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A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay

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Baseline DNA damage in spermatozoa from fertile and infertile men was compared using a modified alkali single cell gel electrophoresis (comet) assay. Semen from normozoospermic fertile, normozoospermic infertile and asthenozoospermic infertile (World Health Organization criteria, 1992) samples were studied. No significant difference was observed in levels of baseline damage between the three groups. A median value for baseline damage of ~20% (80% head DNA) was obtained in all samples. Irradiation with X-rays (5–30 Gy) produced no additional damage in fertile samples when median values were examined. However, irradiation with 30 Gy X-rays produced significant damage in both infertile groups. Hydrogen peroxide (40 μM) treatment induced significant damage in the asthenozoospermic group, whereas 100 μM H_2O_2 was required to cause significant damage in the normozoospermic fertile and infertile samples. Within the fertile population a subgroup in which percentage head DNA was greater than 80% was observed in both treated and untreated specimens. This subgroup significantly decreased with treatment in both infertile groups. We conclude that the asthenozoospermic infertile group is more susceptible to damage than the normozoospermic infertile group, which in turn is more susceptible than the fertile group. The fertile group contains a resistant subpopulation of spermatozoa with relatively intact DNA.

Key words: DNA damage/human spermatozoa/modified comet assay

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Introduction

There is still very little understanding of the major causes of male infertility. This is surprising since male infertility constitutes the primary problem for up to 30% of couples (Oehninger *et al.*, 1992), with a further 30% of couples having a contributory male factor problem. Assessment of male infertility has traditionally been based on semen analysis classified according to the World Health Organization standards (WHO, 1992). This analysis is based on gross visual determination of sperm number and motility as measured by light microscopy and it has remained relatively unchanged for many years. Its criteria for normality have limited prognostic value in predicting pregnancy (Irvine, 1992). As a result, tests for morphology (Kruger *et al.*, 1988a), acrosome status (Liu and Baker, 1988), sperm zona pellucida binding (Liu and Baker, 1992), and free hamster egg sperm penetration (Kruger *et al.*, 1988b) have emerged to provide additional information to the traditional tests.

Once in the oolemma, the sperm DNA must decondense and interact with the DNA of the oocyte to form a genome (Zirkin *et al.*, 1985). Several approaches have been used to assess nuclear material in spermatozoa. Electron microscopy studies have been carried out on spermatozoa from fertile and infertile men (Lipitz *et al.*, 1992; Zamboni, 1992), correlating patterns of nuclear staining with infertility. This technique is time-consuming and results in a picture of DNA pattern limited to only one plane of the nucleus. Flow cytometry studies have also attempted to assess fertility (Evenson *et al.*, 1991; Engh, 1993) and to determine ploidy through various stages of spermatogenesis (Lee and Choo, 1991; Spano and Evenson, 1991; Hittmair *et al.*, 1994). Abnormalities of chromatin structure measured by this technique have been related to infertility and are useful in giving an overall picture of the nuclear damage. However, it cannot determine whether chromatin aberrations are due to DNA damage.

Levels of DNA damage may be measured using alkaline elution which detects single strand breaks in DNA (Van Loon *et al.*, 1993). Filters are used to impede the passage of long DNA strands and so if single strand breaks have been induced the smaller DNA fragments will pass more easily through the filter and thus be measured. DNA integrity can also be measured using the enzyme linked immunosorbent assay (ELISA) (Van Loon *et al.*, 1991). Such techniques have been used to assess DNA damage and repair during spermatogenesis but no results have been published concerning DNA damage in mature spermatozoa and its association with fertility status.

The alkali single cell gel electrophoresis (SCGE or comet) assay (Singh *et al.*, 1988; McKelvey-Martin *et al.*, 1993) has also been used to assess strand breakage in DNA. It has an advantage over alkaline elution and the ELISA assay, since DNA integrity in individual cells can be measured, as opposed to obtaining an overall value for an entire sample. Rydbert and Johanson (1978) were the first to perform a quantitation of damage in single cells. Ostling and Johanson (1984)

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improved the technique by developing an electrophoretic microgel technique under neutral conditions. The alkaline SCGE technique of Singh *et al.* (1988) with minor modifications (McKelvey-Martin *et al.*, 1993) is widely used and is the basis of the protocol used in this study.

Intact nuclei appear in the comet assay to have brightly fluorescent heads. Strand breaks induced in DNA release the supercoiling and allow migration of DNA to occur in the direction of the anode. This migrated DNA forms a comet tail in which the fluorescence intensity is related to the number of strand breaks.

This present study employs a slightly modified alkaline comet assay to compare baseline DNA damage in spermatozoa from fertile and infertile men. The effects of DNA damaging agents, X-irradiation and hydrogen peroxide, have been measured in each group in order to assess the ability of spermatozoa to withstand induced damage and to determine the relationship, if any, between DNA integrity and DNA status.

Materials and methods

Semen analysis

Semen samples were obtained from 20 normozoospermic (sperm number $\geq 20\times10^6$/ml, motility >50%, normal morphology >30%) men of recently proven fertility, 20 normozoospermic infertile (sperm number $\geq 20\times10^6$/ml, motility >50%, normal morphology >30%), and 20 asthenozoospermic infertile (sperm number $\geq 20\times10^6$/ml, motility <50%, normal morphology >30%) (WHO criteria, 1992) men after abstinence from sexual activity for 3 days. The infertile men were patients at the Regional Infertility Centre, Belfast. They were the partners of women who had failed to conceive after 2 years of unprotected intercourse. Routine semen analysis was carried out by light microscopy providing details of count, motility and morphology. In order to select the spermatozoa which showed the best motility and morphology from the seminal plasma and the less motile subpopulations, the samples were prepared by two-step discontinuous Percoll gradient centrifugation (Krausz *et al.*, 1994). Freshly liquefied semen was treated as follows: (i) sperm separation by two-layer Percoll (95.0–47.5%) centrifugation at 500 *g* for 20 min; (ii) concentration by centrifugation at 250 *g* for 10 min; and (iii) the resulting sperm pellets were diluted with Biggers–Whitten–Whittingham medium (BWW; Biggers *et al.*, 1971) to a concentration of 1×10^5/10 µl.

Damaging agents

Spermatozoa from each preparation were divided into seven aliquots so that a control group could be compared to cells from the same sample which had been treated with damaging agents at different concentrations. To induce damage spermatozoa from each sample were irradiated with doses of 5, 10, or 30 Gy at room temperature, or treated with 40, 100 or 200 µM hydrogen peroxide (Sigma Chemical Company, Poole, Dorset, UK) for 1 h at 4°C. Spermatozoa have a lack of DNA repair mechanisms (Chandley and Kofman-Alfaro, 1971; Van Loon *et al.*, 1991) and hence it was anticipated that irradiation with X-rays at room temperature would reflect the induced damage without the complication of repair. As expected, preliminary experiments carried out at 4°C and at room temperature on sperm cells indicated no differences in the extent of induced damage (data not shown). X-ray irradiation was carried out using a 300 kV Siemens Stabilipan X-ray source at a dose rate of 2.6 Gy min^{-1}.

Single cell gel electrophoresis assay

The alkaline comet assay was carried out using a method described by McKelvey-Martin *et al.* (1993) which is based on that initially reported by Singh *et al.* (1988). For each sample seven slides were prepared, one control and one for each of the damaging agents at each dose. Fully frosted slides (Richards Supply Company Limited, London, UK) were covered with 100 µl of 0.5% normal melting point agarose (Sigma), a coverslip added and the agarose allowed to solidify. The coverslips were removed and 1×10^5 spermatozoa in 10 µl BWW were mixed with 75 µl of 0.5% low melting point agarose (Sigma) and this was used to form the second layer. A final layer of 75 µl of 0.5% low melting point agarose was then added and allowed to solidify. The slides with coverslips removed were then placed in lysis buffer for 1 h (2.5 M NaCl, 100 mM NaEDTA, 10 mM Tris pH 10, 1% Triton X, and 10% dimethylsulphoxide (DMSO; Sigma) at a pH of 10). To lyse the spermatozoa the McKelvey-Martin (1993) protocol was modified by incubating the slides at 37°C in 0.1 mg/ml of proteinase K (Sigma) overnight. 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) for 20 min to allow the DNA from the cells to unwind. Electrophoresis was performed at room temperature, at 25 V (0.714 v/cm) and 300 mA, obtained by adjusting the buffer level (pH 13), for the shorter time of 10 min. 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 of 20 µg/ml ethidium bromide (Sigma) and covered with a coverslip. All steps were carried out under yellow light to prevent further DNA damage.

Analysis of cells

A total of 50 cells from each slide were selected randomly and analysed by image analysis using Hewlett and Packard Super VGA and Fenestra Comet Software (version 2.2). Observations were made at magnification ×400 using an epifluorescent microscope (Olympus BH2). Several features of each cell were calculated by the software package, including percentage head and tail DNA, physical area, optical density of the head and tail, tail length and tail moment (percentage tail DNA multiplied by tail length measured from the edge of the comet head).

Statistical analysis was carried out using non-parametric statistics with Arcus Pro 2 (Medical Computing, Aughton, UK) statistics package on the values obtained for the percentage head DNA of each cell. Comparisons were made between the baseline values obtained in the spermatozoa from the fertile men with those from each of the infertile groups using the non-parametric Mann–Whitney *U* test. Further analysis was carried out to determine whether the damage induced by the X-ray irradiation and hydrogen peroxide treatments was significantly different from the control values within each sample using the Wilcoxon Signed Rank test.

Results

Untreated spermatozoa from both fertile (Figure 1a) and infertile men appear to have a comet head significantly smaller than that observed in somatic cells such as lymphocytes at the same magnification (Figure 1b). This reflects the haploid nature of the sperm nucleus compared to the diploid complement of DNA in somatic cells and also the tight DNA packaging present in sperm cells. On average, ~20% of total DNA fluorescence (representing damaged DNA) was found to be in

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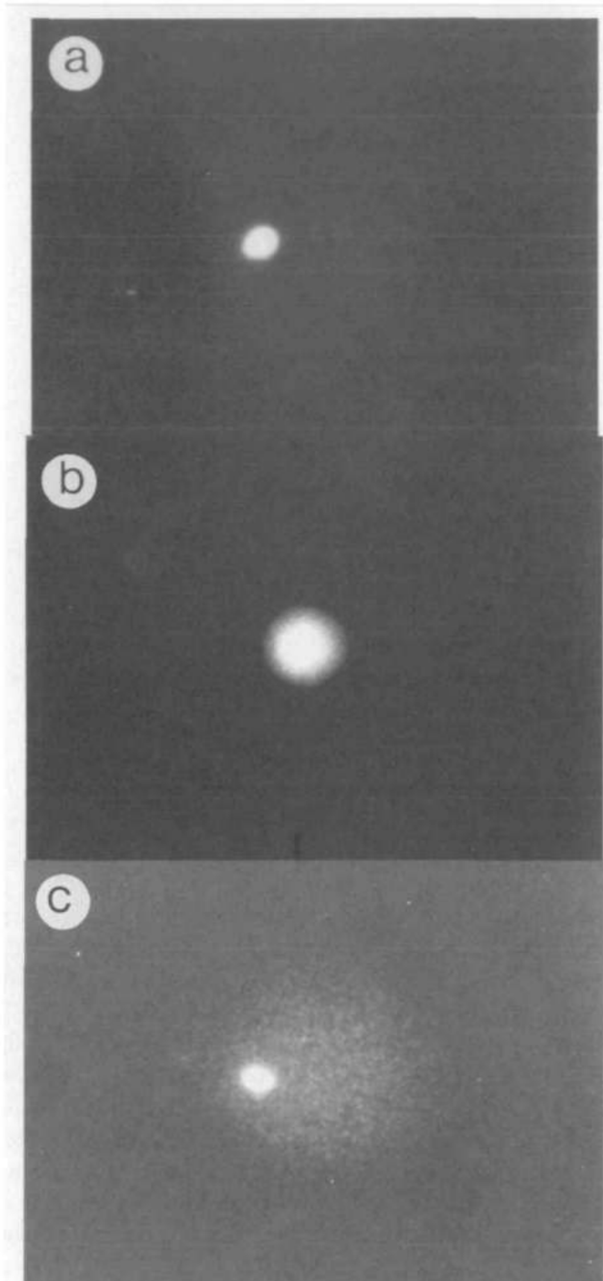

Figure 1. (**a**) An untreated spermatozoon from a fertile donor processed in the comet assay. (**b**) An untreated human lymphocyte processed in the comet assay. (**c**) A spermatozoon from a fertile donor processed in the comet assay following 30 Gy irradiation. Original magnification ×400.

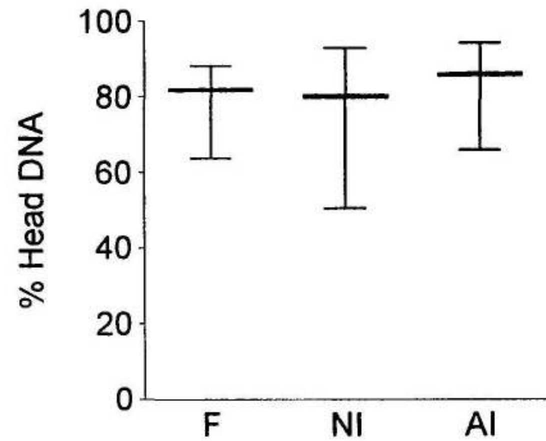

Figure 2. Median baseline values with interquartile values for percentage head DNA in spermatozoa from fertile (F), normozoospermic infertile (NI), and asthenozoospermic infertile (AI) men.

the comet tail of untreated sperm cells. Median percentage head DNA was therefore ~80% in control spermatozoa (Table I). This degree of damage is notably higher than that observed in somatic cells where <5% tail DNA baseline damage (95% head DNA) is usually observed. Each damaged spermatozoon had a comet tail which was deposited in a circular pattern (Figure 1c). No increase in tail length was observed with increased damage; instead the tail fluorescence became more intense.

Percentage head DNA values were compared within the untreated normozoospermic fertile, normozoospermic infertile and asthenozoospermic infertile groups. Figure 2 shows that there was no significant (P >0.05) difference in baseline median head DNA values between the spermatozoa from fertile men and those from the infertile men.

Median percentage head DNA values were obtained for each population after in-vitro treatment with X-rays or hydrogen peroxide. These values were compared with the median control values (Table I). There was a clear dose-dependent response following either treatment for both fertile and infertile groups with decreasing values of percentage head DNA accompanying increasing dose. X-ray irradiation at 30 Gy of the infertile groups produced significant amounts of damage when compared with the control. The asthenozoospermic infertile group was statistically significantly damaged by 40 µM H_2O_2, whereas 100 µM H_2O_2 was required to statistically significantly damage the DNA from normozoospermic fertile and normozoospermic infertile spermatozoa (Table I).

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Table I. Percentage head DNA after X-ray and hydrogen peroxide treatment. Values are median (interquartile range)

	X-ray irradiation (Gy)				Hydrogen peroxide (µM)			
	0 (control)	5	10	30	0 (control)	40	100	200
Normozoospermic fertile	81.7 (24.5)	74.4 (27.6)	69.2 (28)	72.5 (31.4)	81.7 (24.5)	74.2 (26.7)	63.6 (12.5)*	53.8 (26.5)*
Normozoospermic infertile	79.9 (42.3)	69.5 (53.2)	68.9 (48.3)	58.5 (59.6)*	79.7 (42.3)	82.5 (44.8)	64.6 (50.7)*	56.1 (68.3)*
Asthenozoospermic infertile	85.1 (28.6)	82.9 (37.6)	76.2 (47.7)	63.0 (36.9)*	85.1 (28.6)	72.54 (42)*	54.3 (43)*	45.0 (36.7)*

*Significantly different (P <0.05 by Wilcoxon Signed Rank test).

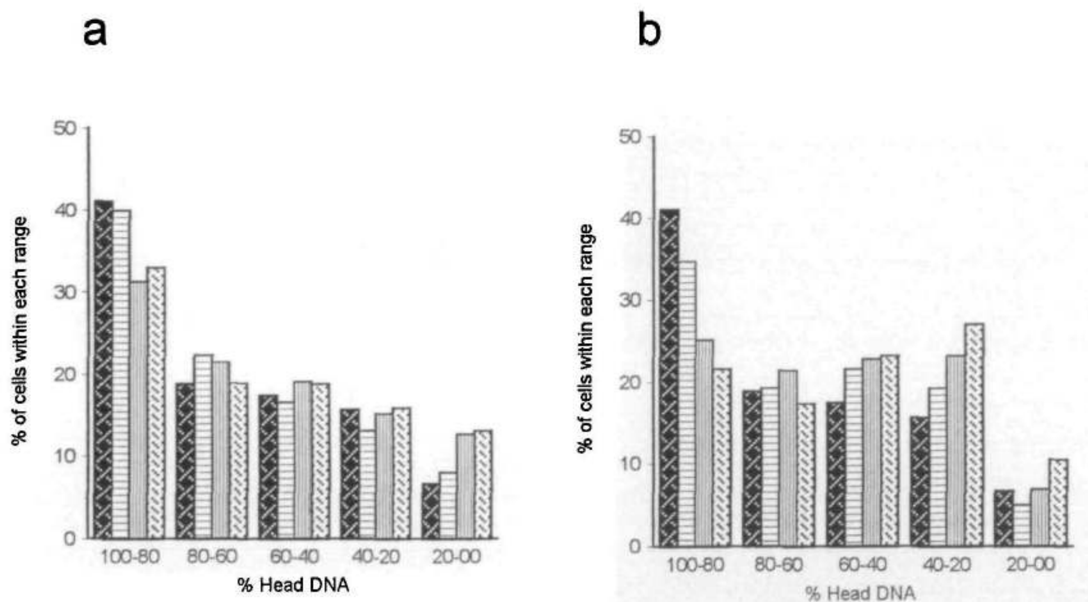

Figure 3. (**a**) Distribution of DNA damage in the fertile group showing control (▩) values and following 5 Gy (▤), 10 Gy (▥) and 30 Gy (▧) irradiation. (**b**) Distribution of DNA damage in the fertile group showing control (▩) values and following treatment with 40 μM H_2O_2 (▤), 100 μM H_2O_2 (▥) and 200 μM H_2O_2 (▧).

To determine whether all the cells in the sample were affected to the same extent by the damaging agents, or whether certain subgroups within the ejaculate were more susceptible, the data was examined by dividing the percentage head DNA values into ranges of 20%, i.e. the percentage of cells whose percentage head DNA lay between the range of 0–20%, 21–40% and so on. Analysis of the control spermatozoa from the normozoospermic fertile men (Figure 3) showed that >40% of the cells had a percentage head DNA value of 80–100%. Irradiation with X-rays or treatment with hydrogen peroxide at each dose caused an increase in DNA damage to the cell population as there were fewer cells with a percentage head DNA of 80–100%. However this decrease was not significant ($P > 0.05$).

However, both of the infertile groups showed a significant decrease ($P < 0.05$) in the number of spermatozoa having percentage head DNA >80% after 30 Gy irradiation (Figures 4a and 5a) and after treatment with 40 μM, 100 μM and 200 μM hydrogen peroxide (Figures 4b and 5b).

Discussion

Although there has been interest in DNA damage and repair in spermatogenesis (Van Loon *et al.*, 1991; 1993), the relationship between DNA status in mature spermatozoa and the possible effect on fertility status in man has been neglected in the literature. However, there is increasing awareness of the importance of DNA integrity in spermatozoa. Intracytoplasmic sperm injection (ICSI; Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1994) has given previously untreatable men the chance to have a child. Using this method, oocytes have been shown to be capable of processing spermatozoa with intact acrosomes, spermatozoa with no motility, tailless sperm heads and immature spermatozoa (Baker, 1993) which would have previously been rejected as candidiates for in-vitro fertilization (IVF). When penetration of such spermatozoa has been achieved, the subsequent development of the embryo is dependent on the DNA present. In this study we have proposed a method by which the alkaline single cell gel electrophoresis assay may be adapted to assess DNA damage in human spermatozoa. We found it was necessary to adapt the assay used for somatic cell for use with spermatozoa, due to the tight packaging of DNA in sperm nuclei.

During spermiogenesis the nucleus elongates and the chromatin becomes highly condensed, with somatic-type histones being replaced by basic proteins which are in turn replaced by protamines enriched in arginine (Sidney and Grimes, 1986). The DNA is organized into loops with an average length of 27 kb, attached at their bases to the nuclear matrix and anchored to the base of the sperm tail by the nuclear annulus. The loops are also stabilized by disulphide bonds (Ward and Coffey, 1991; Ward, 1993; Barone *et al.*, 1994), causing the condensed chromatin to be inert and resistant to the standard lysis agents. After ejaculation three phases have been described in the evolution of chromatin stability (Molina *et al.*, 1995). Initially, some thiol groups are oxidized to form S–S bonds while a large number remain non-covalently bound. Secondly, chromatin is stabilized by zinc present in the seminal plasma which binds to free thiol groups. Thirdly, hyperstability is introduced by depletion of zinc which allows the formation of additional interchromatin bonds. These workers found that addition of sodium dodecyl sulphate (SDS) 1 min after ejaculation caused decondensation of sperm chromatin, whereas 45 min after ejaculation disulphide reducing agents such as dithiothreitol (DTT) were required for decondensation. Other studies have also shown the need to use DTT to reduce the protein bonds for techniques such as fluorescence in-situ hybridization (FISH, Rousseaux and Chevret, 1995).

Because of this compact structure the strands of DNA cannot migrate during electrophoresis, so a proteinase enzyme is

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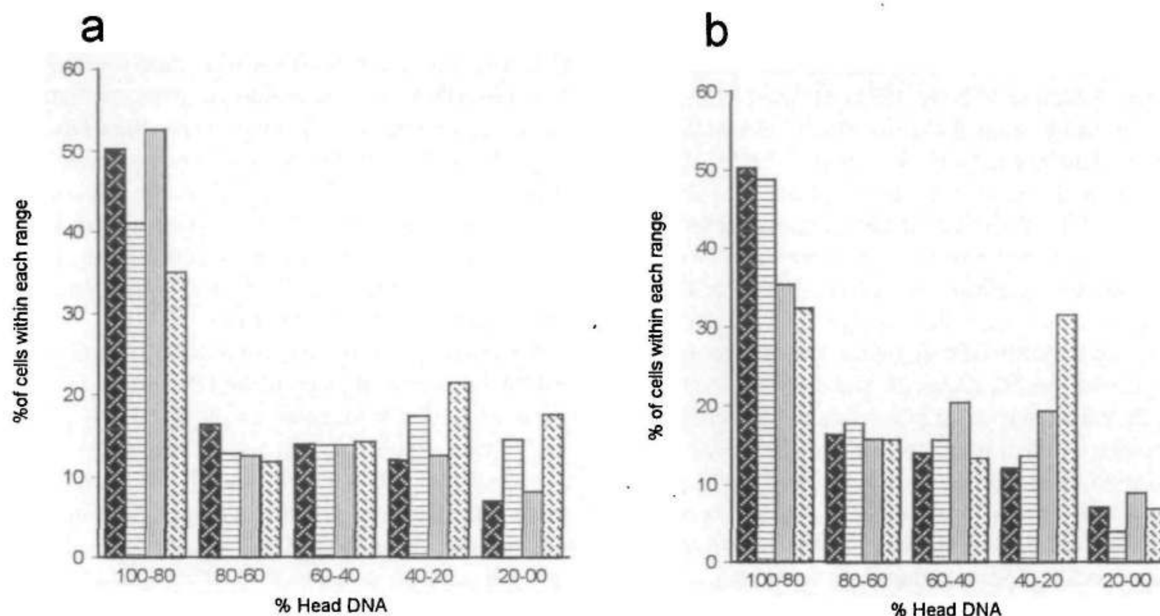

Figure 4. (a) Distribution of DNA damage in the normozoospermic infertile group showing control (▣) values and following 5 Gy (▤), 10 Gy (▥) and 30 Gy (▧) irradiation. (b) Distribution of DNA damage in the normozoospermic infertile group showing control (▣) values and following treatment with 40 μM H_2O_2 (▤), 100 μM H_2O_2 (▥) and 200 μM H_2O_2 (▧).

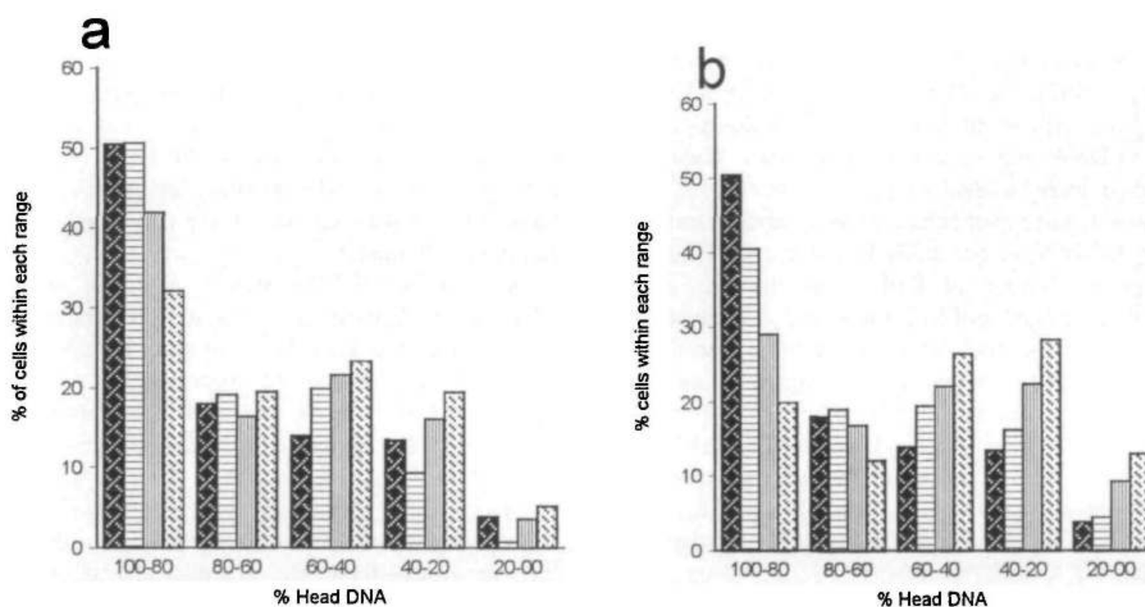

Figure 5. (a) Distribution of DNA damage in the asthenozoospermic infertile group showing control (▣) values and following 5 Gy (▤), 10 Gy (▥) and 30 Gy (▧) irradiation. (b) Distribution of DNA damage in the asthenozoospermic infertile group showing control (▣) values and following treatment with 40 μM H_2O_2 (▤), 100 μM H_2O_2 (▥) and 200 μM H_2O_2 (▧).

added to break down the protamines. The concentration of proteinase K (0.1 mg/ml) used here was lower than that used by Singh *et al.* (1989) in the only previously published attempt to examine mature spermatozoa with the SCGE assay. This group observed high levels of damage with 400 rads irradiation, in which the total DNA complement had migrated out of the head. In contrast, the present study found that baseline damage in spermatozoa from both fertile and infertile men was ~20% (Figure 2). This is still a high degree of baseline damage compared with the much lower levels found in somatic cells (McKelvey-Martin *et al.*, 1996).

Other workers have found higher levels of damage in spermatids than in somatic cells using alkali elution and ELISA techniques (Van Loon *et al.*, 1991; 1993). These techniques have all shown that damage induced in elongated spermatids cannot be repaired (Sega *et al.* 1986; Sega and Generoso, 1990; Van Loon 1991, 1993), probably due to loss of the cytoplasm which contains the repair machinery (Sega, 1976). This lack of repair may partially explain the high levels of damage found in spermatozoa which might appear to be a cause for concern. However, it has been shown that some repair of sperm DNA may take place within the oocyte after fertilization (Ashwood-Smith and Edwards, 1996). The reduction in cytoplasm within the spermatozoon also leads to

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a deficiency in antioxidant enzymes including glutathione peroxidase, superoxide dismutase or catalase that protect most other cell types (Aitken and Fisher, 1994). However, this might be compensated for by seminal plasma which has a high chain breaking antioxidant capacity (Lewis *et al.*, 1995), since it contains ascorbic and uric acid and α-tocopherol (unpublished data). Therefore, in the absence of seminal plasma spermatozoa may be vulnerable to oxidative stress leading to DNA strand breakage. In addition, spermatozoa, unlike somatic cells, may contain a large number of alkali-sensitive sites (Singh *et al.*, 1989). This group hypothesized that these sites reflect physiologically important modifications of gamete DNA compared to somatic cells, which may assist in the packing of spermatozoa DNA and therefore play a protective role, or may play a role in decondensation, replication or transcription.

Chromosomal aberrations, which may be a direct consequence of DNA lesions (Brandriff *et al.*, 1990), have recently been correlated with repeated abortions following a study using the sperm–hamster egg test (Rosenburch and Sterzik, 1994), where the chromosome breaks were found to be significantly increased in spermatozoa from men whose partners repeatedly aborted. Sperm chromatin structure assays (SCSA) using fluorescent microscopy or flow cytometry have also suggested that poor quality chromatin is highly indicative of male subfertility (Tejada *et al.*, 1984; Evenson *et al.*, 1991; Liu and Baker, 1992). The SCSA assay measures denatured DNA by staining with Acridine Orange which fluoresces red with denatured DNA, and green with intact DNA. The semen samples studied were washed or prepared by the swim-up technique and may have contained a larger number of immotile spermatozoa. In contrast, our study has shown that baseline DNA damage is similar in fertile and infertile groups (Figure 2). We examined motile spermatozoa after separation by a Percoll gradient. As these represent both the spermatozoa most likely to reach the oocyte *in vivo* and the same subpopulation used routinely in assisted conception procedures, male fertility may be more closely associated with the DNA status within this group of spermatozoa. It would, however, be of interest to study the 41.5% Percoll layer to assess whether there was any association between DNA status and sperm motility.

The decline in human semen quality as assessed by sperm concentration over the past 50 years has been blamed on environmental pollution. Chemicals, reactive oxygen species and radiation have been implicated as major culprits. To determine whether sperm DNA may be susceptible to such pollutants we treated spermatozoa with X-rays or hydrogen peroxide. We found sperm DNA surprisingly resistant to damage compared to somatic cells. Spermatozoa from fertile donors remained undamaged after exposure to 30 Gy radiation. Significant damage to control spermatozoa was induced only with 100 μm H_2O_2 (Table I). Even at this dose of H_2O_2, a subpopulation of 20–30% remained relatively intact with 80–100% head DNA (Figure 3). This is in contrast to the DNA of somatic cells which is significantly damaged with doses of <10 μm H_2O_2 (McKelvey-Martin *et al.*, 1993), and the DNA in cells in the earlier stages of spermatogenesis which require less than 10 Gy radiation to induce damage (Wiger *et al.*, 1995). This is no doubt a consequence of the marked condensation which protects the DNA of mature spermatozoa.

Sperm DNA from the normozoospermic infertile group were more susceptible to damaging agents than DNA from fertile men (Table I) as 30 Gy X-rays caused statistically significant damage to this group. This infertile group contained a subpopulation of resistant cells similar to that observed in the fertile group; however, the number of cells in the 80–100% DNA head range significantly decreased following treatment with 30 Gy radiation or 40 μM H_2O_2.

Spermatozoa from the asthenozoospermic infertile group exhibited statistically significant DNA damage following treatment with 30 Gy radiation or 40 μM H_2O_2 (Table I). There was also a significant decrease in the number of cells in the subpopulation of 80–100% head DNA range after either treatment. In previous studies a higher incidence in the activity of reactive oxygen species was observed in asthenozoospermic infertile men in comparison with asthenozoospermic fertile men (Lewis *et al.*, 1995; McKinney *et al.*, 1995). This was induced following sperm removal from the protective seminal plasma. This correlates with the greater susceptibility of asthenozoospermic samples to undergo DNA damage, and suggests that this damage may be oxidative. In addition, the seminal plasma of asthenozoospermic men has reduced chain-breaking antioxidant capacity (Lewis *et al.*, 1995) caused in part by a reduced ascorbate concentration (unpublished data). Although the baseline levels of DNA damage are similar in the fertile and infertile groups, the DNA may be at greater risk of oxidation. The greater susceptibility of the DNA present in infertile samples to damaging agents may lead to fertilization failure.

Our modified SCGE assay is a simple visual technique which may be used to assess the fertilization potential of spermatozoa. We have shown that the baseline DNA damage is similar in spermatozoa from fertile and infertile men. Spermatozoa from fertile men are less susceptible to damaging agents and these samples also contain a distinct subpopulation with DNA which is resistant to damage and thus remains relatively intact. Although this subpopulation was present in both normozoospermic infertile and asthenozoospermic infertile groups there was a statistically significant decrease following treatment with damaging agents. We conclude that the greater susceptibility of DNA to damage in the normozoospermic and asthenozoospermic infertile groups may be associated with their infertility.

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