Effects of age on DNA double-strand breaks and apoptosis in human sperm

Narendra P. Singh, M.B.B.S., M.S.,[a] *Charles H. Muller, Ph.D.,*[b] *and Richard E. Berger, M.D.*[b]

University of Washington, Seattle, Washington

Objective: This study was designed to explore the relationship between men's age and DNA damage and apoptosis in human spermatozoa.

Design: Semen samples were collected from men between the ages of 20 and 57 years. Sperm DNA double-strand breaks were assessed using the neutral microgel electrophoresis (comet) assay, and apoptosis was estimated using the DNA diffusion assay.

Setting: Academic medical center.

Patient(s): Sixty-six men aged 20 to 57 years were recruited from infertility laboratory and general populations and consented to donate a semen sample. Recruitment was determined by time and day of analysis; the only exclusions were for azoospermia, prostatitis, or prior cancer therapy.

Intervention(s): None.

Main Outcome Measure(s): DNA damage and apoptosis in human sperm.

Result(s): Age correlated with an increasing percentage of sperm with highly damaged DNA (range: 0–83%) and tended to inversely correlate with percentage of apoptotic sperm (range: 0.3%–23%). For example, percentage of sperm with highly damaged DNA, comet extent, DNA break number, and other comet measures was statistically significantly higher in men aged 36–57 years than in those aged 20–35 years, but percentage apoptosis was statistically significantly lower in the older group. Semen analysis showed percentage motility to be significantly higher in younger age groups.

Conclusion(s): This study clearly demonstrates an increase in sperm double-stranded DNA breaks with age. Our findings also suggest for the first time an age-related decrease in human sperm apoptosis. These novel findings may indicate deterioration of healthy sperm cell selection process with age. (Fertil Steril® 2003;80: 1420–30. ©2003 by American Society for Reproductive Medicine.)

Key Words: DNA double-strand breaks, apoptosis, human sperm, aging, Comet assay

Maintenance of sperm DNA integrity is crucial to the health of future generations (1, 2). In contrast to the relatively dormant female gametes, spermatozoa are continuously produced by the testes as male germ cells and undergo lifelong cell replication, meiosis, and spermiogenesis. Male gametes (compared with female gametes) have a greater possibility of damage to nuclear DNA of Y chromosomes because of lack of recombination repair process, as there is only one Y chromosome available during meiosis. DNA in sperm is sixfold more compacted and has 40-fold less volume than does somatic cell DNA (3–6). To achieve this compactness there is disulfide bonding between DNA and protamines. Protamines constitute approximately 85%, and histones, about 15% of proteins in sperm cells (7). DNA-protamine disulfide bonding is used to fold DNA like a collapsible ladder at specific sites and at specific and regular intervals, according to the linear side-by-side arrays model (3, 4, 8).

This packaging is useful for several reasons: [1] to reduce the volume of the spermatozoa during their travel through the genital tract, [2] to minimize damage by exogenous agents before fertilization, and [3] to keep the genome transcriptionally inactive. In addition to these protective measures, sperm are immersed in fluids containing high levels of antioxidants. Despite these extensive defenses, DNA damage does occur in both developing and mature sperm. A high proportion of sperm with DNA damage may be a cause of infertility (9). Among fertile couples, sperm already selected through apoptotic pathways compete to fertil-

ize a single ovum. Although ideally the healthiest sperm (with intact DNA) will fertilize the ovum, sperm with damaged DNA may accomplish fertilization; this is suggested to result in poor pregnancy outcomes (10, 11). Intracytoplasmic sperm injection and IVF make it more likely that sperm with damaged DNA will fertilize the egg (12–14). Thus, evaluating sperm nuclear DNA damage and apoptosis is of clinical importance (15). Our goal is to establish and standardize a protocol for the sensitive estimation of DNA damage and apoptosis in human spermatozoa.

Although age-related changes in the male reproductive system are universally recognized, the question of declining fecundity with male age is controversial (16). Among couples who ultimately conceive, a man aged >35 years has twice the likelihood of requiring more than 12 months to impregnate his female partner than does a man aged <25 years (16). A recent multicenter study suggests that paternal age of ≥40 years significantly increases the risk of miscarriage in couples where the wife is ≥35 years old (17).

One possible explanation of these findings is that older men may have more sperm with damaged DNA. Age-dependent accumulation of DNA damage in sperm cells has only been assessed by chromosomal damage (18) using the premature chromosome condensation technique. To date, no sensitive highly quantitative method has been used to examine the relationship between subject's age and sperm DNA damage. Methods such as filter elution assays (19–21), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (22), comet assay, or microgel electrophoresis technique (23–32) have been used to quantify DNA damage in sperm cells. The neutral microgel electrophoresis technique is a sensitive and simple method of evaluating double–strand break DNA damage in individual sperm cells (24, 33–35). The technique involves making microgels with cells suspended in agarose solution on special microscopic slides, lysing cells with high salt and detergents, digestion and removal of proteins from nuclear DNA with proteinase K, electrophoresis under relatively neutral conditions, staining of electrophoresed DNA in microgels with an intense fluorescent dye, and analysis of images under epifluorescence by image analysis software. Various measurements made by the software are used as indices of DNA damage.

Apoptosis is a normal event that occurs both during and after embryonic development. Germ cell loss (36, 37), now recognized as apoptosis, is a dominant process during spermatogenesis and is regulated by p53, p21, caspases, bcl2, and Fas expression levels, with many alternate pathways (38–42). Mild to moderate genotoxic and cytotoxic insults also induce apoptosis. Although apoptosis is considered a mechanism to ensure selection of sperm cells with undamaged DNA, sperm with DNA damage that are not eliminated by apoptosis do sometimes manage to fertilize ova (10, 11).

The TUNEL assay (22, 43–48) and annexin V/PI staining (49–53) are two commonly used methods for detecting spontaneous and induced apoptosis in sperm. The DNA diffusion assay is simple, and it differentially measures apoptotic and necrotic cells accurately. This method is based on the fact that apoptotic cells have numerous alkali-labile sites, and these sites, once exposed to alkaline conditions, should yield small pieces of DNA. These pieces can easily diffuse in the agarose matrix, giving the appearance of a halo with a hazy outline. This unique pattern of DNA gradient diffused in agarose is characteristic of apoptotic cells and is distinguishable from necrotic cells (54). The DNA diffusion assay has been used previously for detection of apoptosis (55, 56). This assay involves mixing cells with agarose and making a microgel on a microscopic slide, then lysing the embedded cells with salt and detergents and treating them with pH >13 alkaline solution (to allow breakage of DNA at an abundant alkali labile site found in apoptotic cells and allowing diffusion of small molecular weight DNA in agarose), and finally visualizing the DNA using a sensitive fluorescent dye.

MATERIALS AND METHODS

Subjects

Men were recruited from both infertile and general populations. Excess seminal fluid from men who had clinical appointments at the infertility laboratory for semen analysis as part of an infertility workup was included. Other healthy men were recruited to be sperm donors from the general population or from a group of men in a prevasectomy cryopreservation program, after signing an institutional review board–approved consent. Men who were azoospermic, were diagnosed with chronic pelvic pain syndrome (prostatitis), or had received prior cancer therapy were excluded. Participants were requested to abstain from ejaculation for 2 to 5 days before sample collection.

Semen Analysis

Subjects were provided sterile polypropylene specimen containers that were not toxic to sperm. Semen samples were collected in a room next to the male fertility laboratory at our institution and were incubated at 37°C for 20 minutes before analysis. A portion of semen sample was taken at this time and processed for assessment of DNA damage and apoptosis as described in "Sperm DNA Analysis." The remaining semen sample was analyzed for standard semen quality parameters according to World Health Organization protocols (57) and for computerized strict criteria morphology using the Hamilton Thorne Research (Beverly, MA) Dimensions program (58). Semen smears were made and later stained with Bryan-Leishman stain and DiffQuick stain (Dade Behring Inc., Newark, DE) for leukocyte differential by one trained technologist and for assessment of sperm morphology. Analysis time, liquefaction, viscosity, color, volume, and pH were recorded. A 7-μL wet drop was examined using phase-contrast microscopy for progressive motility, rapid and linear motility, subjective speed and

progress, "round cells" per high-power field, and sperm agglutination, by counting ≥ 100 sperm or 25 fields. If motility was $<25\%$, a live-dead stain was performed. Concentration was measured using immobilized sperm in a hemocytometer. Monthly quality control was performed using cryopreserved aliquots of semen or by semen samples from normal donors.

Sperm DNA Analyses

Small volumes of semen (100–250 μL) were placed in a 1.5-mL polypropylene tube within 30 minutes of collection. Mineral oil was placed over the seminal fluid to prevent further contact with air, and the tubes were kept in the dark and immersed in ice until use. Approximately 10,000 sperm cells were mixed with 50 μL of agarose, and the mixture was used for making microgels as described in the following section. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Double-Strand Breaks in DNA

Making Agarose

This methodology is described in detail elsewhere (59). Briefly, 70 mg of high resolution agarose 3:1 (Amresco, Solon, OH) was boiled in 9 mL of distilled water in a microwave oven and 1 mL of 10× modified PBS (for 1 L: 80 g of NaCl, 2 g of KCl, 2 g of KH_2PO_4, 11.5 g of anhydrous Na_2HPO_4 or 29 g of $Na_2HPO_4 \cdot 7H_2O$, 32 g Tris HCl, pH 7.4). After adjusting volume to 10 mL by adding distilled water, the solution was just boiled once more. The volume was adjusted to 10 mL by adding distilled water, and the solution was well mixed to provide a concentration of 0.7%. The agarose was then dispensed in small aliquots and maintained at 55°C for 24 hours before use.

Preparing Slides

Fifty microliters of 0.7% agarose, 3:1, was used to coat MGE slides (Erie Scientific Co., Portsmouth, NH). The first layer of the microgel was made in two parts. First, 50 μL of agarose was pipetted on the top end of the frosted part of the slide while the slide was held horizontally in the left hand between thumb and index finger and the agarose was smeared in one motion using a pipet tip held horizontally in the right hand. The slide was then air-dried. Second, 200 μL of 1% agarose was pipetted onto the center of a clear-window frosted slide, and a cover glass (24 × 50 mm^2; Corning Glass Works, Corning, NY) was placed over it. After keeping the slides for 5 minutes at room temperature, the cover glass was removed, and approximately 10,000 sperm cells in 5 μL of PBS were mixed well with 50 μL of 0.7% agarose, 3:1, for each slide. Fifty microliters of this mixture was layered onto the slides precoated with the first layer of microgel to make a second layer of microgel. The slides were put in a cold steel tray kept on ice. After removing the cover glass, a third layer of 200 μL of agarose was layered on top of the second layer.

X-Ray Dose Response

As a positive control, aliquots of semen from a young donor were irradiated with 0, 50, 100, or 200 rads of X rays at a rate of 100 rads/min using a Kelley-Koett X-ray machine (Covington, CT). These sperm were then processed for the neutral microgel electrophoresis assay.

Lysis and Electrophoresis

Slides with microgels were incubated in a prewarmed (37°C) lysing solution (containing 1.25 M NaCl, 0.01% sodium N-lauroyl sarcosinate, 50 mM tetrasodium ethylenediaminetetraacetic acid, 100 mM Tris, pH 10, 2 mg/mL reduced glutathione [crystalline free acid], and 0.5 mg/mL of DNAse free proteinase K [Amresco]) for 2 hours at 37°C and then placed on a horizontal electrophoretic unit (Ellard Instrumentation, Monroe, WA) that had been modified to allow electrical input from power supply to both ends of an electrode. The unit was filled with 1 L of 500 mM NaCl, 100 mM Tris HCl, pH 9, and 1 mM EDTA. Slides with microgels were allowed to equilibrate for 20 minutes and electrophoresed for 20 minutes at 12 V and ~250 mA while the solution was recirculated at ~100 mL/min.

Neutralization, DNA Precipitation, and Staining of Microgels

Slides for neutralization and DNA precipitation were immersed in freshly prepared 20 mM Tris, pH 7.4 in 50% ethanol with 1 mg/mL of spermine for 15 minutes. This step was repeated twice with fresh solution. Slides were air-dried. One slide at a time was stained with 50 μL of 0.25 μM YOYO-1 in 2.5% dimethyl sulfoxide and 0.5% sucrose.

Image Analysis

Images at ×400 magnification were captured using a charge-coupled device (CCD) camera GW525x (Genwac Inc., Orangeburg, NY) attached to a DMLB epifluorescence microscope (Leica, Germany) with an excitation filter of 490 nm, a 500-nm dichroic filter, and an emission filter of 515 nm. For estimation of DNA damage, a minimum of 50 spermatozoa from each sample was analyzed using Vis-Comet image analysis software (Impulse Bildanalyse GmbH, Gilching, Germany). Spermatozoa were distinguished from somatic cells by their size. The diameter of somatic cells is more than twice that of spermatozoa. Ten computed measurements of the images were used as indices of DNA double-strand breaks: comet extent, comet total intensity, comet total area, tail extent, tail extent moment, tail (Olive) moment, tail integrated intensity (Singh), tail integrated intensity ratio (Singh), tail area (Singh), and DNA break number (Singh). Most of these measurements were highly correlated with each other. We chose two primary computed measures: comet extent and DNA break number. Comet extent, which measures total image length, is highly correlated with all parameter measures ($r^2 = 0.68$–0.99) and poorly correlated with total comet intensity and DNA break

number. DNA break number measures pieces of fragmented and migrated DNA and is poorly correlated with all other measures ($r^2 \leq 0.5$).

The software could not analyze comets that were >625 pixels in length. We designated these as sperm with highly damaged DNA and manually calculated the percentage of these images found per 100 sperm. Because these comets could not be analyzed by computer, the average comet extent and other computerized comet measures were underestimated in samples, particularly those in which there was a large proportion of sperm with highly damaged DNA. The percentage of sperm with highly damaged DNA did not correlate with any other measures ($r^2 \leq 0.5$). Thus, our three primary measures for DNA damage (comet extent, DNA break number, and percentage of sperm with highly damaged DNA) were relatively independent of each other.

Apoptosis Assessment

Making Agarose and Slide Preparation

The methodology is similar to that described in the preceding "Double-Strand Breaks in DNA" section, except that only one layer of agarose (50 μL dried) was used, and a third layer was of 2% SFR agarose (Amresco). The second layer of microgel was made on slides (already having first layer of microgel) by pipetting 50 μL of 0.7% agarose 3:1 with approximately 10,000 to 500,000 sperm cells. This agarose was immediately covered with a 24 × 50 mm^2 cover glass (no. 1; Corning Glass Works) and was cooled in a steel tray on ice for 1 minute. The cover glass was removed, and 200 μL of 2% SFR agarose solution was layered as before to make a third layer. Use of 2% SFR agarose is essential for controlling against too much diffusion of DNA from apoptotic cells in agarose. After keeping slides for 2 minutes on ice, cover glasses were removed, and the slides were immersed and maintained for 10 minutes in a freshly made and cold lysing solution (1.25 M NaCl, 1 mM tetrasodium ethylenediaminetetraacetic acid, 5 mM Tris, 0.01% sodium lauroyl sarcosine, 0.2% dimethyl sulfoxide, and 300 mM NaOH). Neutralization and staining of microgels were done as described in our section, "Double-Strand Breaks in DNA." Details of the methodology for apoptosis assessment in sperm are described elsewhere (60). The percentage of apoptotic cells with diffuse DNA and a hazy outline were calculated from a total of 1,000 cells.

Statistics

Data were analyzed using StatView version 5.01 (SAS Institute Inc., Cary, NC) on a Macintosh G4 computer (Apple Computer Inc., Cupertino, CA). Unpaired two-group comparisons were made using nonparametric Mann-Whitney U tests. Correlations were performed using the nonparametric Spearman rank correlation test; regression plots and analyses were generated using simple linear regression after natural logarithmic transformation of apoptosis and highly damaged DNA data. To assess effects of clinical group and age group on semen analysis, comet, and apoptosis data, a factorial design analysis of variance was employed. Before analysis of variance, measurements not normally distributed were transformed to approximate normal distribution. In particular, percentage apoptosis and percentage of sperm with highly damaged DNA were logarithmically transformed after adding 1 to the decimal representation of the data; and percentage motility, total sperm number, tail integrated intensity, tail integrated intensity ratio, and DNA break number were transformed to their square root values.

Age was treated as a continuous variable for regression and correlation analysis. For two-group comparisons, different ages were used as cut points to divide the subjects into two groups ("younger" and "older"). The following ages were used as cut points: 25, 27, 30, 32, 33, 34, 35, 36, 38, 40, and 43 years. At least nine subjects remained in the smaller of the two groups.

RESULTS

Sixty-six men were recruited to the study; 40 were patients appearing for an infertility evaluation, and 26 were controls, either healthy sperm donors (n = 21) or men seeking to cryopreserve their sperm (n = 5). These subjects ranged in age from 20 to 57 years (infertility subjects: range, 25–57 years; mean, 36 years; controls: 20–54 years; mean, 31 years; $P<.01$). Because the subjects were recruited from two separate populations (clinical groups) whose mean ages were statistically different, analyses included statistical evaluation of the contribution of clinical group to the outcomes. Abstinence from ejaculation ranged from 1 to 240 days and was significantly longer in the infertility subjects ($P<.02$). Men younger than 24 years of age had shorter abstinence times (median 2 vs. 4 days; $P<.025$) than did older men. However, days of abstinence did not correlate with any of the DNA damage measures and were not a significant factor in the analysis of variance model using age and clinical group as factors (data not shown). Only 8 of the 66 men abstained longer than the recommended 5 days.

Semen Analysis

Age-related differences in semen analysis results were of interest. Men aged 20–25 years had significantly higher percentage motility than did older men ($P<.003$). When all men were divided into two groups using different age cut points for the younger vs. older group, percentage motility was higher in the younger group for all but one of the cut points examined. Three age group comparisons are shown in Figure 1. This difference between younger and older groups was significant ($P<.0003$ to $P<.04$) for all cut points up to age 40 years. The difference was not significant at the age breakpoint of 43 years (Fig. 1). Total sperm per ejaculate, percentage strictly normal morphology, and neutrophil concentration did not significantly differ with age group. Semen analysis results differed between the two clinical groups only

Percentage motile sperm in age groups. *Bars* represent mean percentage motility of sperm, and *vertical lines* are 1 standard error. Number of subjects in each group is given within the bar. Three age breakpoints are compared: men aged ≤25 years vs. men aged >25 years; men aged ≤35 years vs. men aged >35 years; and men aged ≤43 years vs. men >43 years. Percentage motility for the older of the first two pairs is significantly lower than the younger group of the pair (**P<.006; NS = not significant; Mann-Whitney U test).
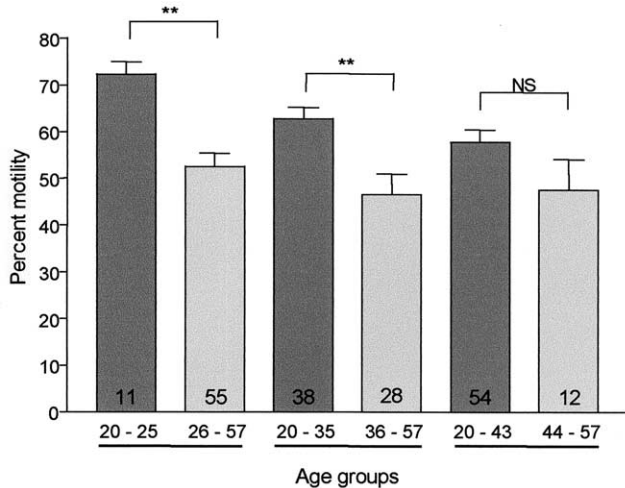
Singh. DNA damage and apoptosis in human sperm. Fertil Steril 2003.

for percentage motility (controls, 66.7 ± 16.0 [mean ± SE]; infertility, 48.9 ± 20.5; P<.0025), without significant interaction with age group.
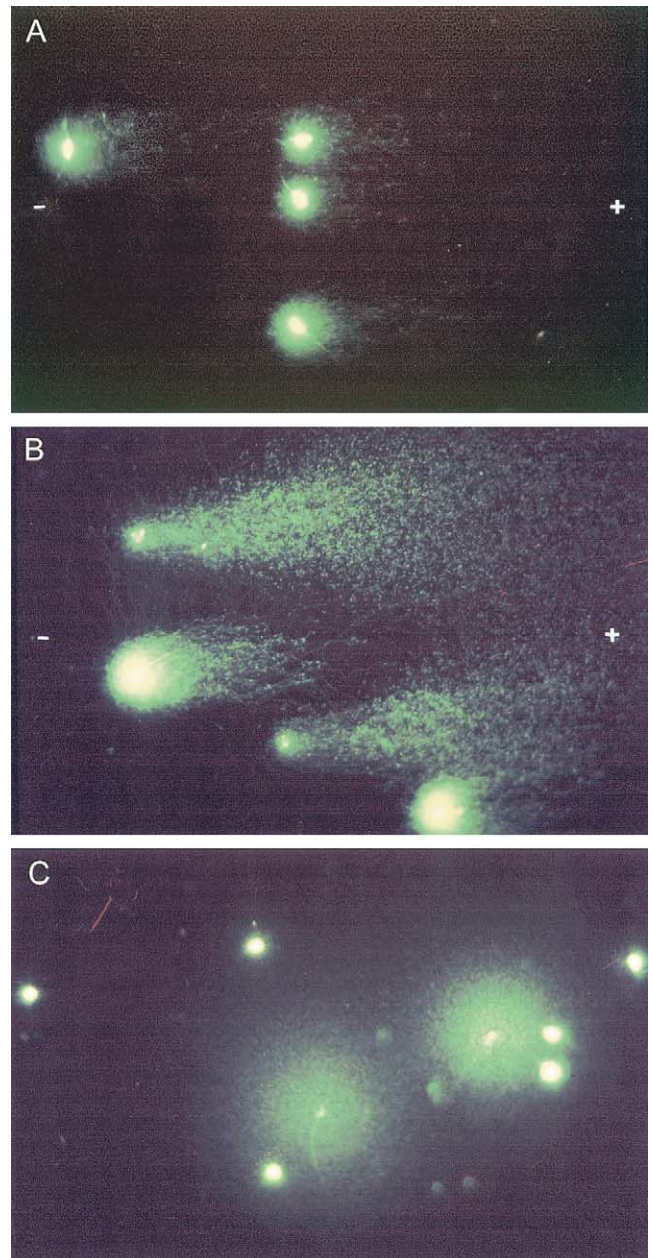
Sperm DNA Damage

DNA damage in four typical sperm cells from a young subject, assayed by neutral microgel electrophoresis with minimal DNA migration from nuclei is shown in Fig. 2A. In contrast, four sperm cells from an older subject show significant DNA migration from the nuclei, including two cells with highly damaged DNA (Fig. 2B). Figure 2C shows two typical apoptotic cells with diffuse halos and shows six normal cells with no halos in DNA diffusion assay done on semen samples.

We exposed sperm to external genotoxic insult, ionizing radiations to generate a standard X-ray dose–response (0 to 200 rads) curve (Fig. 3). This resulted in a linear ($R^2 = 0.95$; P<.025) increase in comet extent.

Two distinct types of DNA damage measurements were recorded: percentage of cells with highly damaged DNA (with comet extent >625 pixels) and image analysis data of comets <625 pixels. Because the highly damaged cells could not be included in the image analyses, these image analysis–recorded measurements are an underestimation of DNA damage. Percentage of sperm with highly damaged

(**A**) A photomicrograph showing the DNA migration patterns from four typical sperm cells of young subject. The symbols + and − represent cathode and anode, respectively, during electrophoresis of negatively charged DNA. Magnification: ×400. Dye: YOYO-1. (**B**) A photomicrograph showing the DNA migration patterns from four sperm cells of an older subject. Two sperm cells with highly damaged DNA and two sperm cells with significant DNA damage are shown. The symbols + and − represent cathode and anode, respectively, during electrophoresis of negatively charged DNA. Magnification: ×400. Dye: YOYO-1. (**C**) A photomicrograph of sperm processed for the DNA diffusion assay. Two apoptotic and six normal sperm are shown. Magnification: ×400. Dye: YOYO-1.
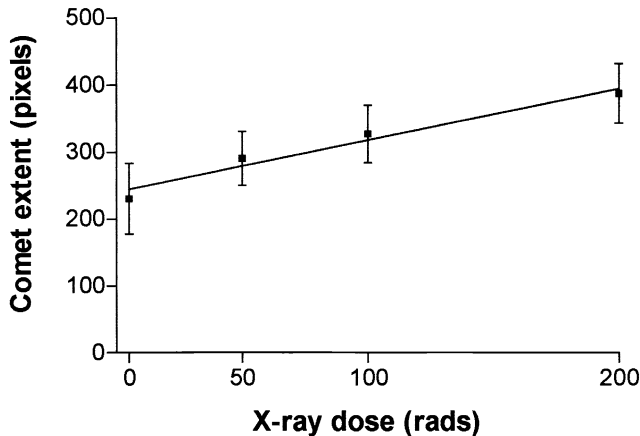

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X-ray dose–response relationship in human sperm cells. Sperm in seminal fluid were exposed to the indicated rads of X rays before neutral microgel electrophoresis. *Points* represent the mean comet extent of 50 analyzed sperm, and *error bars* represent SD. A highly linear relationship is demonstrated between rads and comet extent, indicating a quantitative relationship between DNA-damaging insult and response measured as comet extent.

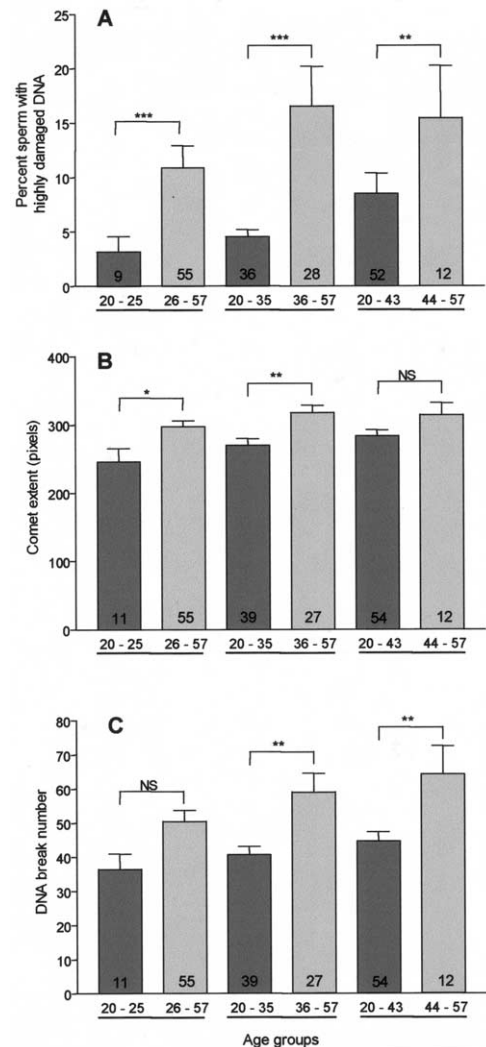*Singh. DNA damage and apoptosis in human sperm. Fertil Steril 2003.*

(**A**) Percentage of sperm with highly damaged DNA in age groups. Three age breakpoints are compared, as in Figure 1. Percentage sperm with highly damaged DNA for the older of all pairs is significantly higher than that for the younger group of the pair (***P<.005; **P<.02; Mann-Whitney U test). (**B**) Comet extent. Three age breakpoints are compared, as in Figure 1. Comet extent for the older of the first two pairs is significantly higher than that for the younger group of the pair (*P<.02; **P<.005; NS = not significant; Mann-Whitney U test). (**C**) DNA breaks. Three age breakpoints are compared, as in Figure 1. Number of DNA breaks for the older of the first two pairs is significantly higher than that for the younger group of the pair (**P<.02, NS = not significant). In each graph, *bars* represent mean and error *bars* represent SE. Numbers of subjects in each group is shown within the bar.

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DNA in subjects ranged from 0 to 83% (mean ± SE, 9.7 ± 1.8%). Comet extent ranged from 122.4 to 454 pixels (289 ± 8 pixels), and DNA break number ranged from 11 to 129 (48 ± 3).

All comet measurements increased significantly with subject age, except comet total intensity (data not shown). When results were divided into two groups (younger and older) using the different age cut points, percentage of sperm with highly damaged DNA was significantly higher by Mann-Whitney U test in each older group (Fig. 4A). Similar patterns were found for Comet extent (Fig. 4B) and DNA break number (Fig. 4C). Regression analysis demonstrated a highly significant increase in sperm DNA damage with increasing age, a significant increase in mean comet extent with age (P<.004, Spearman's rho = 0.36). Similar results were found for DNA break number (P<.02; Spearman's rho = 0.29). Percentage of sperm with highly damaged DNA also exhibited a significant positive regression with age (Fig. 5A).

Clinical group was not a significant factor, nor was the interaction between age and clinical group for the computed comet measures using factorial analysis of variance (P>.05). However, the natural log of the percentage of sperm with highly damaged DNA was significantly affected by both age (P<.004) and clinical group (P<.02), without significant interaction, in this analysis. Unpaired Mann-Whitney U tests

also revealed that of the comet measures, only the percentage of sperm with highly damaged DNA differed with high statistical significance between the two clinical groups (P<.0001).

FIGURE 5

(**A**) A scatter plot showing the relationship between age of subject and sperm with highly damaged DNA. Percentage of highly damaged cells are plotted as a natural log against age (Spearman rank correlation coefficient = 0.56; $P<.0001$). (**B**) A scatter plot showing the relationship between age of subject and percentage apoptotic sperm. Percentage apoptotic sperm are plotted as a natural log against age. Spearman rank correlation coefficient (rho) = -0.275; $P=.028$). (**C**) Percentage of apoptotic sperm in age groups. *Bars* represent mean percentage apoptosis, and *error bars* represent SE. Number of subjects in each group is given within the bar. Three age breakpoints are compared, as in Figure 1. Percentage apoptotic sperm is significantly lower for the older age groups of the last two pairs (20–35 years vs. 36–57 years, and 20–43 years vs. 44–57 years; **$P<.02$–.001, NS = not significant).
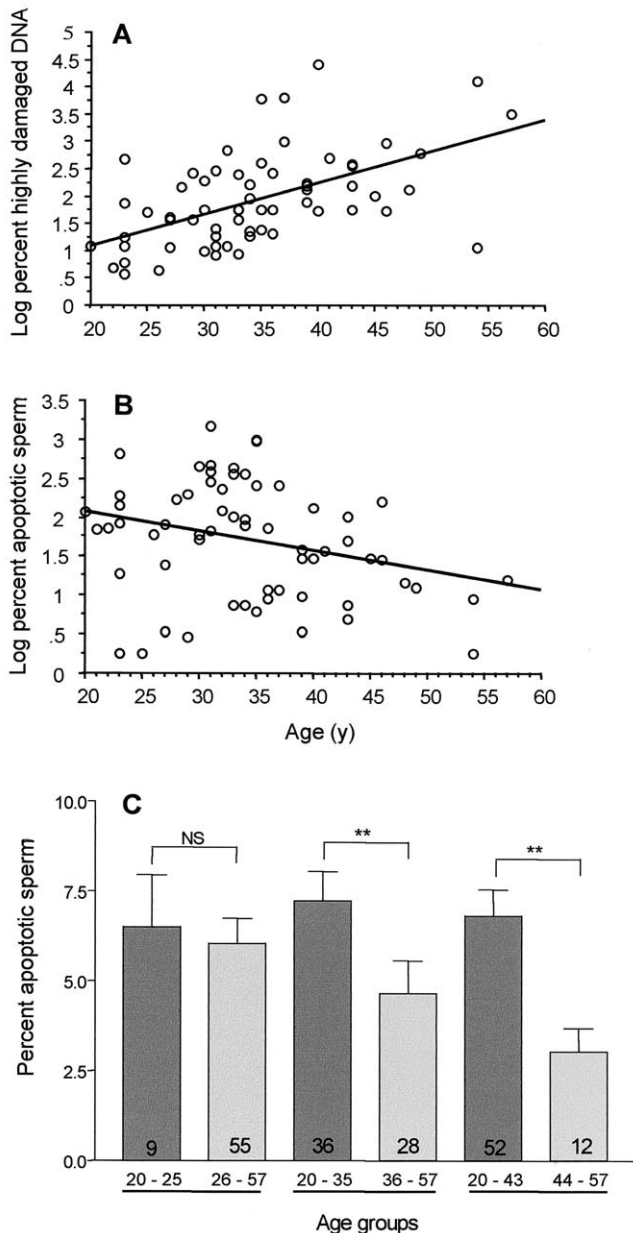
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Sperm Apoptosis

Sperm exhibiting diffuse halos in the DNA diffusion assay were considered apoptotic. Overall, 6.5% ± 5.1% of sperm exhibited this pattern (range, 0.3% to 23%). The percentage of apoptotic sperm was significantly and inversely related to age (Spearman's rho = -0.27, $P=.029$, Fig. 5B). Incidence of apoptosis showed age-related changes when subjects were divided into age groups. The older age groups (>35 years and >43 years) showed a highly significant decrease in apoptosis compared with the younger group (Fig. 5C). However, there was no significant difference when subjects aged <25 years were compared with those aged >25 years. We examined other semen analysis and comet parameters and found that none were highly correlated with percentage apoptosis, except that a significant inverse relationship existed between percentage apoptosis and DNA break number ($P<.001$, Spearman's rho = -0.43). Using analysis of variance, there was no overall effect of clinical group on percentage apoptosis.

DISCUSSION

Our results demonstrate several novel findings. First, sperm DNA double-strand breaks assessed by neutral microgel electrophoresis significantly increase with age of subjects. Second, we observed a decrease in apoptosis with age. Taken together, these results suggest an increase in damaged DNA of sperm as men age, possibly partly as a result of a less efficient cell selection system (apoptosis) operating during or after spermatogenesis.

Third, in this population of men, including both infertility patients and normal healthy men, sperm motility was the only semen analysis measure that exhibited a relationship with age. Both decreased sperm motility and increased sperm DNA damage can result from high levels of reactive oxygen species produced by leukocytes in semen (2, 27). However, we found no relationship between age and number of leukocytes in these men, and reactive oxygen species production also did not correlate with age (data not shown). The nature of these relationships requires further study.

Fourth, increasing exposure of sperm to X rays results in a linear increase in comet extent and other comet parameters. This demonstrates that the DNA of ejaculated sperm in seminal fluid can be damaged quickly by ionizing radiation. It also shows that our measurements of DNA damage can be directly and linearly related to a quantifiable amount of radiation-induced DNA damage. This X-ray dose response, apart from serving as a positive control, allows us to extrapolate the X-ray dose equivalent of the level of DNA damage observed in individual subjects. Thus, it is possible to calibrate the amount of damage observed in a sperm sample to a standardized amount of radiation. However, it is important to realize that quantitatively equating DNA damage with age in sperm and DNA damage induced by X rays may not be

accurate, as these two forms of damage can be very different qualitatively.

We also observed an age-related increase in sperm cells with highly damaged DNA. However, these are not apoptotic cells, as most apoptotic cells are lost during lysis and electrophoresis in the microgel electrophoresis assay because of their small molecular weight DNA. We postulate that these cells may have single-stranded DNA and may be those detected as abnormal sperm in the sperm chromatin structure assay (61) and the sperm chromatin dispersion test (62). These two tests depend on acid-mediated DNA breakage and probably are able to detect only sperm cells with highly damaged DNA. Our analysis is unique and novel in part because we have chosen three relatively independent variables as measures of sperm DNA damage. The neutral microgel electrophoresis technique also allows determination of the percentage of cells with highly damaged DNA.

We observed both intraindividual and interindividual variation in DNA damage among men of any age range. Thus, age is not the only factor influencing sperm DNA damage. In fact, some younger men had more sperm with highly damaged DNA than some older men. Also, some men had a wider range of sperm damage in their semen sample, indicating possible higher individual baseline of DNA damage or possible variable sensitivities of individual sperm to damage. This suggests that a sperm selection method might prove of value in assisted reproduction.

Gorxzyca et al. (63) and Sailer et al. (64) used terminal deoxynucleotide assays to study the levels of strand breaks in dead sperm cells from human semen samples. Saga et al. (65) used alkaline elution assays for studying the induction of DNA single-strand breaks in sperm from mice exposed to methylmethanesulfonate. Using alkaline elution, Saga and Generoso (66) detected DNA single-strand breaks in sperm cells from mice treated with ethylene oxide. Singh et al. (67), looking for levels of strand breaks in human and mouse sperm by using microgel electrophoresis, found abundant alkali labile sites. Because of extensive single-strand breakage resulting from the alkali lability, they were unable to quantify the levels. More recently, Fraga et al. (68) studied oxidative products of DNA from smokers in sperm cell lysates using high-performance liquid chromatography.

We have previously estimated DNA double-strand breaks in human sperm using the neutral microgel electrophoresis assay (24). Neutral electrophoresis conditions are required for estimation of DNA double-strand breaks. There are abundant alkali labile sites in DNA of human sperm (23); therefore, alkaline electrophoresis conditions are not suitable for sensitive estimation of DNA damage in this cell type as the number of alkali-induced DNA breaks is very high. Thus, in the current study we chose to use the neutral microgel electrophoresis assay to measure spontaneous DNA double-strand breaks. An increase in DNA damage with age in ejaculated human sperm has been shown elsewhere (11). The

current work, however, is the first to show an age-related increase in DNA double-strand breaks and also the first to show an age-related decrease in apoptosis in human sperm.

Cell selection is a dominant and efficient process during early reproductive years to ensure healthy offspring (41, 69–73). DNA damage can be caused by a variety of agents; for example, it has been correlated with reactive oxygen species (2, 25, 74–76). We speculate that when DNA damage is induced in cells of a young individual, levels of apoptosis also increase to eliminate cells with irreparable DNA damage. However, when DNA damage is induced in cells of an older individual, levels of apoptosis do not increase, indicating that with aging, the ability to remove cells with highly damaged DNA decreases. We further speculate that the inability to eliminate sperm cells with DNA damaged beyond repair may be attributable to age-related damage to the genes involved in the apoptotic pathway.

Our results clearly show that this cell selection process declines as a function of age, which may be responsible for adverse outcomes of pregnancy (lowered fertility or increased postfertilization failure and fetal malformations) in sperm from older subjects. Possible reasons for such a decline in cell selection process may be the loss of scavenging of free radicals during aging, continued exposure to damaging environmental factors, or mutations in genes responsible for apoptotic pathways. Less likely reasons for the decline in putative apoptotic cells with age could be lower apoptotic rate or the loss of apoptotic cells during longer abstinence. Sakkas et al. (22) have proposed that apoptosis is an event that is triggered early in spermatogenesis due to defects in cytoplasmic remodeling. However, cytoplasmic remodeling is unlikely to be a triggering event because apoptosis is considered to be initiated by DNA damage (49, 77) or its misrepair (78).

Possible mechanisms of apoptosis occurring in sperm cells (postcondensation) have been explored by several researchers. Gorczyca et al. (63), Maione et al. (79), and McCarthy and Ward (80) have speculated that the presence of DNAse, after activation, can result in sperm-specific apoptotic degradation, although no caspases have been shown to be involved (44). Most research on apoptosis in the male germ line has been focused on cell elimination during spermatogenesis. Recote et al. (81) found elevated levels of P53 and P21 in spermatogonial and primary spermatocytes in marble-newt testes, and Blanco-Rodriguez (82) observed elevated levels of apoptosis and DNA synthesis in spermatogonia and primary spermatocytes of cat testes. Similar findings have been reported in rats (83) and rabbits (84). Approximately three fourths of spermatogonial cells undergo apoptosis during spermatogenesis (37), indicating thorough levels of cellular proofreading (85). Also, Woolveridge and Morris (86) have reported extensive cell death during human spermatogenesis. This massive apoptosis during spermato-

genesis signifies the importance of healthy sperm cell selection.

Why, then, is there such a late-stage selection (apoptosis occurring in sperm)? Is there a need for quality control at every step of the way during spermatogenesis before the final product is complete? The late-stage selection (apoptosis in sperm) is perhaps the most crucial step to ensure the quality of fertilizing sperm. There are several reports of apoptosis in ejaculated semen, indicating that late-stage selection, although not massive, is by no means rare. Anzar et al. (42) reported an incidence of 12% to 25% apoptosis in fresh bull semen using the TUNEL assay. Baccetti et al. (43), using TUNEL assay, reported an incidence of 0.1% to 10% in human ejaculated sperm, whereas Oosterhuis et al. (47) found that 20% of sperm from infertile men were TUNEL positive. Using Fas as a marker of apoptosis in human sperm, Sakkas et al. (87) found that almost all normozoospermic men had <10% apoptotic sperm, whereas almost 60% of oligozoospermic men had >10% of their sperm exhibiting this marker. Kemal Duru et al. (88), along with other researchers (52, 89) have used annexin V binding to sperm as an indicator of early apoptosis and TUNEL. They found that 9%–11% of sperm were positive for either TUNEL or annexin V binding, although much higher levels were found in the low motility fractions from infertile men. Our results showing 0.3%–23% apoptosis (mean, 6.5%) using the diffusion assay agree with these previous findings.

Work elsewhere (24, 90–92) has shown that sperm DNA damage is not repaired, largely because of the chromatin structure. On the basis of these previous observations, we speculate that in sperm cells with damaged DNA, apoptosis is the only feasible option to prevent transmission of faulty genetic information. Thus, apoptosis in sperm can be assumed to be a parallel pathway to DNA repair in somatic cells because both serve identical functions (namely, to ensure a zero-error transmission either during cell replication or reproduction). We have shown elsewhere an increased level of DNA damage and decreased rate of its repair with aging in somatic cells (93, 94). Therefore, our current findings, that DNA damage increases with age and apoptosis decreases with age in sperm, are in accordance with our above speculation that apoptosis and DNA repair are parallel pathways.

We propose that apoptosis may be the only means by which a male germ cell with DNA damage is prevented from propagating its flaws. We also propose that environmental conditions play a role in the accumulation of such damage. These conditions may cause or allow DNA breakage, may decrease or prevent DNA repair (during spermatogenesis), or may prevent apoptosis in damaged cells. Thus, sperm in an ideal environment from a young individual should show minimal DNA damage and thus lower apoptosis. High levels of antioxidants in seminal fluid protect sperm from environmental DNA damage. Thus, an antioxidant-rich environment may help to provide optimal conditions for maintaining integrity of sperm DNA, both in the testes and during the journey of sperm to the ovum for fertilization. Conversely, sperm existing in a poor environment in a young individual may show high apoptosis and high DNA damage.

As shown in the current research, age is one likely factor in the increase in DNA damage to sperm and the decrease in apoptotic means of eliminating such damaged sperm. However, there are many environmental factors that may be responsible for observed decreases in sperm quality, independent of age, and that may have significant reproductive consequences, including birth defects (95), cancer (96–98), and mental disorders (99) in offspring. These environmental insults include cigarette smoking (97, 100), consumption of alcohol (98), and caffeine intake (101). Other environmental factors having a detrimental effect on sperm quality include exposure to industrial chemicals such as phthalates (35) and diagnostic radiation (96).

The possible relevance of our findings to the health of future generations is also significant. In the face of DNA damage and possibly of low levels of DNA repair, apoptosis may be the process of last resort to ensure against transmission of DNA defects to the embryo. Overexpression of apoptotic events in germ cells could lead to oligozoospermia or azoospermia and thus infertility (102, 103). Conversely, underexpression of apoptotic events will lead to survival and thus fertilization by sperm with faulty DNA (73), potentially resulting in early embryonic loss or unhealthy offspring (104). Research to elucidate and achieve a healthy DNA-protective environment during spermatogenesis, sperm maturation and transport, and fertilization is warranted to ensure healthy offspring and eventually, healthy adults.

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