X-ray-induced DNA double-strand breaks in human sperm

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A methodology for quantifying DNA double-strand breaks in human sperm is described. Sperm from three healthy human donors on three separate days each were irradiated with 12.5, 25, 50 and 100 cGy X-rays. Linear dose–response effects were observed in migrated DNA from sperm nuclei when electrophoresed under neutral conditions. RNase and proteinase K treatments for longer duration were necessary, to decondense the chromatin and presumably to release the broken DNA for migration in the electrophoretic field in a dose-dependent manner. An increase in DNA migration was observed with as low as 12.5 cGy, but damage was observed in all samples at 25 cGy. No evidence of repair of these X-ray-induced DNA double-strand breaks was observed during a 2 h period.

Introduction

Evans (1996), in his award-winning lecture, reviewed the consequences of DNA damage in present generations and their offspring. Realizing the importance of environmental DNA damage to parents and its effects in following generations, several investigators have studied DNA damage in sperm cells. Gorczyca et al. (1993) and Saier et al. (1995) used terminal deoxynucleotidyl assays to distinguish between dead (labeled) and live (non-labeled) sperm cells from human semen samples. Sega et al. (1986) used alkaline elution assays to study induction of DNA single-strand breaks in sperm from mice exposed to methyl methanesulfonate. Using alkaline elution, Sega and Generoso (1988) detected DNA single-strand breaks in sperm cells from mice treated with ethylene oxide. Singh et al. (1988) found abundant alkali-labile sites using the microgel electrophoresis technique while looking for levels of strand breaks in human and mouse sperm. Due to extensive single-strand breakage, they were unable to quantify the levels of breaks even in control samples. More recently, Fraga et al. (1996) studied oxidative products of DNA from smokers in sperm cell lysates using HPLC.

Knowing the importance of measuring DNA damage in sperm cells, it is surprising that there is no literature concerning the estimation of DNA double-strand breaks in human sperm. Also, the presence of abundant alkali-labile sites in sperm obviates the detection of single-strand breaks; we attempted to standardize the microgel electrophoresis technique to quantify DNA double-strand breaks in sperm cells. Due to the unique packaging of DNA (condensed chromatin) in sperm cells compared with other cells, it is difficult to free the DNA from proteins. Different strategies have been adopted to decondense the DNA. Here we have standardized a protocol that allows dissociation of other macromolecules from DNA. Cells were lysed in microgels at 4°C for 1 h and then treated with RNase A for 4 h at 37°C and proteinase K for at least 15 h at 37°C. For detection of DNA double-strand breaks, microgels were electrophoresed at neutral pH. This study deals with the basal levels of DNA double-strand breaks in human sperm and the response of these cells to X-rays and H2O2 in vitro.

Materials and Methods

Most of the chemicals were purchased from Sigma (St. Louis, MO). Three samples were collected from each of three healthy young adult donors who were asked to abstain for 2 days before providing each sample. Lymphocytes were isolated from the blood of one of the sperm donors. After viability testing (which was always >97% by dye exclusion test), these samples were immediately irradiated with 0, 12.5, 25, 50 and 100 cGy X-rays using a Kelley-Koent device (Covington, CT) at a rate of 100 cGy/min. Approximately 0.5 ml semen were well mixed with 50 µl 0.7% 1:3 high resolution agarose (Amresco, Solon, OH). Fifty microliters of the sperm cell suspension in agarose was layered onto the fully frosted and already agarose-coated (dried) slides (coating the slides with 100 µl 10× saline) for 1 h.

For detection of DNA double-strand breaks, microgels were lysed in microgels at 4°C for 1 h and then treated with RNase A (Amresco, Solon, OH). The slides were incubated at 37°C for 4 h. The slides were transferred to the solution for enzyme treatment containing 1 mg/ml DNase-free proteinase K (Amresco, Solon, OH) and incubated at 37°C for 15 h. For hydrogen peroxide experiments, slides were exposed to differing concentrations of the chemical for 1 h at room temperature in the solution for enzyme treatment. The slides were placed on a horizontal slab in an electrophoretic unit and microgels equilibrated with 300 mM sodium acetate, 100 mM Tris, pH 9, for 20 min and electrophoresed at 12 V (0.4 V/cm) and ~100 mA for 1 h. The slides were immersed in 1 M ammonium acetate in ethanol for 30 min and then in absolute ethanol for a minimum of 2 h. The slides were transferred to 70% ethanol for 30 min and air dried. The slides were stained with a freshly prepared 1 µM solution of YOYO (Molecular Probe, Eugene OR) in 5% DMSO. The stock solution of YOYO usually comes at 1 mM concentration and should be diluted 1:1000. To achieve uniform staining, slides may be prestained with a freshly prepared 50 µl solution of 5% DMSO, 10 mM NaH2PO4, 5% sucrose. Observations were made using a Reichert fluorescent microscope having a FITC filter combination (excitation 490, dichroic 500, emission 510 nm). Lymphocytes were also processed in similar ways and analysis was performed under identical conditions as for sperm cells.

Total length (µm), which included the nucleus and migrated DNA up to the last pixel, was measured using an eyepiece micrometer. Data were subjected to one way ANOVA. Student–Newman–Keuls test was used for all pairwise multiple comparisons (significant at P < 0.05).

Results

Lysed cells when treated first with RNase and then with proteinase K in a solution containing 2.5 M NaCl, 0.05%
sodium lauryl sarcosine, 5 mM Tris, pH 7.4, for adequate times show a linear dose-response effect to 12.5, 25, 50 and 100 cGy X-rays (Figures 1 and 2). We observed significant differences in response in samples collected on different days. Data from the same individual for each of three days was combined. With the combined data, for each individual, significant differences were detected between all doses except between the control and 12.5 cGy for subject 3.

Due to the compact chromatin, sperm cells do not reveal any strand breaks when exposed to X-rays, lysed and electrophoresed under neutral or alkaline conditions (Figure 3). After lysis in regular lysis solution containing 2.5 M NaCl, 100 mM EDTA, 1% sodium lauryl sarcosine, 10 mM Tris, pH 10, Triton X-100, DNA migration show an inverse X-ray dose–response relationship (data not shown). Furthermore, when we used a lysing solution having 2.5 M NaCl, 0.05% sodium lauryl sarcosine, 5 mM Tris, pH 7.4, for extensive RNase (10 μg/ml for 4 h) and proteinase K (1 mg/ml for 15 h) treatment, either alone or in combination with cysteine, a linear response to X-ray dose was observed (Figure 4). However, when the above protocol of extensive enzymatic treatment was combined with heparin and glutathione, the response to X-rays was insignificant (Figure 4).

No evidence of repair of these double-strand breaks was observed when cells, after exposure to 100 cGy, were allowed to repair at 37°C for up to 2 h. However, human lymphocyte repair is almost complete under these conditions (Figure 5).

When lysed cells were exposed to RNase followed by 1 mg/ml l-cysteine and 0.5 mg/ml proteinase K, a non-linear dose–response relationship was achieved (Figure 6). When proteinase K was replaced with 3 mg/ml heparin and 3 mg/ml glutathione, a non-linear dose–response relationship was obtained (Figure 6). In this case we observed very small halos or nucleoids, indicating incomplete decondensation of chromatins.

Sperm cells lysed, exposed first to RNase and proteinase K and then to 0, 17.6, 35.2, 70.4, 140.8 or 281.6 μM hydrogen peroxide showed a linear dose–response relationship in length of DNA migration (Figure 7).

**Discussion**

Protamines constitute ~85% and histones ~15% of proteins in sperm cells (Tanphaichitr et al., 1978). Packaging of DNA in sperm cells is 6-fold more compact and has 40-fold less volume than that in somatic cells (Wyrobek et al., 1976; Ward and Coffey, 1991; Ward, 1994; Ward and Zalensky, 1996). To achieve this compactness there is disulfide bonding between DNA and protamines. DNA–protein (protamine) disulfide bonding is used to fold the DNA like a folded ladder at specific sites and at specific and regular intervals. This is called a linear side-by-side arrays model (Balhorn, 1982; Ward and Coffey, 1991; Ward, 1994). This packaging is useful in sperm cells to reduce volume for easier passage through the genital tract, to minimize damage by exogenous agents before fertilization and to keep the genome inactive during travel. This sleeping genome (Ward, 1994) is incapable of repairing damage. The compact packaging does not allow easy dissociation of the DNA from proteins and thus it is difficult to perform assays for DNA strand breaks. Investigators have achieved decondensation of mouse sperm using cysteine and proteinase K in combination (Sega et al., 1986) and in human sperm using proteinase K (Singh et al., 1988) heparin and glutathione in combination (Reyes, 1989). We tried all these protocols with limited success. Addition of the RNase digestion step and the change in lysing solution for enzyme digestion allowed chromatin decondensation. We also noted that the presence of ionic (sodium lauryl sarcosine) and non-ionic (Triton X-100 or NP-40) detergents in the lysing solution for 1 h prior to enzymatic treatment was necessary for adequate DNA decondensation.

The decondensation step is essential for the demonstration of an X-ray-induced linear dose–response relationship with a reasonable sensitivity of 12.5 cGy. We observed a high variability in DNA migration in control cells and cells exposed to X-rays. We currently do not understand the reasons for this. One reason may be that large numbers of sperm cells carry various levels of chromosomal aberrations (>10% cells showing abnormality; Genesca et al., 1992). The other reason for this variability may be inadequate DNA decondensation in
some cells. A reduction in variability or analyzing a larger number of cells will further enhance sensitivity.

We did not observe any dose–response relationship using a combination of heparin and reduced glutathione (replenishing glutathione every hour for 4 h). Small or incomplete halos with a compact central core were seen. Complete decondensation of DNA was observed in control samples when digested with 0.5 mg/ml proteinase K and 1 mg/ml cysteine in combination. However, samples irradiated with 50 and 100 cGy did not show complete decondensation of DNA, which resulted in a non-linear dose–response relationship. We feel that some proteins were left undigested due to a low concentration of proteinase K in the protocol (0.5 versus 1 mg/ml). We were surprised at the effectiveness of RNase treatment but note that sperm cells have been shown to have 0.1 pg RNA (Passot et al., 1989). The authors reported various sizes of molecules of RNA, some in the form of nucleoproteins, and speculated that the RNA is probably needed soon after fertilization. Because of the effectiveness of RNase in our system, we speculate that the RNA may be bound to the DNA like proteins and play a role in packaging of DNA in sperm cells. RNase A digestion may be necessary for dissociation of the DNA from other macromolecules. We also found that elimination of EDTA and Triton X-100 from the lysis solution during enzymatic digestion of chromatin and higher concentration of agarose (0.7 rather than 0.5%) provided us with a better X-ray-induced linear dose–response relationship ($r^2 > 0.9$).

Sperm cells lack an effective DNA repair capacity, as has been shown by Sega (1974) and Genesca et al. (1992) using unscheduled DNA synthesis and a cytogenetic technique respectively. We also did not observe any repair of DNA double-strand breaks in sperm cells over a period of 2 h. This could be due to the compactness and thus inactivity of the genome. Contrary to this, we noticed complete repair of
Fig. 3. Photomicrograph of lysed and electrophoresed (but not digested with RNase or proteinase K) sperm cells showing a lack of decondensation of sperm DNA. Cells were stained with YOYO 1 (×400).

Fig. 4. XY line plot demonstrating the efficacy of various protocols to decondense sperm DNA. RNase plus proteinase K and RNase plus cysteine plus proteinase K are effective. Each point is represented by the mean and standard deviation of DNA migration from 50 cells.

Fig. 5. XY line plot showing repair kinetics of DNA double-strand breaks in human lymphocytes and human sperm cells. Each point is represented by the mean and standard deviation of DNA migration from 50 cells. DNA double-strand breaks in lymphocytes seem to be repaired in 120 min but not in sperm cells.

Fig. 6. XY line plot showing X-ray induced dose–response effects in human sperm cells lysed and decondensed using various protocols. In all three protocols RNase digestion was used after the lysis step. Each point is represented by the mean and standard deviation of DNA migration from 50 cells.

Fig. 7. XY line plot showing a dose-dependent increase in DNA migration in human sperm cells lysed, treated with RNase and proteinase K and then exposed to differing concentrations of hydrogen peroxide in microgels. Each point is represented by the mean and standard deviation of DNA migration from 50 cells.

X-ray-induced DNA double-strand breaks in human lymphocytes. Similar results on DNA repair in human lymphocytes have been reported previously (Singh and Stephens, 1997).

Preliminary results indicate that sperm cells treated with hydrogen peroxide in vitro for 1 h do not show a significant increase in DNA double-strand breaks even at the highest dose tested of 281.6 μM (data not shown). However, DNA from lysed and RNase- and proteinase K-treated sperm cells in microgels does show a linear dose–response relationship when exposed to 35.2, 70.4, 140.8 and 281.6 μM hydrogen peroxide (Figure 7). Together, these findings suggest that folding of sperm DNA, with the help of other macromolecules, like proteins, into condensed chromatin allows the cell to maintain the integrity of its DNA against chemicals like hydrogen peroxide which are generated intracellularly (Bize et al., 1991). Also, this may imply that chemically induced DNA damage can be detected in sperm cells if progenitor cells (spermatogonia and spermatocytes) are exposed to DNA damaging agents in vivo and the damage is not repaired or not allowed to repair while these cells are progressing into the sperm stage. Progenitor cells are more vulnerable to DNA damage because their chromatin is not condensed.

The development of this method to quantify DNA strand breaks in human sperm provides another biomonitoring tool. This technique can now be used to screen for the genotoxic
effects of various environmental agents and possibly for risk assessment in individuals as it might relate to the genotoxic or even mutagenic effects in their offspring.

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References


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