewis et al., 1997). It is actively secreted into the seminal plasma by the seminal vesicle during ejaculation (Berg et al., 1941) and it has been found to have a greater protective effect on sperm DNA in vitro than α-tocopherol, urate or acetylcysteine (Hughes et al., 1998).

α-Tocopherol is also a chain breaking antioxidant, but is found in only small quantities in seminal plasma (Lewis et al., 1997). However, these low concentrations do not reflect the obvious importance of this antioxidant, as α-tocopherol also significantly prevented DNA damage to baseline values by X-irradiation. The low concentrations found in the seminal plasma may be sufficient to prevent oxidative attack because it is rapidly regenerated by ascorbic acid, as in other cells (Kagan et al., 1992).

As the ELISA and Comet assays provide similar overall results for sperm DNA, other aspects of the assays should be considered. Both of the assays are simple, although technically time consuming, and data can be obtained after 32 h for the Comet assay and 48 h for ELISA. The automated measurement of absorbance for ELISA by a plate reader is faster than analysis of 50 individual cells for each slide in the Comet assay, but ELISA does require a larger population of cells. The Comet assay requires only 50 cells from each population, a number that has been found to be sufficient for reproducibility of the assay (Hughes et al., 1997). Also, as subpopulations can be assessed separately in the Comet assay, resistant subgroups may be identified in a heterogeneous population such as that found in semen (Hughes et al., 1996).

It is possible to freeze the cells for analysis by ELISA assay following alkaline unwinding with no induction of additional damage. This confers the advantage of storing a large number of samples for analysis at a later stage. Although sperm may be analysed by the Comet assay following freezing with a cryopreservative (Anderson et al., 1997a), there is no intermediate stage at which the assay may be stopped. However, once the slides are prepared for the Comet assay, they can be dried and stored for analysis at a later date. After the initial set-up expense of purchasing equipment for each, the running cost of the ELISA assay is approximately three times that of the Comet assay.

In conclusion, the ELISA and Comet assays are both useful in the detection of DNA damage in sperm as they provide comparable results of total damage in a given population of cells. This confirms our belief that the Comet assay is as accurate as established methods. The main advantage of the Comet over the ELISA assay is that DNA damage can be assessed in individual cells and its distribution assessed giving detailed information on the pattern of damage.
The Comet assay has numerous applications. The assay may have a future in the determination of DNA integrity prior to ICSI, especially since the technique may be carried out using few sperm. This would be an advantage as ICSI is often carried out using samples with few sperm present. The addition of antioxidants to incubation media for ICSI requires additional investigation. The present study is in agreement with other work carried out using the Comet assay, that antioxidants such as ascorbic acid, α-tocopherol and urate (Hughes et al., 1998) and flavonoids (Anderson et al., 1997b) have a protective effect against sperm DNA damage. This may increase the probability of fertilization following ICSI.

The Comet assay could also be applied in the biomonitoring of mutagen exposure as the assay has been shown both here and in previous publications to be useful in the detection of damage by chemicals such as hydrogen peroxide (Hughes et al., 1996) and estrogen-like compounds (Anderson et al., 1997c) to sperm DNA. We conclude that the Comet assay is a preferred technique over ELISA for the detection of damage in a population and the technique has many possible applications.

Acknowledgements

This study was supported by Wellcome Project Grant No. 049993/2/97/Z. The authors would like to thank Dr Van der Schans for kindly allowing us to use his antibody for the ELISA assay. The skilful technical assistance of Mrs M. Kennedy is gratefully acknowledged.

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Received on February 11, 1998; accepted on August 25, 1998

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