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Abstract

BACKGROUND: The trend for men to have children at older ages 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 (age: 22-80) with no known fertility problems using the sperm Comet analyses.

RESULTS: The average percent 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 % tail DNA under neutral but not alkaline conditions compared to men who consumed no caffeine ($p=0.005$).

CONCLUSIONS: Our findings indicate that (a) older men have increased sperm DNA damage associated with alkali-labile sites or single-strand DNA breaks, and (b) 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 for developmental defects and genetic diseases among offspring.

Introduction

The societal trend for older parents to have children raises public health concern about age-associated risks for abnormal pregnancies and birth defects. It is well known that female fecundity declines precipitously by the 4th decade of life due to oocyte loss, and that older mothers have increased risks for 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 for 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 increase 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 etiology such as prostate cancer (Zhang et al. 1999). However, 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. 2005) and growing evidence links sperm DNA damage with risks for 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). For example, older men seem to produce more sperm with chromosomal aberrations, mutations for achondroplasia and Apert sydrome, and DNA damage (Tiemann-Boege et al. 2002; Glaser et al. 2003; Singh et al. 2003), but not with more sperm aneuploidy (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 (intracytoplasmic sperm injection) and IVF (in vitro fertilization) 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 increases in genetic damage in blood cells (Park and Kang 2004; Glei et al. 2005; Wyrobek et al. 2005), but little is known about their effects on genetic damage in sperm (Wyrobek et al. 2005). 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 (a) 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-

stranded DNA breaks and double-stranded DNA breaks (Haines et al. 1998; Hughes et al. 1999; Morris et al. 2002) and (b) 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.

Material 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 six month; had no vasectomy or a history of an undescended testicle or prostata cancer; or had no previous semen analysis with zero sperm count. At least 15 men were enrolled for each age decade between 20 and 60 years; 25 men were recruited above the age of 60. The 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, education), occupation, possible exposures, diet and lifestyle habits. Semen samples were analyzed for count and motility upon collection (Eskenazi et al. 2003) and immediately stored at -80°C.

Sperm Comet analyses

Frozen sperm aliquots were shipped on dry ice to the University of Bradford, United Kingdom, for analysis by single-cell electrophoresis (sperm Comet). Each specimen was analyzed 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 PBS at a 50 C and dried at room temperature for three days. Approximately 10,000 sperm were mixed with 100 uL 0.5% low melting agarose to form a cell suspension of which 90 μL were 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% DMSO) for 2 hours. Then 10 mM DTT was added to the lysing solution for an additional 30 minutes. Slides were then incubated in either 1 mM EDTA and 300 mM NaOH buffer (pH 13.0) for the alkaline analysis or in 1x 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 neutralise the alkaline buffer for 5 min. Fifty μL of ethidium bromide (EtBr, 20 μg/ml) were added, the slides coverslipped and analysed within 3 hours. Slides were examined at x400 under a fluorescent microscope. Fifty cells were scored from each of two replicate slides for each specimen (100 cells total). A computerized image analysis system (Comet 3.0, Kinetic Imaging, Nottingham, UK) was used to measure the percent tail DNA (% tail DNA, the average percent 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 ANOVA and t-tests. Trends by age decade were calculated by a nonparametric test across ordered groups (Cuzick 1985) and correlations were determined using Pearson's correlation.

Multiple linear regression models were used 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 past history of infertility; fatherhood history; body mass index; ethnicity; diet characteristics, and vitamin supplement use. Covariates were retained in the final models (see Table 2 footnotes) if they changed the parameter estimate of age by at least 10% or if they were statistically significant ($p<0.1$). We checked regression assumptions with residual vs. 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 correlations and multiple linear regression. Age and duration of abstinence were examined as potential covariates. For these analyses, conventional semen quality outcomes (% motile sperm, 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, Texas, USA).

Results

Characteristics of study population

The 80 participants were on 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 1, we found that older age was associated with increased consumption of vitamin supplements and prior history of urinary tract infections ($p<0.05$) and with trends towards increased duration of sexual abstinence, prior tobacco use, lower regular alcohol usage, and lower kilocalorie intake ($p<0.1$).

The study volunteers were well distributed among age decades (Table 2). 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 higher alkaline % tail DNA in men of the 60-80 versus 20-29 year groups ($p = 0.03$). There was a 0.18% increase in % tail DNA per year of age ($p=0.006$; Table 2) after adjusting for covariates (vitamin C use, kilocalorie intake, urinary infections, and season of collection). Age explained approximately 10% (partial $r =0.32$) of the total variance of % tail DNA under alkaline conditions. While the unadjusted alkaline Comet data appears to follow a quadratic or parabolic shape, a linear model (Fig. 1a) provided a satisfactory fit after adjusting for covariates. Two other measures of DNA strand damage obtained by sperm Comet analysis (olive tail moment, tail extend 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 driven by four men with high comet values. Three of the four men were over 65 years of age (28, 66, 68, 71) and all four had alkaline % tail DNA values > 60. All 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 of the time of semen collection. When we exclude the four men with alkaline % tail DNA values over 60 from the regression analyses, the association with age becomes marginally significant (slope = 0.07, p=0.18).

There was no significant effect of age on neutral sperm DNA damage (Fig. 1b). Neutral % tail DNA decreased non-significantly 0.02% per year of age (p=0.7) after adjusting for covariates (total caffeine use in last 3 months, any urinary tract infections, vitamin E use, kilocalorie intake).

Association with lifestyle, medical factors and caffeine consumption

Sperm Comet results were associated with several lifestyle and medical factors obtained from questionnaire data (Table 1). Higher values of alkaline % tail DNA were associated with prior history of urinary tract infections ($p<0.05$) and prior 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 assigned to tertiles based on caffeine consumption and men with more than 308 mg of caffeine intake per day (equivalent to ~2.9 cups of coffee) had approximately 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 history of urinary tract infections) (Figure 2).

Correlations among sperm Comet, 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; 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 sperm chromatin structure assay (SCSA) (Wyrobek et al. 2006). Alkaline % tail DNA results were not correlated with DNA fragmentation (%DFI) nor with the percent of cells with immature chromatin (HDS, high DNA stainability). 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 associated double strand DNA breaks. 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.

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 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 males. Our finding of no association between age and sperm DNA damage under neutral conditions is in contrast with the study of Singh and colleagues (Singh et al. 2003), who studied 66 men, aged 20 to 57 years, from an infertility clinic as well as a non-clinical group. However, Singh et al. (2003) did not investigate sperm DNA damage under alkaline conditions in sperm and Morris at al. (2002) did not investigate sperm damage under neutral conditions.

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. 1998; Barroso et al. 2000). Oxidative stress can damage sperm DNA as well as mitochondrial and nuclear membranes (Aitken et al. 2003). Kodama and colleagues (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. 1998). 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 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 prior 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 percent of cells with immature chromatin (HDS), as measured by the sperm chromatin structure assay (SCSA) (Wyrobek et al. 2006). During spermiogenesis endogenous nuclease activity induces DNA strand breaks ("nicks") as part of normal chromatin remodeling 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 2005). 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 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 aging males.

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Table 1. Associations between study population characteristics and sperm DNA damage measured by single-cell electrophoresis (sperm Comet) under both alkaline and neutral conditions.

	Donors[a]		Age	% tail DNA Alkaline	% tail DNA Neutral
	Number	in %	Mean ± SD	Mean ± SD	Mean ± SD
Abstinence					
2-5 days	61	76	42.3 ±14.3	42.4 ± 8.9	35.5 ± 8.0
> 5 days	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					
≤ 1731 kcal/day	42	53	46.7 ±14.0	40.8 ± 8.8	35.6 ± 8.3
> 1731 kcal/day	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

[a] n=80 for neutral, n=79 for alkaline conditions
[b] kidney, bladder or ureter infections
(p values for t-test or ANOVA)

Table 2. Effects of age on sperm DNA damage measured by single cell electrophoresis (sperm Comet) under both alkaline and neutral conditions.

		Alkaline conditions		Neutral conditions	
Age Group	**Number of men[a]**	**% tail DNA[b]**	**(SD)**	**% tail DNA[b]**	**(SD)**
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		0.28		0.40	
p value					
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	

[a] n=14 in 60-80 yr group for alkaline assay

[b] % tail DNA: percent of 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, total daily kilocalorie intake.

Figure legend

Figure 1. Relationship between male age in years and sperm DNA damage measured under both alkaline and neutral electrophoresis conditions.

Individual data points are shown as well as the adjusted linear regression lines.

Panel A. Alkaline conditions: 0.18% change per year after adjusting for vitamin C use, kilocalorie intake, kidney/bladder/urinary infections, and season of collection: (r^2 = 0.26; p = 0.006).

Panel B. Neutral conditions: -0.02% change per year after adjusting for total caffeine use in last 3 months, any urinary tract infections, vitamin E use, kilocalorie intake: (r^2 = 0.19; p = 0.73).

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 vs. none, unadjusted p=0.01).

Figure 3. Relationship between total sperm count and sperm DNA damage measured under alkaline electrophoresis conditions.

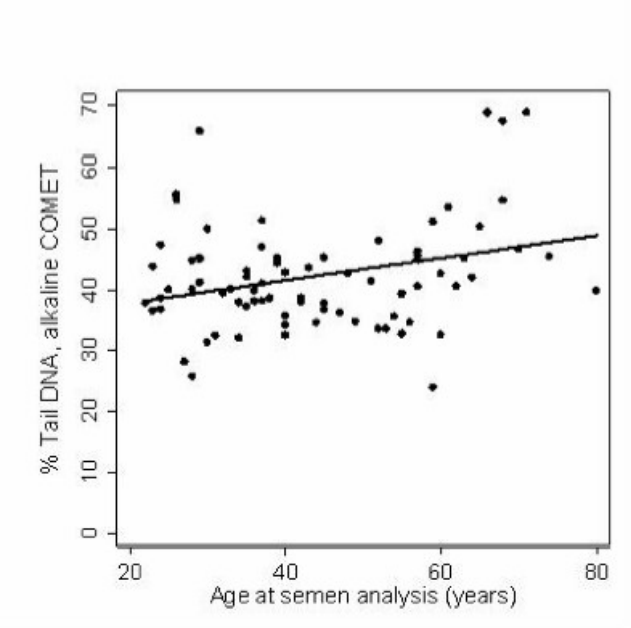
Individual data points are shown as well as linear regression line for square-root transformed sperm count, adjusted for age and abstinence (β= -0.24, p = 0.03).

Figure 4. Relationship between the frequencies of sperm with immature sperm chromatin (high DNA stainability, HDS) in the SCSA assay and sperm DNA damage under neutral electrophoresis conditions.

Individual data points are shown as well as the regression line for log-transformed HDS adjusted for age and abstinence (2.6% relative change in HDS per unit increase in % tail DNA, $r^2 = 0.11$; $p = 0.002$).

Figure 1.

a. Alkaline conditions



b. Neutral conditions

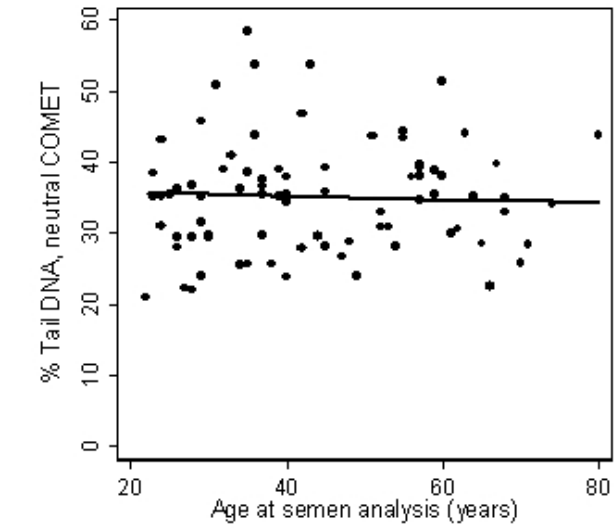


Figure 2.

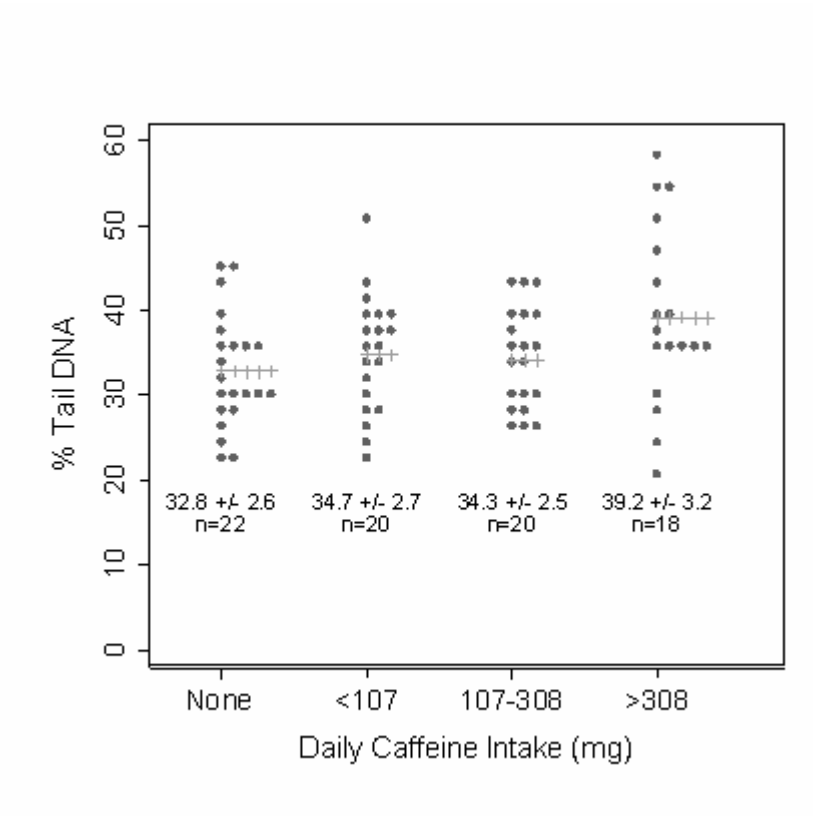

% Tail DNA
0
10
20
30
40
50
60
32.8 +/- 2.6
n=22
34.7 +/- 2.7
n=20
34.3 +/- 2.5
n=20
39.2 +/- 3.2
n=18
None
<107
107-308
>308
Daily Caffeine Intake (mg)

Figure 3.

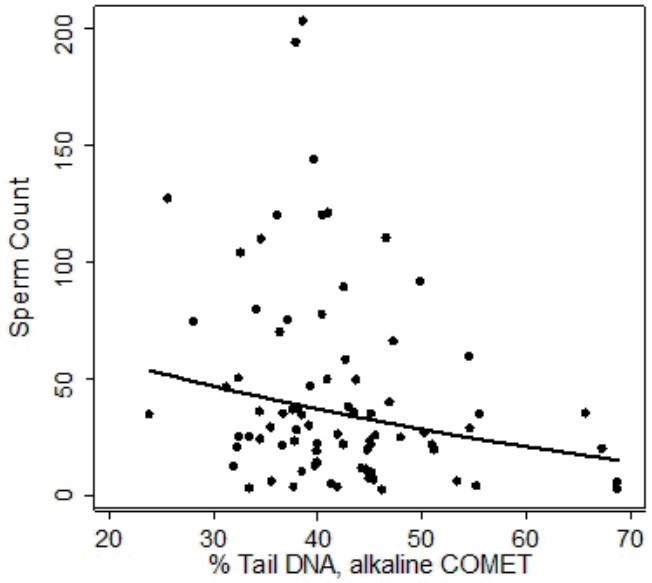


Figure 4.