Human Reproduction Vol.22, No.1 pp. 180–187, 2007 doi:10.1093/humrep/del338
Advance Access publication October 19, 2006.

The effects of male age on sperm DNA damage in healthy non-smokers

T.E.Schmid[1,2,4], B.Eskenazi[2], A.Baumgartner[3,4], F.Marchetti[1,4], S.Young[2], R.Weldon[2], D.Anderson[3] and A.J.Wyrobek[1,4,5]

[1]Biosciences Directorate, Lawrence Livermore National Laboratory, Livermore, [2]School of Public Health, University of California in Berkeley, Berkeley, CA, USA and [3]Department of Biomedical Sciences, University of Bradford, Bradford, UK

[4]Present address: Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

[5]To whom correspondence should be addressed at: Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA. E-mail: ajwyrobek@lbl.gov

BACKGROUND: The trend for men to have children at older age raises concerns that advancing age may increase the production of genetically defective sperm, increasing the risks of transmitting germ-line mutations. METHODS: We investigated the associations between male age and sperm DNA damage and the influence of several lifestyle factors in a healthy non-clinical group of 80 non-smokers (mean age: 46.4 years, range: 22–80 years) with no known fertility problems using the sperm Comet analyses. RESULTS: The average percentage of DNA that migrated out of the sperm nucleus under alkaline electrophoresis increased with age (0.18% per year, $P = 0.006$), but there was no age association for damage measured under neutral conditions ($P = 0.7$). Men who consumed >3 cups coffee per day had ~20% higher percentage tail DNA under neutral but not alkaline conditions compared with men who consumed no caffeine ($P = 0.005$). CONCLUSIONS: Our findings indicate that (i) older men have increased sperm DNA damage associated with alkali-labile sites or single-strand DNA breaks and (ii) independent of age, men with substantial daily caffeine consumption have increased sperm DNA damage associated with double-strand DNA breaks. DNA damage in sperm can be converted to chromosomal aberrations and gene mutations after fertilization, increasing the risks of developmental defects and genetic diseases among offspring.

Key words: ageing/caffeine/Comet/DNA damage/sperm

Introduction

The societal trend for older parents to have children raises public health concern about age-associated risks of abnormal pregnancies and birth defects. It is well known that female fecundity declines precipitously by the fourth decade of life due to oocyte loss and that older mothers have increased risks of miscarriage, trisomies and chromosomal defective offspring (Lansac, 1995). Male fecundity also seems to decline with age, although spermatogenesis continues well into male senescence and some men of advancing age can father children (Kidd *et al*., 2001; Sloter *et al*., 2004). However, the risks of abnormal pregnancies and heritable effects associated with advancing paternal age are poorly understood.

There is suggestive epidemiological evidence that the incidence of abnormal reproductive outcomes and heritable defects increases with paternal age (Tarin *et al*., 1998; de la Rochebrochard and Thonneau, 2002), including pregnancy loss (Risch *et al*., 1987; de la Rochebrochard and Thonneau, 2002), developmental and morphological birth defects (Lian *et al*., 1986), gene mutations (Crow, 2000; Tiemann-Boege *et al*., 2002), various aneuploidy and chromosomal syndromes (Sloter *et al*., 2004) and diseases of complex aetiology such as prostate cancer (Zhang *et al*., 1999). However, the epidemiological studies of abnormal reproductive outcomes require large numbers of pregnancies and have inherent difficulties in distinguishing between the impact of maternal and paternal age. New methods for measuring genetic and chromosomal defects in human sperm provide more direct approaches to identifying paternal risk factors (Wyrobek *et al*., 2005a,b), and growing evidence links sperm DNA damage with the risks of developmental defects and mutations in the offspring, including childhood cancer and infertility (Aitken *et al*., 2003).

Advancing male age has been associated with increased frequencies in sperm of certain genetic and chromosomal defects (Crow, 2000; Shi and Martin, 2000; Tiemann-Boege *et al*., 2002; Bosch *et al*., 2003; Sloter *et al*., 2004), but there remain unexplained differences in age dependencies among the major categories of sperm damage (Sloter *et al*., 2004). Older men seem to produce more sperm with mutations associated with achondroplasia and Apert syndrome (Tiemann-Boege *et al*., 2002; Glaser *et al*., 2003; Wyrobek *et al*., 2006) and with certain types of sperm DNA damage measured by the Comet and the sperm chromatin structure assay (SCSA) analyses (Spano

et al., 1998; Morris *et al*., 2002; Singh *et al*., 2003; Wyrobek *et al*., 2006), but not more aneuploid sperm (Wyrobek *et al*., 2006). Understanding the effects of male age on sperm DNA damage is especially relevant for men attending reproductive clinics because of the increasing reliance on modern technologies, especially among marginally fertile older men. ICSI and IVF enhance the probability of achieving fatherhood, yet they also circumvent the natural barriers against fertilization by damaged sperm (Maher *et al*., 2003; Singh *et al*., 2003).

Lifestyle factors such as smoking, alcohol and caffeine consumption have been associated with the increase in genetic damage in blood cells (Park and Kang, 2004; Glei *et al*., 2005; Wyrobek *et al*., 2005a), but little is known about their effects on genetic damage in sperm (Wyrobek *et al*., 2005b). Cigarette smoking and alcohol consumption have uncertain associations with sperm aneuploidy (Robbins *et al*., 1993; Rubes *et al*., 1998; Shi *et al*., 2001) and no detectable associations with sperm DNA damage as measured by single-cell electrophoresis (sperm Comet) (Belcheva *et al*., 2004). Also, no associations were detected between vitamin consumption and sperm damage as measured by SCSA (Silver *et al*, 2005).

The purpose of our study was to (i) investigate the association between male age and DNA damage in sperm within a group of generally healthy non-smokers, using sperm Comet analyses performed under both alkaline and neutral conditions to detect alkali-labile sites, single- and double-stranded DNA breaks (Haines *et al*., 1998; Hughes *et al*., 1999; Morris *et al*., 2002) and (ii) characterize associations between sperm Comet results and previously reported data on conventional semen quality and sperm DNA fragmentation as measured by SCSA for the same group of men (Eskenazi *et al*., 2003; Wyrobek *et al*., 2006). Additionally, we investigated the influence of several common lifestyle factors on sperm DNA damage using questionnaire data.

Materials and methods

Study population

A group of 80 healthy male volunteers, aged 22–80, were recruited for the Age and Genetic Effects on Sperm (AGES) study (Eskenazi *et al*., 2003). Men were eligible to participate if they had no current fertility or reproductive problems, had not smoked cigarettes in the last 6 months, had no vasectomy or a history of an undescended testicle or prostata cancer or had no previous semen analysis with zero sperm count. Male participants were recruited over a 10-month period through posted advertisements, e-mail listings and newsletters targeting current workers and retirees. To achieve a balanced age distribution, we targeted to enrol at least 15 men for each age decade from 20 to 70 years. Recruitment for each decade ceased once we achieved the enrollment goal for that decade. Our recruitment strategy was also designed to address the concern that enrolment rates might be biased by age as men in their reproductive years might be more interested in semen analysis results for reproductive purposes, especially if they had previous problems in conceiving a child. To minimize this potential bias, we excluded men with fertility or reproductive problems and men who ever had a semen analysis with zero sperm count. After preliminary screening, 20 men were excluded because of smoking (n = 11), varicocele (n = 3), one testicle (n = 2), undescended testicles (n = 1), valium use (n = 1), chemotherapy (n = 1) and hepatitis B infection (n = 1). Eighteen men recruited for our study refused to participate; their mean age was 48.7 years which was not statistically different from the mean age of all participants, 46.7 years. This AGES study was approved by the Institutional Review Boards of each participating institution (UC Berkeley and LLNL), and all volunteers gave written consent to participate.

Men were mailed a semen collection container with instructions and a questionnaire on medical and reproductive history, sociodemographic characteristics (age, race and education), occupation, possible exposures, diet and lifestyle habits. Semen samples were analysed for count and motility upon collection (Eskenazi *et al*., 2003) and immediately stored at –80°C.

Sperm Comet analyses

The frozen sperm aliquots were shipped on dry ice to the University of Bradford, UK, for analysis by sperm Comet analyses. Each specimen was analysed under both alkaline and neutral conditions (Anderson *et al*., 1997; Duty *et al*., 2003). Briefly, fully frosted microscope slides were covered with 110 µl of 0.5% normal melting point agarose in phosphate-buffered saline (PBS) at 50°C and dried at room temperature for 3 days. Approximately 10 000 sperm were mixed with 100 µl of 0.5% low melting point agarose to form a cell suspension of which 90 µl was spread onto the slide surface and solidified on ice. A third layer of 0.5% low melting point agarose was then added and again allowed to solidify on ice (5 min). Slides were immersed in lysing solution [2.5 M sodium chloride, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% dimethylsulphoxide (DMSO)] for 2 h. Then 10 mM dithiothreitol (DTT) was added to the lysing solution for an additional 30 min. Slides were then incubated in either 1 mM EDTA and 300 mM NaOH buffer (pH 13.0) for the alkaline analysis or 1× Tris–borate EDTA (TBE) (pH 8.0) for the neutral analysis. Electrophoresis was performed at 4°C for 20 min at 24 V. For the alkaline assay, 0.4 M Tris (pH 7.5) was used to neutralize the alkaline buffer for 5 min. Fifty microlitres of ethidium bromide (EtBr, 20 µg/ml) was added, and the slides were coverslipped and analysed within 3 h. Slides were examined at ×400 under a fluorescent microscope. Fifty cells were scored from each of the two replicate slides for each specimen (100 cells in total). A computerized image analysis system (Comet 3.0, Kinetic Imaging, Nottingham, UK) was used to measure the percentage tail DNA (% tail DNA, the average percentage of DNA staining outside the area of the sperm nucleus in the electrophoresis). We limit our analysis to % tail DNA because it is linearly related to the DNA break frequency in human sperm over a wide range of levels of damage (Anderson *et al*., 1998; Hartmann *et al*., 2003). Tail extent moment and olive tail moment were also measured and found to be highly correlated with % tail DNA ($P < 0.01$).

Statistical analysis

Age was examined as a categorical variable, by decade, and as a continuous variable. Differences in % tail DNA among covariates were examined using analysis of variance (ANOVA) and t-tests. The trends by age decade were calculated by a non-parametric test across the ordered groups (Cuzick, 1985), and the correlations were determined using Pearson's correlation. Three models were applied to examine the shape of the association between age and Comet results: linear, quadratic and spline. All the three models fit equally well; therefore, we applied multiple linear regression modelling to examine the relationships of continuous age with % tail DNA for both alkaline and neutral conditions after controlling for potential confounding factors. The following variables were evaluated as potential covariates: time from sample collection to sample processing; duration of sexual abstinence before semen collection; season of sample collection; exposure to occupational chemicals and radiation; history of working with radioisotopes; history of tobacco use; alcohol and caffeine intake;

prescription and non-prescription medication use; history of chronic disease such as high blood pressure, heart problems or diabetes; history of genitourinary disease including urinary tract or other genitourinary infection, sexually transmitted diseases or history of infertility; fatherhood history; BMI; ethnicity; diet characteristics and vitamin supplement use. Covariates were included if: (i) they were related to Comet parameters or age in univariate analyses ($P < 0.2$) and (ii) they changed the parameter estimate of age by at least 10% or they were statistically significant ($P < 0.1$) in controlled bivariate models. Once the full models were built, covariates were again evaluated using a stepwise backward elimination process and retained for the final model if they changed the parameter estimate of age by at least 10% or if they were statistically significant ($P < 0.1$). Normally, covariates with a P value of < 0.1 in the full models were not tested to determine whether their deletion changed the parameter estimate of age unless their P value increased >0.1 during the stepwise elimination process. We checked regression assumptions with residual versus fitted plots, and quantile–quantile plots. Regression results are displayed graphically for alkaline and neutral Comet outcomes, with covariates set to the mean value of the population. One subject was excluded from the final regression analyses because his dietary information was contradictory and incomplete.

Relationships between sperm Comet and conventional semen quality and SCSA outcomes were determined using Pearson's correlations and multiple linear regressions. Age and duration of abstinence were examined as potential covariates. For these analyses, conventional semen quality outcomes (motile sperm percentage, sperm concentration and sperm count) were square-root transformed and SCSA outcomes (%DFI and %HDS) were log-transformed. All analyses were performed using Stata 8.0 (Stata Corp LP, College station, TX, USA).

Results

Characteristics of study population

The 80 participants were on an average 46.4 years old (range 22 to 80 years), generally healthy and had not smoked cigarettes during the previous 6 months. As summarized in Table I, we found that older age was associated with the increased consumption of vitamin supplements and the history of urinary tract infections ($P < 0.05$) and with trends towards increased duration of sexual abstinence, previous tobacco use, lower regular alcohol usage and lower kilocalorie intake ($P < 0.1$).

The study volunteers were well distributed among age decades (Table II). The average % tail DNA was 42.1 (SD 8.9) and 35.1 (SD 7.9) under alkaline conditions and neutral conditions, respectively, with no significant correlation between the results of the two analysis conditions ($P > 0.1$).

Effects of age

There was a significant association between age and % tail DNA under alkaline conditions ($P = 0.002$ for ANOVA and $P < 0.05$ for correlation), but not under neutral conditions ($P > 0.1$). Figure 1a shows the alkaline Comet data for the individual men and for the linear, quadratic and spline regression models. Although all the three models show that older men have higher % tail DNA than the younger men, none of the models were significantly better fit than the others (linear model: $r^2 = 0.22$; quadratic model: $r^2 = 0.23$; and spline model: $r^2 = 0.26$). The spline model shows a non-significant decrease in Comet analyses with age until 55 and then a sharp increase after. Given the substantial variation in Comet values among men, we have no evidence in support of an age threshold and, for simplicity, present the following results using linear modelling.

Using the linear regression model, we found a 0.18% increase in % tail DNA per year of age ($P = 0.006$; Table II) after adjusting for covariates (vitamin C use, kilocalorie intake, urinary infections and season of collection). Age explained ~10% (partial $r = 0.32$) of the total variance of % tail DNA under alkaline conditions. Two other measures of DNA strand damage obtained by sperm Comet analysis (olive tail moment and tail extent moment) were highly correlated with % tail DNA ($P < 0.01$) and showed similar age effects on % tail DNA data (data not shown).

The association between age and alkaline % tail DNA seems to be partly driven by four men with high Comet values. Three of the four men were >65 years of age (28, 66, 68 and 71), and all the four had alkaline % tail DNA values >60. All the four men had fathered children earlier in life, and none had ever been diagnosed with fertility problems. Three were relatively healthy, and one was a previous smoker and was currently treated for high blood pressure at the time of semen collection. When we exclude the four men with alkaline % tail DNA values >60 from the linear regression analyses, the association with age approaches significance (slope = 0.07, $P = 0.08$).

There was no significant effect of age on neutral sperm DNA damage (Figure 1b). Using the linear regression model, there was a non-significant −0.02% change per year in neutral % tail DNA after adjusting for total caffeine use in last 3 months, any urinary tract infections, vitamin E use and kilocalorie intake ($r^2 = 0.19$; $P = 0.73$).

Association with lifestyle, medical factors and caffeine consumption

Sperm Comet results were associated with several lifestyle and medical factors obtained from questionnaire data (Table I). Higher values of alkaline % tail DNA were associated with the history of urinary tract infections ($P < 0.05$) and previous tobacco use ($P < 0.1$), although both associations were attenuated after adjusting for age ($P > 0.1$).

Higher caffeine intake was associated with higher values of neutral % tail DNA ($P < 0.1$, unadjusted), but not alkaline % tail DNA. Men were then assigned to tertiles based on caffeine consumption, and men with >308 mg of caffeine intake per day (equivalent to ~2.9 cups of coffee) had ~20% higher neutral % tail DNA than men with no caffeine intake ($P = 0.01$ unadjusted; $P = 0.005$ after adjusting for the covariates total kilocalorie intake and the history of urinary tract infections) (Figure 2).

Correlations among sperm Comet analysis, semen quality and DNA fragmentation

Our specimen set was previously evaluated for conventional semen quality (Eskenazi *et al*., 2003). There were several significant correlations between semen quality and sperm Comet results. After adjusting for age, alkaline % tail DNA was negatively correlated with sperm concentration ($r = –0.25$; $P = 0.03$), total sperm count ($r = –0.28$; $P = 0.01$) and total progressively motile sperm count ($r = –0.29$; $P = 0.01$), but not with % motile ($r = –0.18$; $P = 0.1$) or % progressive motile sperm ($r = –0.15$;

Table I. Associations between study population characteristics and sperm DNA damage measured by single-cell electrophoresis (sperm Comet analysis) under both alkaline and neutral conditions

	Donors *n* (%)[a]	Age (mean ± SD)	Percentage tail DNA	
			Alkaline (mean ± SD)	Neutral (mean ± SD)
Abstinence (days)				
2–5	61 (76)	42.3 ± 14.3	42.4 ± 8.9	35.5 ± 8.0
>5	19 (24)	49.4 ± 16.1 ($P < 0.10$)	41.1 ± 8.9	33.6 ± 7.7
Tobacco use				
Never	63 (79)	42.1 ± 13.9	41.3 ± 9.0	34.7 ± 8.1
Ever	17 (21)	50.8 ± 16.9 ($P < 0.10$)	45.3 ± 7.9 ($P < 0.10$)	36.5 ± 7.0
Regular alcohol use				
Never	30 (38)	47.5 ± 14.5	42.8 ± 10.0	35.5 ± 7.9
Ever	50 (62)	41.8 ± 14.9 ($P < 0.10$)	41.8 ± 8.2	34.8 ± 7.9
Daily caffeine intake (mg)				
None	22 (28)	45.6 ± 13.9	42.8 ± 10.6	32.8 ± 6.7
1–107	20 (25)	40.4 ± 16.2	40.7 ± 7.0	34.7 ± 7.2
107–308	20 (25)	48.6 ± 16.1	44.3 ± 10.7	34.2 ± 6.0
308–1070	18 (22)	40.9 ± 12.5	40.4 ± 5.5	39.1 ± 10.5 ($P < 0.10$)
Urinary tract infection[b]				
Never	69 (86)	42.4 ± 14.7	41.4 ± 9.0	34.9 ± 8.4
Ever	11 (14)	53.5 ± 13.0 ($P < 0.05$)	47.4 ± 6.0 ($P < 0.05$)	36.2 ± 2.8
Vitamin supplement use				
No	33 (42)	39.9 ± 14.1	43.2 ± 8.2	35.1 ± 8.1
Yes	46 (58)	46.9 ± 15.1 ($P < 0.05$)	41.5 ± 9.4	35.2 ± 7.8
Dietary kilocalorie intake (kcal/day)				
≤1731	42 (53)	46.7 ±14.0	40.8 ± 8.8	35.6 ± 8.3
>1731	37 (47)	40.9 ± 15.7 ($P < 0.10$)	43.9 ± 8.8	34.7 ± 7.4
Season of collection				
Fall	35 (44)	48.1 ± 13.1	40.0 ± 7.5	35.2 ± 8.0
Winter	27 (34)	40.0 ± 13.2	43.2 ± 8.5	34.9 ± 8.4
Spring/summer	18 (22)	41.9 ± 19.1 ($P < 0.10$)	44.9 ± 11.2	35.1 ± 7.2

Percentage tail DNA (% tail DNA, the average percentage of DNA staining outside the area of the sperm nucleus in the electrophoresis).
[a]*n*, 80 for neutral; *n*, 79 for alkaline conditions.
[b]Kidney, bladder or ureter infections.
P values for *t*-test or analysis of variance (ANOVA).

$P = 0.2$). Figure 3 illustrates the relationship between total sperm count (square root-transformed) and alkaline % tail DNA after adjusting for the covariates of age and abstinence. We found no significant correlations between semen quality and neutral % tail DNA results.

This specimen set was also previously evaluated for sperm DNA fragmentation by the SCSA (Wyrobek *et al*., 2006). Alkaline % tail DNA results were neither correlated with DNA fragmentation (%DFI) nor with the percentage of cells with immature chromatin [high DNA stainability (HDS)]. Although neutral % tail DNA was not correlated with DNA fragmentation, it was significantly correlated with log HDS ($r = 0.34$; $P = 0.002$). As illustrated in Figure 4, after adjusting for age and abstinence, there was a 2.6% relative change in HDS per unit increase in neutral % tail DNA ($P = 0.002$).

Discussion

We found associations between male age and sperm DNA strand damage in a non-clinical sample of active healthy non-smoking workers and retirees. Sperm of older men had significantly higher frequencies of sperm with DNA damage measured under alkaline conditions, which is thought to represent alkali-labile DNA sites and single-strand DNA breaks. However, age was not associated with sperm DNA damage under neutral conditions, which is thought to represent double-strand DNA breaks. The observations of differential effects of age on genomic damage is consistent with the recent finding of Wyrobek *et al*. (2006) who reported age-related effects on DNA fragmentation and achondroplasia mutations but not aneuploidy, Apert syndrome mutations or sex ratio. Our finding of age effects on DNA fragmentation and alkaline Comet in the context of no correlation between these two sperm endpoints suggests that these two types of DNA damage are induced by different molecular mechanisms.

We also found that men with high caffeine consumption (~3 cups per day or more) had significantly higher frequencies of sperm with DNA damage as measured under neutral, but not alkaline conditions compared to men with less caffeine consumption.

Table II. Effects of age on sperm DNA damage measured by single-cell electrophoresis (sperm Comet analysis) under both alkaline and neutral conditions

	Number of men[a]	Alkaline conditions	Neutral conditions
		Percentage tail DNA[b] (SD)	Percentage tail DNA[b] (SD)
Age group			
20–29	18	43.4 (9.9)	32.2 (7.1)
30–39	19	40.4 (5.5)	37.5 (9.3)
40–49	14	38.0 (4.0)	33.8 (8.7)
50–59	14	39.3 (7.5)	37.1 (5.1)
60–80	15	49.8 (11.5)	34.8 (7.7)
Total	80	42.1 (8.9)	35.1 (7.9)
ANOVA test			
P value		0.002	0.26
Test for trend			
p value		0.28	0.40
Correlation with age			
Correlation coefficient, *r*		0.22	0.06
P value		0.05	0.58
Adjusted change per year			
% change per year		0.18[c]	–0.02[d]
95% confidence interval		0.06, 0.31	–0.16, 0.11
P value		0.006	0.73
r^2		0.26	0.19

ANOVA, analysis of variance.
[a]*n* = 14 in 60–80 year group for alkaline assay.
[b]Percentage tail DNA (% DNA staining outside the area of the sperm nuclei after electrophoresis).
[c]Model was adjusted for vitamin C use, kilocalorie intake, urinary tract infections and season of collection.
[d]Model was adjusted for total caffeine use in last 3 months, any urinary tract infections, vitamin E use and total daily kilocalorie intake.

The finding of age-related increases in DNA strand damage under alkaline conditions is consistent with the findings of Morris and colleagues (Morris *et al*., 2002), who studied 60 men participating in an IVF program. They reported that sperm DNA damage was positively correlated with donor age and with impairment of post-fertilization embryo cleavage following ICSI, indicating an overall decline in the integrity of sperm DNA in older men. Our finding of no association between age and sperm DNA damage under neutral conditions is in contrast with the study of Singh and colleagues (2003), who studied 66 men, aged 20–57 years, from an infertility clinic and a non-clinical group. However, Singh *et al*. (2003) did not investigate sperm DNA damage under alkaline conditions in sperm, and Morris *et al*. (2002) did not investigate sperm damage under neutral conditions. Using a different assay for measuring DNA strand damage in sperm, the SCSA, Spano *et al*. (1998) found a strong association of DFI with age among men 18–55 year olds, a finding that was confirmed by Wyrobek *et al*. (2006) using a larger group of men that spanned 20 to 80 years of age.

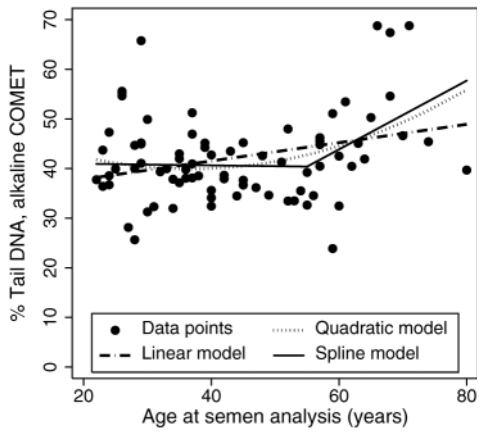
Older men may produce more sperm with DNA damage as a consequence of age-associated increased oxidative stress in their reproductive tracts (Barnes *et al*., 1998b; Barroso *et al*., 2000). Oxidative stress can damage sperm DNA as well as mitochondrial and nuclear membranes (Aitken *et al*., 2003). Kodama *et al*. (1997) reported an association between oxidative DNA damage in sperm and male infertility. Consistent with the hypothesis of the importance of oxidative damage to sperm, our laboratory recently reported that high antioxidant intake was associated with better semen quality, especially motility within the same study group (Eskenazi *et al*., 2005).

Alternatively, apoptotic functions of spermatogenesis may be less effective in older males resulting in the release of more sperm with DNA damage (Brinkworth *et al*., 1997; Print and Loveland, 2000). The testes of older male mice have lower apoptotic frequencies than young adults (Brinkworth and Schmid, 2003). Also, oxidative stress significantly increased the frequencies of apoptotic spermatocytes in young male mice while reducing testicular apoptosis in older males (Barnes *et al*., 1998a). While apoptosis has been identified in the testes of elderly men (Brinkworth *et al*., 1997), there have been no comparisons on rates of apoptosis among men of different ages.

Increased sperm DNA damage has been associated with chromosomal abnormalities, developmental loss and birth defects in mouse model systems (Marchetti *et al*., 1997; Sun *et al*., 1997; Haines *et al*., 1998; Hughes *et al*., 1999; Marchetti *et al*., 2004) and with increases in the percentage of human embryos that failed to develop after ICSI (Morris *et al*., 2002). Experimental evidence in mice indicates that fertilized eggs are capable of repairing damage in paternal DNA induced by UV radiation of sperm before fertilization (Pedersen and Cleaver, 1975; Brandriff and Pedersen, 1981; Sakkas *et al*., 2000). However, the extent to which the DNA repair capacity of early embryos contributes to preventing adverse pregnancy outcome is poorly understood (Generoso *et al*., 1979; Harrouk *et al*., 2000).

Our analysis found a novel association between daily caffeine intake and sperm DNA damage of the type associated with double-strand DNA breaks, but not alkali-labile sites or single-stranded DNA damage. Caffeine, an alkaloid, has two biological activities that may explain our findings. Its catabolic products, theobromine and xanthine, can reduce copper, which is associated with ubiquitous amino groups in cells, from Cu(II) to Cu(I), leading to the generation of oxygen radicals (Shamsi and Hadi, 1995). The generation of oxygen radicals can increase oxidative stress resulting in double-strand DNA

a. Alkaline conditions



b. Neutral conditions

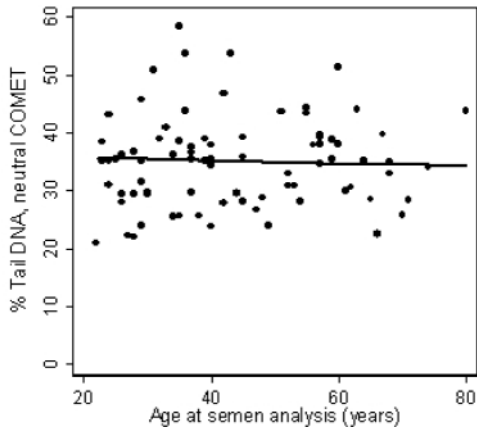


Figure 1. Relationship between male age (years) and sperm DNA damage measured under both alkaline and neutral electrophoresis conditions. (**a**) Alkaline conditions: individual data points are shown as well as the regression lines for the linear, quadratic and spline models. (**b**) Neutral conditions: individual data points are shown as well as the linear regression line.

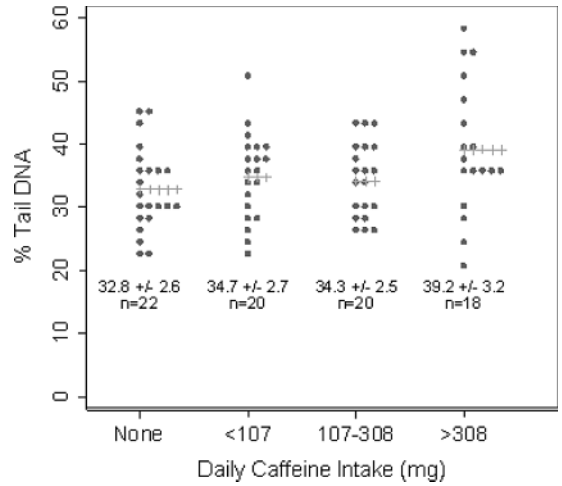


Figure 2. Relationship between daily caffeine intake and sperm DNA damage measured under neutral electrophoresis conditions. The figure shows the individual data points by caffeine intake category. The means of each group are indicated by '+++'. Mean, SE and sample size are included for each intake category. (>308 mg versus none, unadjusted $P = 0.01$).

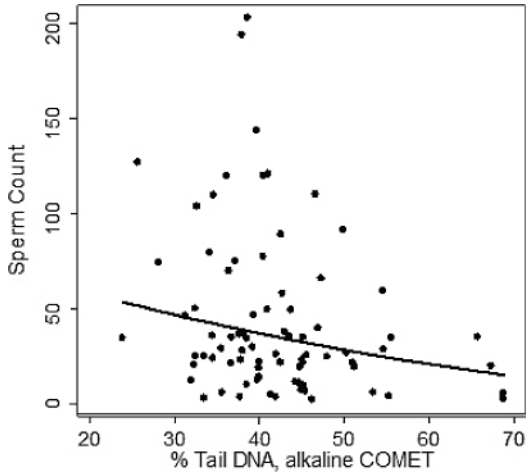


Figure 3. Relationship between total sperm count and sperm DNA damage measured under alkaline electrophoresis conditions. Individual data points are shown and linear regression line for square-root transformed sperm count, adjusted for age and abstinence ($\beta = -0.24$, $P = 0.03$).

breaks (Azam *et al*., 2003). Additionally, caffeine is an efficient inhibitor of DNA double-strand repair (Sarkaria *et al*., 1999), which may explain the increased double-strand DNA damage in sperm after high-dose caffeine consumption.

Our study found significant associations between DNA damage and conventional semen quality within our study group. The correlations between alkaline DNA strand damage and sperm concentration and total sperm count is consistent with the previous reports (Evenson *et al*., 1991; Larson *et al*., 2000; Donnelly *et al*., 2001; Silver *et al*., 2005). However, our finding of no significant association between DNA damage and sperm motility is in contrast with several previous studies (Giwercman *et al*., 2003; Sills *et al*., 2004; Wyrobek *et al*., 2006), which may be due to the different types of DNA damage endpoints evaluated in these studies.

Our study found significant associations between sperm DNA damage measured by sperm Comet under neutral conditions and the percentage of cells with immature chromatin (HDS), as measured by the SCSA (Wyrobek *et al*., 2006). During spermiogenesis, endogenous nuclease activity induces DNA strand breaks ('nicks') as part of normal chromatin remodelling involving DNA supercoiling (McPherson and Longo, 1993; Marcon and Boissonneault, 2004). Nicks are normally repaired during sperm maturation (Marcon and Boissonneault, 2004), and the release of immature sperm has been associated with underprotamination (Evenson and Wixon, 2006). However, our study did not find an association between neutral DNA strand damage and DNA fragmentation endpoints measured by SCSA. These findings are consistent with the

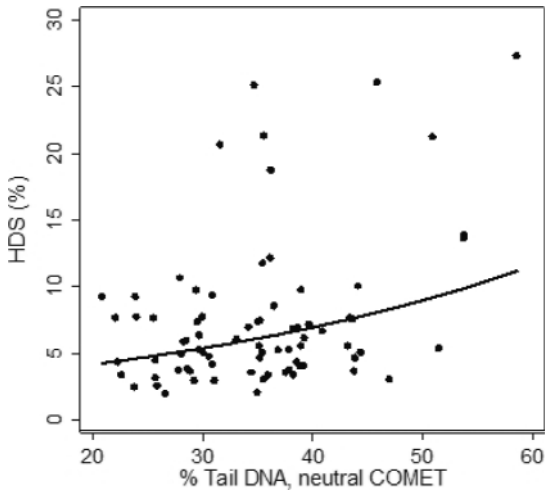


Figure 4. Relationship between the frequencies of sperm with immature sperm chromatin (high DNA stainability, HDS) in the sperm chromatin structure assay analysis and sperm DNA damage under neutral electrophoresis conditions. Individual data points are shown and the regression line for log-transformed HDS adjusted for age and abstinence [2.6% relative change in HDS per unit increase in percentage tail DNA (% DNA staining outside the area of the sperm nucleus after electrophoresis), $r^2 = 0.11$; $P = 0.002$].

likelihood that sperm Comet and SCSA measure different aspects of sperm DNA strand damage (Morris, 2002).

Our findings of age-related increases in sperm DNA damage predict that men who delay fatherhood may have increased risks of unsuccessful and abnormal pregnancies as a consequence of fertilization with damaged sperm. In addition, our finding that higher daily caffeine intake is associated with increased frequencies of damaged sperm, suggests the need for future studies to investigate the roles of dietary factors on sperm DNA damage in ageing males.

Acknowledgements

This work was performed in part under the auspices of the US Department of Energy by Lawrence Livermore National Laboratory, contract W-7405-ENG-48, with funding from NIEHS Superfund P42ES04705. A.B. was a Marie Curie fellow, EC Contract No: QLG4-CT-2002-51611.

References

Aitken RJ, Baker MA and Sawyer D (2003) Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. Reprod Biomed Online 7(1),65–70.

Anderson DN, Basaran MM, Dobrzynska AA, Basaran and Yu TW (1997) Modulating effects of flavonoids on food mutagens in human blood and sperm samples in the comet assay. Teratog Carcinog Mutagen 17(2),45–58.

Anderson D, Dobrzynska MM, Basaran N, Basaran A and Yu TW (1998) Flavonoids modulate comet assay responses to food mutagens in human lymphocytes and sperm. Mutat Res 402(1–2),269–277.

Azam S, Hadi N, Khan NU and Hadi SM (2003) Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. Med Sci Monit 9(9),BR325–30.

Barnes CJ, Covington BWT, Cameron IL and Lee M (1998a) Effect of aging on spontaneous and induced mouse testicular germ cell apoptosis. Aging (Milano) 10(6),497–501.

Barnes CJ, Hardman WE, Maze GL, Lee M and Cameron IL (1998b) Age-dependent sensitization to oxidative stress by dietary fatty acids. Aging (Milano) 10(6),455–462.

Barroso G, Morshedi M and Oehninger S (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Hum Reprod 15(6),1338–1344.

Belcheva A, Ivanova-Kicheva M, Tzvetkova P and Marinov M (2004) Effects of cigarette smoking on sperm plasma membrane integrity and DNA fragmentation. Int J Androl 27(5),296–300.

Bosch M, Rajmil O, Egozcue J and Templado C (2003) Linear increase of structural and numerical chromosome 9 abnormalities in human sperm regarding age. Eur J Hum Genet 11(10),754–759.

Brandriff B and Pedersen RA (1981) Repair of the ultraviolet-irradiated male genome in fertilized mouse eggs. Science 211(4489),1431–1433.

Brinkworth MH and Schmid TE (2003) Effect of age on testicular germ cell apoptosis and sperm aneuploidy in MF-1 mice. Teratog Carcinog Mutagen 2(Suppl),103–109.

Brinkworth MH, Weinbauer GF, Bergmann M and Nieschlag E (1997) Apoptosis as a mechanism of germ cell loss in elderly men. Int J Androl 20(4),222–228.

Crow JF (2000) The origins, patterns and implications of human spontaneous mutation. Nat Rev Genet 1(1),40–47.

Cuzick J (1985) A Wilcoxon-type test for trend. Stat Med 4(1),87–90.

Donnelly ET, Steele EK, McClure N and Lewis SE (2001) Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod 16(6),1191–1199.

Duty SM, Singh NP, Silva MJ, Barr DB, Brock JW, Ryan L, Herrick RF, Christiani DC and Hauser R (2003) The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay. Environ Health Perspect 111(9),1164–1169.

Eskenazi B, Wyrobek AJ, Sloter E, Kidd SA, Moore L, Young S and Moore D (2003) The association of age and semen quality in healthy men. Hum Reprod 18(2),447–454.

Eskenazi B, Kidd SA, Marks AR, Sloter E, Block G and Wyrobek AJ (2005) Antioxidant intake is associated with semen quality in healthy men. Hum Reprod 20(4),1006–1012.

Evenson DP and Wixon R (2006) Clinical aspects of sperm DNA fragmentation detection and male infertility. Theriogenology 65(5),979–991.

Evenson DP, Jost LK, Baer RK, Turner TW and Schrader SM (1991) Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. Reprod Toxicol 5(2),115–125.

Generoso WM, Cain KT, Krishna M and Huff SW (1979) Genetic lesions induced by chemicals in spermatozoa and spermatids of mice are repaired in the egg. Proc Natl Acad Sci USA 76(1),435–437.

Giwercman A, Richthoff J, Hjollund H, Bonde JP, Jepson K, Frohm B and Spano M (2003) Correlation between sperm motility and sperm chromatin structure assay parameters. Fertil Steril 80(6),1404–1412.

Glaser RL, Broman KW, Schulman RL, Eskenazi B, Wyrobek AJ and Jabs EW (2003) The paternal-age effect in Apert syndrome is due, in part, to the increased frequency of mutations in sperm. Am J Hum Genet 73(4),939–947.

Glei M, Habermann N, Osswald K, Seidel C, Persin C, Jahreis G and Pool-Zobel BL (2005) Assessment of DNA damage and its modulation by dietary and genetic factors in smokers using the Comet assay: a biomarker model. Biomarkers 10(2–3),203–217.

Haines G, Marples B, Daniel P and Morris I (1998) DNA damage in human and mouse spermatozoa after in vitro-irradiation assessed by the comet assay. Adv Exp Med Biol 444,79–91; discussion 92–3.

Harrouk W, Codrington A, Vinson R, Robaire B and Hales BF (2000) Paternal exposure to cyclophosphamide induces DNA damage and alters the expression of DNA repair genes in the rat preimplantation embryo. Mutat Res 461(3),229–241.

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V and Tice RR (2003) Recommendations for conducting the in vivo alkaline Comet assay. Fourth International Comet Assay Workshop. Mutagenesis 18(1),45–51.

Hughes CM, McKelvey-Martin VJ and Lewis SE (1999) Human sperm DNA integrity assessed by the Comet and ELISA assays. Mutagenesis 14(1),71–75.

Kidd SA, Eskenazi B and Wyrobek AJ (2001) Effects of male age on semen quality and fertility: a review of the literature. Fertil Steril 75(2),237–248.

Kodama H, Yamaguchi R, Fukuda J, Kasai H and Tanaka T (1997) Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. Fertil Steril 68(3),519–524.

Lansac J (1995) Delayed parenting. Is delayed childbearing a good thing? Hum Reprod 10(5),1033–1035.

Larson KL, DeJonge CJ, Barnes AM, Jost LK and Evenson DP (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. Hum Reprod 15(8),1717–1722.

Lian ZH, Zack MM and Erickson JD (1986) Paternal age and the occurrence of birth defects. Am J Hum Genet 39(5),648–660.

Maher ER, Brueton LA, Bowdin SC, Luharia A, Cooper W, Cole TR, Macdonald F, Sampson JR, Barratt CL, Reik W and Hawkins MM (2003) Beckwith–Wiedemann syndrome and assisted reproduction technology (ART). J Med Genet 40(1),62–64.

Marchetti F, Lowe X, Bishop J and Wyrobek J (1997) Induction of chromosomal aberrations in mouse zygotes by acrylamide treatment of male germ cells and their correlation with dominant lethality and heritable translocations. Environ Mol Mutagen 30,410–417.

Marchetti F, Bishop JB, Cosentino L and Moore D, II and Wyrobek AJ (2004) Paternally transmitted chromosomal aberrations in mouse zygotes determine their embryonic fate. Biol Reprod 70,616–624.

Marcon L and Boissonneault G (2004) Transient DNA strand breaks during mouse and human spermiogenesis new insights in stage specificity and link to chromatin remodeling. Biol Reprod 70(4),910–918.

McPherson SM and Longo FJ (1993) Nicking of rat spermatid and spermatozoa DNA: possible involvement of DNA topoisomerase II. Dev Biol 158(1),122–130.

Morris ID (2002) Sperm DNA damage and cancer treatment. Int J Androl 25(5),255–261.

Morris ID, Ilott S, Dixon L and Brison DR (2002) The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) its relationship fertilization embryo development. Hum Reprod 17(4),990–998.

Park E and Kang MH (2004) Smoking and high plasma triglyceride levels as risk factors for oxidative DNA damage in the Korean population. Ann Nutr Metab 48(1),36–42.

Pedersen RA and Cleaver JE (1975) Repair of UV damage to DNA of implantation-stage mouse embryos in vitro. Exp Cell Res 95(2),247–253.

Print CG and Loveland KL (2000) Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioessays 22(5),423–430.

Risch N, Reich EW, Wishnick MM and McCarthy JG (1987) Spontaneous mutation and parental age in humans. Am J Hum Genet 41(2),218–248.

Robbins WA, Segraves R, Pinkel D and Wyrobek AJ (1993) Detection of aneuploid human sperm by fluorescence in situ hybridization: evidence for a donor difference in frequency of sperm disomic for chromosomes 1 and Y. Am J Hum Genet 52(4),799–807.

de la Rochebrochard, E and Thonneau P (2002) Paternal age and maternal age are risk factors for miscarriage; results of a multicentre European study. Hum Reprod 17(6),1649–56.

Rubes J, Lowe X, Moore D, II, Perreault S, Slott V, Evenson D, Selevan SG and Wyrobek AJ (1998) Smoking cigarettes is associated with increased sperm disomy in teenage men. Fertil Steril 70(4),715–723.

Sakkas D, Manicardi G, Bizzaro D and Bianchi PG (2000) Possible consequences of performing intracytoplasmic sperm injection (ICSI) with sperm possessing nuclear DNA damage. Hum Fertil (Camb) 3(1),26–30.

Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM and Abraham RT (1999) Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res 59(17),4375–4382.

Shamsi FA and Hadi SM (1995) Photoinduction of strand scission in DNA by uric acid and Cu (II). Free Radic Biol Med 19(2),189–196.

Shi Q and Martin RH (2000) Aneuploidy in human sperm: a review of the frequency and distribution of aneuploidy, effects of donor age and lifestyle factors. Cytogenet Cell Genet 90(3–4),219–226.

Shi Q, Ko E, Barclay L, Hoang T, Rademaker A and Martin R (2001) Cigarette smoking and aneuploidy in human sperm. Mol Reprod Dev 59(4),417–421.

Sills ES, Fryman JT, Perloe M, Michels KB and Tucker MJ (2004) Chromatin fluorescence characteristics and standard semen analysis parameters: correlations observed in andrology testing among 136 males referred for infertility evaluation. J Obstet Gynaecol 24(1),74–77.

Silver EW, Eskenazi B, Evenson DP, Block G, Young S and Wyrobek AJ (2005) Effect of antioxidant intake on sperm chromatin stability in healthy nonsmoking men. J Androl 26(4),550–556.

Singh NP, Muller CH and Berger RE (2003) Effects of age on DNA double-strand breaks and apoptosis in human sperm. Fertil Steril 80(6),1420–1430.

Sloter E, Nath J, Eskenazi B and Wyrobek AJ (2004) Effects of male age on the frequencies of germinal and heritable chromosomal abnormalities in humans and rodents. Fertil Steril 81(4),925–943.

Spano M, Kolstad AH, Larsen SB, Cordelli E, Leter G, Giwercman A and Bonde JP (1998) The applicability of the flow cytometric sperm chromatin structure assay in epidemiological studies. Hum Reprod 13(9),2495–2505.

Sun JG, Jurisicova A and Casper RF (1997) Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod 56(3),602–607.

Tarin JJ, Brines J and Cano A (1998) Long-term effects of delayed parenthood. Hum Reprod 13(9),2371–2376.

Tiemann-Boege I, Navidi W, Grewal R, Cohn D, Eskenazi B, Wyrobek AJ and Arnheim N (2002) The observed human sperm mutation frequency cannot explain the achondroplasia paternal age effect. Proc Natl Acad Sci USA 99(23),14952–14957.

Wyrobek AJ, Schmid TE and Marchetti F (2005a) Cross-species sperm-FISH assays for chemical testing and assessing paternal risk for chromosomally abnormal pregnancies. Environ Mol Mutagen 45(2–3),271–283.

Wyrobek AJ, Schmid TE and Marchetti F (2005b) Relative susceptibilities of male germ cells to genetic defects induced by cancer chemotherapies. J Natl Cancer Inst Monogr 34,31–35.

Wyrobek AJ, Evenson D, Arnheim N, Jabs EW, Young S, Pearson F, Glasser RLF, Thiegmann I and Eskenazi B (2006) Advancing male age increase the frequencies of sperm with DNA fragmentation and certain gene mutations, but not aneuploidies or diploidies. Proc Natl Acad Sci USA 103(25),9601–9606.

Zhang Y, Kreger BE, Dorgan JF, Cupples LA, Myers RH, Splansky GL, Schatzkin A and Ellison RC (1999) Parental age at child's birth and son's risk of prostate cancer. The Framingham Study. Am J Epidemiol 150(11),1208–1212.

Submitted on March 2, 2006; resubmitted on July 12, 2006; accepted on July 24, 2006