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Micronutrients intake is associated with improved sperm DNA quality in older men

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Objective: To investigate whether lifestyle factors such as increased dietary intake of micronutrients reduce the risks of sperm DNA damage, and whether older men benefit more than younger men.
Design: Cross-sectional study design with equalized assignments into age groups.
Setting: National laboratory and university.
Patient(s): Nonclinical group of 22–80-year-old nonsmoking men (n = 80) who reported no fertility problems.
Main Outcome Measure(s): Sperm DNA damage measured by alkaline and neutral DNA electrophoresis (i.e., sperm Comet assay).
Result(s): Sociodemographics, occupational exposures, medical and reproductive histories, and lifestyle habits were determined by questionnaire. The average daily dietary and supplement intake of micronutrients (vitamin C, vitamin E, b-carotene, zinc, and folate) was determined using the 100-item Modified Block Food Frequency Questionnaire (FFQ). Men with the highest intake of vitamin C had approximately 16% less sperm DNA damage (alkaline sperm Comet) than men with the lowest intake, with similar findings for vitamin E, folate, and zinc (but not β-carotene). Older men (>44 years) with the highest vitamin C intake had approximately 20% less sperm DNA damage compared with older men with the lowest intake, with similar findings for vitamin E and zinc. The older men with the highest intake of these micronutrients showed levels of sperm damage that were similar to those of the younger men. However, younger men (<44 years) did not benefit from higher intakes of the micronutrients surveyed.
Conclusion(s): Men with higher dietary and supplement intake of certain micronutrients may produce sperm with less DNA damage, especially among older men. This raises the broader question of how lifestyle factors, including higher intakes of antioxidants and micronutrients, might protect somatic as well as germ cells against age-associated genomic damage. (Fertil Steril® 2012;■:■–■. ©2012 by American Society for Reproductive Medicine.)
Key Words: Human sperm alkaline and neutral Comet assay, vitamin C, vitamin E, β-carotene, zinc, folate

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The increasing trend for men over 35 years of age to have children raises public health concern about age-associated risks for abnormal pregnancies and birth defects. Male fecundity is known to decline with age, although spermatogenesis continues well into male senescence, and some men of substantially advanced ages have fathered healthy children (1, 2). Nevertheless, men's age has been associated with reduced semen quality (3), increased frequencies of sperm with structural aberrations (4), increased sperm DNA fragmentation, and

increased frequencies of sperm carrying achondroplasia gene mutations (5). Thus, normally aging males are predicted to experience higher frequencies of chromosomally defective pregnancies and higher proportions of offspring carrying heritable genetic defects (5). In addition, male aging has been associated with sperm DNA strand damage (6), but no studies have examined whether lifestyle and diet can be protective against detrimental effects of aging on the sperm genome.

Dietary supplementations with antioxidants and increased intake of fruits or vegetables rich in antioxidants have been shown to decrease the amount of oxidative DNA damage in vivo (7, 8) and to reduce DNA breaks (9) as well as DNA oxidation (7) in human lymphocytes. In vitro evidence shows that vitamin C (ascorbic acid), a scavenger of reactive oxygen species, can reduce oxidative stress in sperm (10). Our laboratory reported that antioxidant intake was associated with better semen quality, in particular, sperm motility (11), and that men with higher folate intake were less likely to produce aneuploid sperm for chromosomes X, Y, and 21 (12). However, no association was observed between antioxidant intake and DNA fragmentation (13). Whether antioxidant intake and general dietary changes can enhance male fertility and improve reproductive outcomes is a research topic in need of better understanding of underlying cellular and molecular mechanisms (14–16).

The present study utilized a population of healthy nonsmokers in which we previously reported age-associated increases of sperm DNA damage using the sperm Comet assay (6) to address two questions: [1] is micronutrient intake differentially associated with either of the two subtypes of sperm DNA damage measured by the sperm Comet assay (alkaline and neutral versions) (17), and [2] does micronutrient intake modify the age-related increases we previously observed? The alkaline version of the sperm Comet assay is thought to detect double-strand breaks (DSB), single strand breaks (SSB), and alkali-labile sites (ALS), whereas the neutral version is thought to predominantly detect DSB and to a lesser extent SSB (17).

MATERIALS AND METHODS

Study Population

The study population consisted of approximately 80 healthy male volunteers, aged 22–80, who were recruited for the California Age and Genetic Effects on Sperm (AGES) Study. Participants were recruited from among employees and retirees of a national laboratory in California, which provides health care plans for its employees. Recruitment was conducted through the University of California, Berkeley, and the sperm analysis was performed in a research setting at the national laboratory. The California AGES Study was approved by the Institutional Review Board of each participating institution, and all volunteers gave their written consent to participate. Preliminary screenings excluded men who had current fertility or reproductive problems; had smoked cigarettes in the last 6 months; had a vasectomy or a history of an undescended testicle or prostate cancer; had received chemotherapy or radiation treatments for cancer; or had a previous semen analysis with zero sperm count. If men at screening had a fever $>38.5^{\circ}C$ in the last 3 months, they were enrolled 3 months from the date of the fever. At least 15 men were enrolled for each age decade between 20 and 70 years; additional men aged >70 years were also enrolled. Of the original set of men in the California AGES Study, one subject missing dietary data was excluded. Additional subjects were excluded if they did not have adequate remaining semen aliquots for Comet analyses, resulting in a total of 79 men for the analysis of neutral sperm Comet assay and 78 for the alkaline sperm Comet assay. Sixty-two percent of the men had fathered a pregnancy. Men who fathered pregnancy were older than men who had not (48.8 vs. 35.3 years, $P<.001$).

Questionnaire

We mailed to each eligible participant a study questionnaire, semen collection instructions, a sterile container, and protective thermos (11, 13). The self-administered questionnaire asked about sociodemographic characteristics (age, race, and education), occupation, possible occupational exposures, medical and reproductive history, and lifestyle habits. We also sent a 100-item Modified Block Food Frequency Questionnaire (FFQ) (18) to estimate average daily dietary and supplemental vitamin intake. The FFQ assessed the frequency and serving size of major food types over the previous year, including specific supplement intake information for multivitamins, antioxidant combination vitamins, calcium, iron, zinc, and selenium. The FFQ responses were converted into average daily nutrient intake estimates using a standardized reference nutrient database (www.nutritionquest.com). Participants completed the FFQ within 1 week of producing the semen sample. Study staff reviewed the FFQs for reasonable levels of calories and number of foods per day. Questionnaires were reviewed with participants over the phone for completion and accuracy.

Semen Collection and Sperm Comet Assay

Men provided a semen sample by masturbation into a sterile collection container after abstinence of 2–7 days and delivered it within an insulated thermos at room temperature to a drop box at the laboratory, noting the actual duration of abstinence. Samples were delivered within 2 hours of collection and immediately analyzed for conventional semen quality and computer-assisted semen analysis (19). Remaining aliquots of semen were stored at $-80^{\circ}C$. For comet analyses, frozen samples were shipped on dry ice to the University of Bradford, United Kingdom and kept at $-80^{\circ}C$ until analysis. The sperm Comet data used in these analyses were previously published (6) and were based on the prior procedures described by Anderson et al. for the alkaline assay (20) and by Duty et al. for the neutral assay (21). Slides were examined at $\times 400$ magnification on a fluorescent microscope. Fifty cells were scored from each replicate slide (100 cells total). A computerized image analysis system (Comet 3.0; Kinetic Imaging) was used to measure the percent tail DNA and other parameters, including tail extent moment and olive tail moment. These different measurements were highly correlated in our dataset and in the literature. We calculated the percent DNA in the comet tail because it is a clear indication of sperm

comets, linearly related to the DNA break frequency over a wide range of levels of damage, the most commonly reported outcome (22), and considered a reliable measurement (23) for human sperm samples.

Statistical Analysis

The main independent variables were estimated daily intake of vitamin C, vitamin E, β-carotene, folate, and zinc, as well as a composite of antioxidant intake. Intake was based on the combination of reported diet and supplements use and was classified as low (<25th percentile), moderate (25th–75th percentile), or high (>75th percentile). Quartiles were created using all AGES study participants with complete FFQ data to ensure that an individual remained in the same category for all AGES study analyses (11–13). The antioxidant composite variable was created a priori to obtain a combined measure of dietary and supplementary antioxidant intake from vitamins C, E, and β-carotene, as described previously (11). Low antioxidant composite intake was defined as low intake for at least two of the antioxidants; high antioxidant composite intake was defined as high intake for at least two of the antioxidants; and moderate intake was defined as all other combinations of intake.

All analyses were performed using Stata 8.0 (24). Separate multivariable linear regression models were constructed to examine the relationship of intake of each antioxidant with sperm DNA damage (i.e., % tail DNA) for the neutral and alkaline Comet assays. For each nutrient, the three intake groups were included as indicator variables into statistical models, with low intake as baseline. The following variables and classes of variables were considered potential covariates: age; 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 nonprescription 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 disease, varicocele, or past history of infertility; fatherhood history; body mass index; and ethnicity. Covariates were first examined in univariate analyses with sperm DNA damage using analysis of variance and *t* tests and were included in the full models if $P<.2$. Covariates were retained in the final models if they changed the nutrient regression coefficient by at least 10% or had a *P* value <.10. We checked regression assumptions with residual vs. fitted plots and quantile–quantile plots. Final regression results are expressed as adjusted means and SEs predicted from the models with covariates set at their mean values. To obtain *P* values for trend, in the final models we replaced the nutrient indicator variables with ordered variables (i.e., 1 = low intake, 2 = moderate, 3 = high). All *P* values presented are two-sided.

To test whether higher micronutrient intake affected the positive relationship of age and alkaline sperm DNA damage reported previously (6), we created interaction terms for age (dichotomized at the median age of 44 years) and nutrient levels (dichotomized at the median intake for each nutrient) and assessed the associations with sperm DNA damage from the alkaline Comet assay. Any model with an interaction term <0.2 was considered statistically significant and was reanalyzed using dummy variables for the different intake/age groups using old age/low intake as the referent group. Results are expressed as adjusted means and 95% confidence intervals.

Four men with high sperm DNA damage (i.e., alkaline % tail DNA values >2.5 SD from mean) were evaluated as potential outliers (6). Three of the four men were >65 years of age, all four had fathered children, and none had a history of fertility problems. All alkaline Comet models were analyzed with and without these four men and results of all models are presented.

RESULTS

Characteristics of Study Population

Men were on average 46.4 years old (range, 22–80 years), generally healthy, and had not smoked cigarettes during the previous 6 months. The magnitude of sperm DNA damage was 42.2% ± 8.9% (% tail DNA, mean ± SD) and 35.2% ± 7.9% under alkaline conditions and neutral conditions, respectively, with no significant correlation between the results of the two analyses (Pearson correlation, $P>.1$) (6). As summarized in Supplementary Table 1 (available online), we found that higher DNA damage in the alkaline assay was associated with age ($P<.05$) and with history of kidney, bladder, and urinary tract infections ($P<.05$). Under neutral conditions, higher sperm DNA damage was associated with higher body mass index ($P<.05$).

Micronutrient Intake and Sperm DNA Damage

The median daily total intake (diet plus supplements) was 162 mg for vitamin C, 23.7 mg α-tocopherol equivalents (αTE) for vitamin E, 2,586 μg for β-carotene, 475 μg for folate, and 12.3 mg for zinc (Table 1). The percentage of men consuming less than the recommended dietary allowance (RDA) for each micronutrient ranged from 22% (vitamin C) to 47% (zinc) for total intake (dietary intake and supplements) and from 39% (vitamin C) to 81% (vitamin E) for dietary intake only. All further analyses were performed by using the measure of combined diet and supplement intakes.

We found no evidence of significant associations between any of the measures of micronutrient intake and sperm DNA damage as measured by the neutral version of the sperm Comet assay. Thus, the further analyses were conducted only between micronutrient intact and sperm DNA damage as measured by the alkaline version of the sperm Comet assay.

Vitamin C, E, and β-carotene. As shown in Table 2, after adjusting for age and other covariates (Materials and Methods), men in the highest quartile of daily vitamin C intake (459–3,370 mg/d) had 16% less sperm DNA damage in the alkaline Comet assay ($P<.01$) than men in the lowest quartile (26–99 mg/d), with a significant test for trend across the three intake categories ($P<.01$). For vitamin E, there was a similar pattern: men with the highest intake had lower

TABLE 1

Self-reported dietary and supplement intake of micronutrients.

Micronutrient	RDA[a]	Median	Minimum	Maximum	% Men below RDA
Dietary intake only					
Vitamin C (mg)	90	98.5	25.5	291.4	39
Vitamin E[b] (mg)	15	8.2	2.2	42.0	81
β-carotene (μg)	–	1,555.6	215.5	11,881.0	–
Folate (μg)	400	359.1	114.5	1,149.9	63
Zinc (mg)	11	8.8	4.4	20.0	77
Total Intake (diet + supplements[c])					
Vitamin C (mg)	90	161.6	25.5	3,369.1	22[d]
Vitamin E[b] (mg)	15	23.7	2.2	833.0	42[d]
β-carotene (μg)	–	2,571.3	400.8	33,443.7	–
Folate (μg)	400	487.3	114.5	1,149.9	41[d]
Zinc (mg)	11	12.3	5.1	74.0	47[d]

[a] RDA values from dietary reference intakes developed by the Institute of Medicine (50–52). No RDA is available for β-carotene.
[b] Vitamin E presented as αTE.
[c] Forty-five men took supplements, 33 reported dietary intake only.
[d] Diet only vs. diet + supplements, $P \leq .05$.

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sperm DNA damage than men with the lowest intake, although the relationship was nonsignificant ($P=.11$, test for trend $P=.12$). In addition, the antioxidant composite measure (vitamin C, vitamin E, β-carotene) showed a decrease in sperm DNA damage between the highest intake group and the lowest intake group (39.1 vs. 45.9, $P=.03$), with a significant trend ($P=.02$). There was no significant association between β-carotene and sperm DNA damage.

When we excluded the four outliers with high values of sperm DNA damage (i.e., alkaline % tail DNA >60%), we still observed a significant inverse relationship between sperm DNA damage and nutrient intake for vitamin C ($P<.01$) and the antioxidant composite variable ($P=.05$), as well as a borderline-significant relationship with vitamin E (test for trend, $P=.10$). Excluding the outliers did not change the relationship of sperm DNA damage with β-carotene.

Folate. Men in the highest quartile of high folate intake (722–1,150 μg/d) had lower levels of sperm DNA damage compared with men in the lowest quartile (114–333 μg/d), albeit nonsignificantly (39.3 vs. 43.5, $P=.13$). When the four outliers were excluded from the analysis, this relationship strengthened and became statistically significant (38.0 vs. 42.1, $P=.05$).

Zinc. As shown in Table 2, after adjusting for covariates, men in the highest quartile of daily zinc intake (22–74 mg/d) had less sperm DNA damage in the alkaline Comet assay ($P=.07$) than men in the lowest quartile (5–8 mg/d) (test for trend, $P=.07$). The relationship was strengthened slightly when the outliers were excluded ($P=.04$ for both high vs. low comparison and test for trend).

Interaction with age. In models in which nutrient intake was related to sperm DNA damage, age also remained significantly related, suggesting that nutrient intake did not eliminate the age relationship with sperm DNA damage reported

TABLE 2

Micronutrient intake and sperm DNA damage.

Variable	Intake range[a]	% Tail DNA, neutral	% Tail DNA, alkaline[b]	% Tail DNA, alkaline (minus 4 outliers)
Vitamin C				
Low	26–99 mg	37.1 (1.6)	44.7 (1.7)	42.3 (1.3)
Moderate	106–400 mg	34.7 (1.2)	43.4 (1.4)	42.3 (1.0)
High	459–3,370 mg	33.9 (1.6)	37.3[c,d] (1.8)	36.6[c,d] (1.4)
Vitamin E				
Low	2–9.6 αTE	35.8 (1.8)	45.4 (2.0)	42.7 (1.7)
Moderate	9.9–118 αTE	35.6 (1.2)	41.3 (1.4)	41.0 (1.1)
High	146–833 αTE	33.6 (1.7)	40.9 (1.9)	38.8[e] (1.6)
β-carotene				
Low	400–1,168 μg	34.6 (1.8)	42.5 (2.1)	42.0 (1.6)
Moderate	1,263–3,943 μg	35.5 (1.2)	42.7 (1.4)	41.0 (1.1)
High	3,972–33,444 μg	34.9 (1.8)	40.6 (2.1)	39.1 (1.7)
Antioxidant composite				
Low	–	35.5 (1.9)	45.9 (2.1)	42.9 (1.7)
Moderate	–	35.6 (1.1)	41.9 (1.3)	41.1 (1.0)
High	–	33.6 (1.9)	39.1[d,e] (2.1)	38.0[d,e] (1.7)
Zinc				
Low	5–8 mg	36.2 (1.9)	45.4 (2.0)	43.0 (1.6)
Moderate	8–22 mg	34.2 (1.3)	41.5 (1.4)	40.9 (1.1)
High	22–74 mg	35.8 (1.6)	40.4[e] (2.0)	38.4[d,e] (1.6)
Folate				
Low	114–333 μg	36.1 (1.8)	43.5 (1.9)	42.1 (1.4)
Moderate	343–653 μg	34.2 (1.2)	43.0 (1.4)	41.6 (1.0)
High	722–1,150 μg	36.1 (1.7)	39.3 (1.9)	38.0[d,e] (1.4)

Note: Data are presented as adjusted mean (SD).
[a] Low (<25th percentile), moderate (25th–75th percentile), and high (>75th percentile).
[b] n = 78 for alkaline Comet.
[c] Adjusted test for trend, $P<.01$.
[d] High vs. low intake, $P \leq .05$.
[e] Adjusted test for trend, $P<.1$.

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previously (6). In further examination of the relationship between age and nutrient intake on sperm DNA damage, there was a consistent finding for vitamin C, vitamin E, zinc, and folate (but not for β-carotene) that the older men with the lower intake (intake less than the median level for the micronutrient) had the highest sperm DNA damage compared with the other groups, including older men with higher intake (Table 3); this association was not significant for folate. Furthermore, older men with higher intake had sperm DNA damage comparable to that in the younger men. Figure 1 shows the relation between age and sperm DNA damage for vitamins C and E and zinc.

The difference in sperm DNA damage for older men with high zinc intake compared with older men with low zinc intake remained statistically significant even after excluding the four outliers. However, sperm DNA damage for older men with high vitamin C and vitamin E intake was now nonsignificantly lower than for older men with low intake ($P=.11$ and .12, respectively) but still similar to values for younger men with high intake even when excluding the four outliers ($P=.04$).

DISCUSSION

We previously reported significant associations between vitamin C, vitamin E, and β-carotene intake and semen quality (11). We also reported that older men had more sperm DNA damage using the Comet assay (6). In the present study, we report that [1] men with the higher total intake of vitamin C, vitamin E, folate, and zinc from diet and supplements had significantly less sperm DNA damage (alkaline Comet) than men with lower intake; and [2] older men who consumed higher levels of certain micronutrients (vitamin C, vitamin E, and zinc) had similar levels of sperm DNA damage as young men. These new findings suggest that for men who are at increased risk of DNA strand damage due to advancing age, a diet that consists of high levels of antioxidants and micronutrients may decrease the risk of producing sperm with DNA damage.

Our findings that high antioxidant intake was associated with lower amounts of DNA breaks may be explained by changes in oxidative stress in the male reproductive tract (25, 26). Anderson et al. (27) showed that vitamin C can be an antioxidant in the sperm Comet assay. Vitamin C, a scavenger of reactive oxygen species (28), is secreted from the seminal vesicles during ejaculation and is the major antioxidant present in seminal plasma of fertile males, contributing to up to 65% of the total antioxidant capacity (29). In a prior study, we showed that dietary intake of vitamin C can protect human sperm against endogenous oxidative DNA damage (30). Hughes et al. (31) reported that in vitro treatment of sperm with α-tocopherol, an antioxidant, reduced the magnitude of DNA damage as measured by the Comet assay.

Folate, another antioxidant, also seems to affect genetic quality of sperm in older men. Folate is an essential B vitamin that occurs naturally in a wide variety of foods, such as broccoli, cabbage, cauliflower, fruit, and nuts. Folate metabolism is critical for proper cell function (32, 33). Its main roles are to provide carbon groups for purine and pyrimidine synthesis

TABLE 3

Interactions between micronutrient intake and age.

Nutrient[a]	Age group[b]	Intake group[c]	Median intake	25th%–75th%	n	% Tail DNA, mean (95% CI)
Vitamin C	Younger	Low	113 mg[d]	75–132 mg	26	42.1[e] (38.9–45.3)
		High	306 mg	206–1,130 mg	18	40.2[e] (36.4–44.0)
	Older	Low	86 mg	51–98 mg	15	49.0 (44.7–53.2)
		High	717 mg	347–1,156 mg	19	39.0[e] (35.3–42.7)
Vitamin E	Younger	Low	9.9 mg	6.9–12.1 mg	25	41.6[f] (38.2–45.0)
		High	44.4 mg[d]	34.6–199.8 mg	19	40.0[e] (36.1–44.0)
	Older	Low	10.5 mg	5.9–12.8 mg	15	47.0 (42.7–51.4)
		High	274.4 mg	104.9–562.4 mg	19	41.4[f] (37.5–45.3)
β-carotene	Younger	Low	1,142 μg	730–1659 μg	24	40.6 (37.0–44.2)
		High	3,871 μg	3,213–5,396 μg	20	41.7 (37.6–45.8)
	Older	Low	1,785 μg	998–2,357 μg	15	44.3 (39.7–48.9)
		High	3,681 μg	3,026–13,741 μg	19	43.1 (39.1–47.2)
Zinc	Younger	Low	8.5 mg	7.0–10.6 mg	22	40.8[e] (37.2–44.4)
		High	18 mg[d]	13.4–23.4 mg	22	40.9[e] (37.3–44.6)
	Older	Low	7.8 mg	6.9–8.9 mg	17	47.2 (43.0–51.3)
		High	25 mg	22.4–28.8 mg	17	40.7[e] (36.6–44.8)
Folate	Younger	Low	344 μg	217–394 μg	23	41.9 (38.2–45.5)
		High	749 μg	562–879 μg	21	41.0 (37.3–44.8)
	Older	Low	316 μg	266–378 μg	16	45.4 (41.1–49.7)
		High	631 μg	593–836 μg	18	41.2 (36.9–45.5)

Note: CI = confidence interval.

[a] Reference group: older men, low intake. All models adjusted for season of sample collection and total daily dietary kilocalories. Vitamin C and folate models also adjusted for kidney, bladder, or urinary tract infections. β-carotene models adjusted for history of smoking.

[b] Younger age group <44 years (median of population), older group ≥44 years.

[c] Low group defined as less than median intake of specific nutrient, high group median or greater intake.

[d] Intake differs from older men in similar intake group, rank-sum $P<.05$.

[e] $P<.05$ vs. reference group (older men, low intake).

[f] $P<.10$ vs. reference group (older men, low intake).

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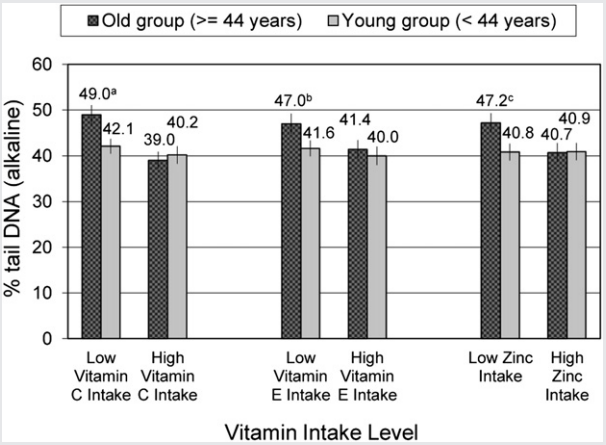
FIGURE 1



Older men with poor micronutrient intake have higher sperm DNA damage. Sperm DNA damage is reported as alkaline % tail DNA with adjusted means and SE; age and nutrient intake are reported as medians. Low vitamin intake defined as less than the median intake for entire of AGES population. Median value for vitamin C is 162 mg, median value for vitamin E is 23.7 mg for α-TE, and median value of zinc is 12.3 mg. [a]$P<.01$ for old/high vitamin C intake and young/high vitamin C intake vs. referent group (old/low vitamin C intake); $P<.05$ for young/low vitamin C intake vs. referent group. [b]$P<.10$ for old/high vitamin E intake and young/low vitamin E intake vs. referent group (old/low vitamin E intake); $P<.05$ for young/high vitamin E intake vs. referent group. [c]$P<.05$ for all groups compared with referent group (old/low zinc intake).

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and to create the vital methyl donors methionine and S-adenosylmethionine (34). A low thymidine/uracil ratio caused by folate deficiency results in uracil being incorporated into DNA during synthesis. The repair of two nearby opposing lesions can cause DNA DSB (35). It was shown that dietary deficiency in folate increased DNA strand breaks in the liver of rats (36). Folate deficiency has been also shown to increase chromosomal instability and aneuploidy (32, 33) in human lymphocytes. Physiologic levels of folate deficiency can cause DNA damage in human lymphocytes, by up-regulation of several excision repair genes and down-regulation of many folate metabolism genes (35). There is an interaction between folic acid deficiency and oxidative stress on DNA damage because the simultaneous excision of an oxidized base and uracil on opposite DNA strands within 12 bases of each other could result in the formation of double-strand breaks (37).

Lopes et al. (38) showed that in vitro exposure of human sperm to reactive oxygen species increased DNA fragmentation as measured by the TUNEL assay, whereas in vitro treatment with antioxidants (including cysteine and glutathione) reduced DNA fragmentation. Oxidative stress can induce oxidative damage in the sperm plasma membrane and DNA damage in mitochondrial and nuclear genomes (39). Kodama et al. (40) reported an association between the level of endogenous oxidative DNA damage in spermatozoa and male infertility linked to sperm DNA damage. The association of oxidative damage and infertility is supported by our previous findings that antioxidant intake is associated with better semen quality, in particular, motility (11). Mechanistically, antioxidants may be important for protecting human sperm from peroxidative damage that may result from the high amount of polyunsaturated fatty acids in sperm (14).

The present study noted an association between zinc intake and sperm DNA damage as measured by the alkaline sperm Comet assay. Ten percent of the US population consumes less than half the RDA for zinc, especially those who eat little meat and/or consume high phytate-containing foods (41). Insufficient zinc intake can impair antioxidant defenses and compromise DNA repair, making the cell susceptible to oxidative damage (42). Zinc deficiency can also lead to increased oxidative damage to testicular DNA (43). There is also evidence from rats that zinc deficiency is associated with increased sperm DNA damage (44). Yang et al. (45) used the sperm Comet assay to show that higher zinc concentrations can prevent human sperm from damage by sodium nitroprusside, a blood pressure–reducing drug. Together, these data suggest that zinc deficiency can compromise DNA integrity via oxidative stress, but the mechanisms by which zinc deficiency induces oxidative stress are still unclear. One possibility is that zinc deficiency may impair mitochondrial function, causing excess free radical species to be leaked through the electron transport chain (43).

The protective effects of antioxidants and micronutrient intake were seen in the alkaline version of the sperm Comet assay but not the neutral version, using the same semen specimens. Oxidative stress, which increases with age, primarily causes SSB and ALS but only very few DSB. The alkaline Comet assay detects DSB, SSB, and ALS, whereas the neutral version predominantly detects DSB and to a much lesser extent SSB (17). This suggests that antioxidant and micronutrient intake protects specifically against SSB and ALS in sperm, which are increased in aging males (as measured by the alkaline assay), but show no detectable protection for DSBs in sperm, which are not increased in aging males (Table 1).

Our biomarker study design had some limitations. First, the AGES study population was composed of healthy nonsmoking volunteers without major reproductive problems. Therefore, our findings may not be generalizable to clinic-based groups or infertile populations. Second, our assessment of micronutrient intake was derived from the FFQ. The advantage of the FFQ is that it can ascertain usual intake during the entire period of spermatogenesis (13). However, the FFQ only provides estimates of intake and does not measure longitudinal, daily dietary, and supplemental vitamin intake. Therefore, we cannot assess variation in intake for the separate phases of sperm development. Additionally, the FFQ was not specifically validated in our population, although it was validated against dietary records in several prior studies (46–48). Third, our cross-sectional design could not determine whether micronutrient intake and sperm DNA damage were causally related; a future randomized clinical trial might address this question. Last, because of the correlation among the intake of different micronutrients, we could not definitively determine whether our results derived specifically from a high-quality diet, from one nutrient or nutrients in

particular, or from one or several associated lifestyle factors. Recently, a position report on sperm DNA organization, protection, and vulnerability also highlighted the importance of micronutrients such as zinc for maintaining sperm chromatin integrity (49). Given the potential importance for older men, our findings merit further scrutiny in future dietary studies, including intervention trials.

Our findings suggest that men who consume higher amounts of antioxidants (vitamin C, vitamin E, and folate) and the micronutrient zinc will, on average, produce sperm carrying less DNA damage, and that this protective effect is most pronounced among older men. The reproductive and heritable implications for individual older men who take higher amounts has not yet been investigated, and it remains to be shown whether these small average changes will enhance reproductive capacity of individuals. Our sperm findings point to a need for future studies to determine whether increased antioxidant intake in older fathers will improve fertility, reduce risks of genetically defective pregnancies, and result in healthier children. Our findings also raise the broader question of whether lifestyle factors, including higher intakes of antioxidants and micronutrients, can protect germ cells as well as somatic cells against age-related DNA damage.

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SUPPLEMENTARY TABLE 1

Study population characteristics and sperm DNA damage.

Characteristic	n	% Tail DNA, neutral (SD)	% Tail DNA, alkaline (SD)
Overall	79[a]	35.2 (7.9)	42.2 (8.9)
Age (y)			
20–29	18	32.3 (7.1)	43.4 (9.9)
30–39	19	37.5 (9.3)	40.4 (5.5)
40–49	13	34.2 (8.9)	38.1 (4.1)
50–59	14	37.1 (5.1)	39.3 (7.5)
60+	15	34.8 (7.7)	49.8[b] (11.5)
Abstinence before collection (d)			
≤5	60	35.7 (8.0)	42.6 (8.9)
>5	19	33.6 (7.7)	41.4 (8.9)
Previous tobacco use (cigarettes, cigars, pipes, chew, or snuff)			
No	71	34.8 (7.8)	41.9 (9.2)
Yes	8	38.5 (8.4)	45.2 (5.0)
Alcohol use in the last 3 mo			
No	31	35.6 (7.8)	43.1 (9.9)
Yes	48	34.9 (8.0)	41.7 (8.2)
Vitamin supplement use in last year			
No	33	35.1 (8.0)	43.2 (8.2)
Yes	46	35.2 (7.8)	41.5 (9.4)
Season of sample collection			
Fall	34	35.4 (8.0)	40.1 (7.6)
Winter	27	34.9 (8.4)	43.2 (8.5)
Spring/Summer	18	35.1 (7.2)	44.9 (11.2)
Body mass index (kg/m^2)			
≤25.0	44	33.8 (8.1)	43.4 (10.7)
>25.0	35	36.9[c] (7.4)	40.7 (5.9)
Ever had high blood pressure			
No	67	34.7 (8.0)	41.5 (8.8)
Yes	12	37.7 (6.9)	46.0 (8.7)
Ever had kidney, bladder, urinary tract infection			
No	68	35.0 (8.4)	41.4 (9.0)
Yes	11	36.2 (2.8)	47.4[c] (6.0)

[a] n = 78 for alkaline Comet.
[b] *F* test, $P < .01$.
[c] *t* test, $P < .10$.

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