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Effect of Antioxidant Intake on Sperm Chromatin Stability in Healthy Nonsmoking Men

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ABSTRACT: Oxidative stress is detrimental to sperm function and a significant factor in the etiology of male infertility. This report examines the association between dietary and supplementary intake of the antioxidants vitamin C, vitamin E, and beta-carotene and sperm chromatin integrity. Eighty-seven healthy male volunteers donated semen samples, completed food-frequency questionnaires, and provided information about their sociodemographic characteristics, medical and reproductive histories, and lifestyle habits. Sperm chromatin integrity was measured using the DNA fragmentation index (DFI) and related parameters, obtained from the sperm chromatin structure assay (SCSA®). SCSA measures the susceptibility of sperm DNA to acid-induced denaturation in situ. After adjusting for age and duration of abstinence, there was no dose-response association between any DFI outcome and any antioxidant intake measure. Non-dose–related associations were found between beta-carotene intake and both the standard deviation of DFI (SD DFI) and the percent of immature sperm. Participants with moderate, but not high, beta-carotene intake had an increase in SD DFI compared with participants with low intake (adjusted means 206.7 and 180.5, respectively; $P = .03$), as well as an increase in the percentage of immature sperm (adjusted means 6.9% and 5.0%, respectively; $P = .04$). If antioxidant intake in the range studied is indeed beneficial for fertility in healthy men, it does not appear to be mediated through the integrity of sperm chromatin. The results of this study do not preclude possible beneficial effects of high antioxidant intake on sperm chromatin integrity for men with fertility problems.

Key words: Fertility, spermatozoa, nutrition, oxidative stress, DNA fragmentation.

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Approximately 15% of all couples experience infertility at some time in their reproductive years (Templeton, 1995), with an estimated 25% (Templeton, 1995) to 40% (Fleming et al, 1995) due to male contribution. Diet may play a role in male infertility (Wong et al, 2000; Rolf and Nieschlag, 2001). Dietary intervention, particularly of dietary antioxidants, such as vitamin C, vitamin E, and beta-carotene, has been proposed as a potential way to improve male reproductive outcomes by reducing the extent of oxidative damage (Wong et al, 2000). Several intervention trials have found a reduction in concentration of oxidized DNA (8-hydroxy-2-deoxyguanosine) in sperm after antioxidant supplementation (Kodama et al, 1997; Comhaire et al, 2000). However, there is still much controversy and no consensus in the literature on whether antioxidant intake indeed reduces male infertility (Taylor, 2001; Agarwal and Saleh, 2002; Agarwal et al, 2004; Sikka, 2004).

The sperm chromatin structure assay (SCSA), which measures the susceptibility of sperm DNA to acid-induced denaturation, is considered to be an indicator of the integrity of sperm chromatin. Susceptibility to acid-induced denaturation depends on preexisting DNA strand breaks or labile sites (Aravindan et al, 1997) that can be quantified by the DNA fragmentation index, or DFI (Evenson et al, 1991, 2002). Increased DFI has been associated with increased risk of miscarriage and increased time to pregnancy in humans (Evenson et al, 1999; Larson et al, 2000; Spano et al, 2000) and in bulls and mice (Evenson et al, 1980a,b; Ballachey et al, 1987, 1988).

While there is some evidence that vitamin C intake may reduce DNA strand breaks (Green et al, 1994) and DNA base oxidation (Duthie et al, 1996) in human lymphocytes, there have been no investigations of the effects

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of antioxidant intake on sperm DNA and chromatin integrity in animals or humans. However, Hughes et al (1998, 1999) reported that in vitro treatment of sperm with antioxidants (300 and 600 μM ascorbic acid, 30 and 60 μM alpha-tocopherol, and 400 μM urate) reduced the magnitude of DNA damage as measured by the comet assay. Similarly, Lopes et al (1998) showed that in vitro treatment of human sperm with reactive oxygen species (ROS) increased sperm DNA fragmentation as measured by the terminal transferase-mediated deoxyuridine triphosphate-biotin end labeling (TUNEL) assay, while in vitro treatment with antioxidants (including cysteine and glutathione) reduced fragmentation. Moustafa et al (2004) found higher levels of ROS in the ejaculate of infertile patients compared with healthy sperm donors, and ROS-positive patients had significantly higher percentage of cells with DFI greater than 247 channels of fluorescence (%DFI), mean DFI, and standard deviation of DFI (SD DFI) in the whole ejaculate than the donors with normal semen parameters.

We have recently demonstrated that antioxidant intake is associated with better semen quality, in particular, motility (Eskenazi et al, 2005). The purpose of the present investigation is to examine the association between dietary and supplementary intake of vitamin C, vitamin E, and beta-carotene and sperm chromatin integrity in healthy men. We hypothesized that increased antioxidant intake would be associated with lower DNA fragmentation in sperm.

Materials and Methods

The Age and Genetic Effects in Sperm (AGES) Study was conducted in 1997 and 1998 at the University of California, Berkeley, and Lawrence Livermore National Laboratory (LLNL) to investigate the effects of age on semen quality and genetic damage in sperm. The study group was composed of 97 healthy male volunteers employed by or retired from LLNL in Livermore, Calif. LLNL was selected as the recruitment site because it is made up of a relatively homogeneous workforce and has an on-site semen-analysis laboratory. Healthy males between 20 and 80 years of age were recruited from advertisements posted at the laboratory, e-mail listings, and in newsletters. At least 15 men were enrolled from each decade of age up to age 60 years, and at least 15 men were enrolled over age 60 years, to assure that there would be a sufficient number of participants across the age spectrum. Study procedures received Institutional Review Board approval and all participants gave written informed consent.

Men were excluded if they had smoked cigarettes in the last 6 months, had current fertility or reproductive problems, had a vasectomy or a history of an undescended testicle or prostate cancer, had received chemotherapy or radiation treatments for cancer, or ever had a previous semen analysis with zero sperm count. Men who, on the day of screening, had a fever over 101°F were scheduled for enrollment 3 months later.

Eligible men were mailed a questionnaire and a semen collection container with instructions. The men were instructed to abstain from ejaculation for at least 2 days before sample collection and to collect the specimen by masturbation. Men were instructed to either mail the completed questionnaire to the University of California, Berkeley, or to deliver it with the semen sample to a confidential drop box at LLNL. After the questionnaire was returned, it was reviewed with the participant over the phone for completeness and accuracy. The self-administered questionnaire ascertained sociodemographic characteristics, reproductive history (including the number and outcomes of the pregnancies they fathered), medical history (including a family history of genetic diseases), and lifestyle habits, such as caffeine, tobacco, and alcohol consumption. The questionnaire also included a 100-item Modified Block Food Frequency Questionnaire (FFQ) (Huang et al, 2002). This FFQ estimates average daily nutrient intake, including total calories, protein, fat, carbohydrate, vitamins, and micronutrients, based on questions about frequency and portion size of a given food and usual eating habits over the past year. The FFQ took about 30 minutes to complete. Out of 97 men, 1 participant, whose nutrient analysis indicated intake of too few foods and calories per day (659 kcal/d), was excluded from the data analysis.

Sperm Chromatin Structure Assay

Numerically coded semen specimens were delivered within 2 hours of collection to the laboratory at LLNL for analysis. Conventional semen analyses (including sperm motility, count, and semen volume) were performed immediately upon receipt of the sample and aliquots of the remaining semen were stored at −80°C. Frozen samples were express mailed on dry ice to South Dakota State University for analysis of DNA fragmentation using the sperm chromatin structure assay (SCSA®) (Evenson et al, 1991, 1999). Laboratory technicians were blinded to the age, dietary intake, and identity of the participants. Upon receipt, each semen sample was placed in a 37°C water bath until thawed and then immediately placed on ice (4°C). An aliquot of thawed semen was diluted in TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA; pH 7.4) to 1–2 × 10^6 sperm/mL, and 0.20-mL aliquots of diluted samples were mixed with 0.40 mL of an acid-detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% Triton-X 100; pH 1.2). After 30 seconds, the cells were stained by adding 1.20 mL acridine orange (AO) staining solution containing 6.0 μg AO (chromatographically purified, Polysciences Inc, Warrington, Pa, cat. # 04539) per mL buffer (0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011 M EDTA [di-sodium], 0.15 M NaCl; pH 6.0) (Darzynkiewicz et al, 1976; Evenson et al, 1985). Three minutes after the staining procedure started, fluorescence measurements were saved to a computer list mode file. Flow-cytometer measurements were made on 5000 cells per sample. Two SCSA measurements were obtained for each sample, and the average of the two measurements was taken.

AO intercalates into sperm DNA so that double-stranded DNA fluoresces in green and single-stranded DNA fluoresces in red. The DFI, which is the amount of red fluorescence divided by the sum of red and green fluorescence, is an approximate measure of the proportion of single stranded to total DNA in each sperm. For each semen specimen, we generated mean (mean

DFI) and standard deviation (SD DFI) of the DFI distribution, expressed in channels of fluorescence; and the percent of cells with detectable DFI, or DFI more than 247 channels of fluorescence (%DFI, formerly known as COMPα_t). The percent of immature cells in a sample is represented by the high DNA stainability index (HDS). Immature sperm cells emit higher amounts of green fluorescence because they have less chromatin condensation, which allows for greater amounts of AO intercalation (higher stainability).

SCSA results could not be obtained from semen samples with no or low sperm counts or from samples collected after the initial shipment to South Dakota. Out of the 96 men with complete food-frequency data, the semen samples of 87 men were analyzed by SCSA.

Statistical Analysis

We evaluated four aspects of DFI: mean DFI, SD DFI, %DFI, and HDS. The main independent variables were vitamin C, vitamin E, and beta-carotene intake as well as a composite of antioxidant intake. Intake was based on the combination of reported diet and supplement use and was investigated as a continuous variable for each individual antioxidant and also as a categorical variable for each antioxidant and for the antioxidant composite variable. Dietary intake alone (not including supplement intake), as a continuous variable, was also investigated for the three antioxidants, as was a binary antioxidant supplement intake variable (yes/no—regular weekly intake of either vitamin C or E or beta-carotene).

For the categorical variables, intake was divided into low (less than 25th percentile), moderate (25th to 75th percentile), and high (greater than 75th percentile). Quartiles were created using all 96 participants in the AGES study with complete FFQ data, although only 87 men were included in the current analyses. This was done so that each individual remains in the same category in all AGES study analyses, even when fewer than 96 men can be included. The three intake groups were included as dummy variables into statistical models, with low intake as baseline, and were also investigated as ordered variables (ie, 1 = low intake, 2 = moderate, 3 = high) to obtain a *P*-value for trend. All *P* values presented are two-sided.

An antioxidant composite variable was created to obtain a combined measure of dietary and supplementary antioxidant intake from vitamins C and E and beta-carotene. Because these antioxidants differ in their units (vitamin C was measured in milligrams [mg], vitamin E in milligrams alpha-Tocopherol equivalents [mg αTE], and beta carotene in micrograms [μg]), we could not combine intake. Instead, low antioxidant composite intake was defined as at least two low intake rankings of the three antioxidants; high antioxidant composite intake was defined as at least two high intake rankings of the three antioxidants; and moderate intake was defined as all other combinations of intake.

Separate multiple linear regression models were constructed to examine the relationship of intake of each antioxidant and the antioxidant composite variable with each DFI parameter. Mean DFI, %DFI, and HDS were log transformed to better fit the model assumptions. Final regression results are expressed as adjusted means and 95% confidence intervals (CI) and are back-transformed for the log-transformed DFI parameters. Logistic regression was also performed to examine the relationship between intake of each antioxidant and having a DFI value considered not compatible with good fertility: more than or equal to 300 channels of fluorescence for mean DFI, more than or equal to 200 channels of fluorescence for SD DFI, and more than or equal to 30% for %DFI (Larson et al, 2000). HDS was not included in this analysis because there were only 7 participants with HDS above the abnormal value of 15%.

All models included age and duration of abstinence (days) before semen collection as independent variables. We also considered as potential covariates in regression models: body mass index; ethnicity; history of tobacco use; alcohol intake; caffeine intake; history of working with occupational chemicals, radiation, or radioisotopes (as measured from LLNL dosimetry records); season in which semen collection occurred; hot-tub use during the last 3 months; time spent bicycling in the last 3 months; history of chronic diseases, including 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. Covariates were considered for the final model if they were significant at $P < .1$ or if they changed the coefficient on nutrient intake by greater than 10%; however, in every model, only age and duration of abstinence meet these criteria. Final models were considered with and without adjusting for total kilocalorie intake per day to examine relative as well as absolute intake. Results are presented without adjusting for total kilocalorie intake because the inclusion of this variable did not change results. Interactions between age and antioxidant intake, age and abstinence, and between antioxidants and abstinence were evaluated but not found to be significant at $P < .1$ in any of the models and are not presented. All analyses were conducted using STATA 7.0 (1999).

Results

The 87 men included in this analysis were predominately white (92%), highly educated (53% had postgraduate education), and had never smoked cigarettes regularly (79%). They ranged in age from 22 to 80 years (mean = 44; SD = 15). Duration of abstinence ranged from 2 to 15 days (mean = 4.7; SD = 2.7). Table 1 summarizes selected sociodemographic characteristics, lifestyle habits, and medical history of the study population by antioxidant composite intake.

Forty-four percent of the study population (38 men) took a vitamin supplement regularly (more than once a week) in the past year. Twenty percent (18 men) consumed less than 90 mg vitamin C per day (the recommended daily allowance [RDA] for men) (Institute of Medicine, 2000) and 44% (38 men) consumed less than the RDA of 15 mg αTE vitamin E per day (Institute of Medicine, 2000). There is no RDA for beta-carotene, but the Institute of Medicine recommends that 3 to 6 mg of beta-carotene daily will maintain plasma beta-carotene

Table 1. *Selected sociodemographic characteristics, lifestyle habits, and medical history of study population, by antioxidant composite intake (N = 87). The Age and Genetic Effects in Sperm Study, Livermore, Calif, 1997–1998*

Characteristic	Total (n = 87)	Composite Index of Antioxidant Intake*		
		Low (n = 19)	Moderate (n = 50)	High (n = 18)
Age of subject				
Mean ± SD	44 ± 15	43 ± 16	42 ± 14	50 ± 13
Ethnic group, N (%)				
White	80 (92.0)	16 (84.2)	47 (94.0)	17 (94.4)
Hispanic	2 (2.3)	1 (5.3)	1 (2.0)	0 (0.0)
Asian	5 (5.8)	2 (10.5)	2 (4.0)	1 (5.6)
Education completed, N (%)				
High-school graduate	4 (4.6)	1 (5.3)	2 (4.0)	1 (5.6)
Some college/vocational school	13 (14.9)	4 (21.0)	7 (14.0)	2 (11.1)
College graduate	24 (27.6)	6 (31.6)	15 (30.0)	3 (16.7)
Graduate school	46 (52.9)	8 (42.1)	26 (52.0)	12 (66.7)
Ever drank alcohol regularly, N (%)				
No	28 (32.2)	8 (42.1)	15 (30.0)	5 (27.8)
Yes	59 (67.8)	11 (57.9)	35 (70.0)	13 (72.2)
Ever smoked cigarettes regularly, N (%)				
No	69 (79.3)	13 (68.4)	42 (84.0)	14 (77.8)
Yes	18 (20.7)	6 (31.6)	8 (16.0)	4 (22.2)
History of high blood pressure, N (%)†				
No	74 (85.1)	13 (68.4)	47 (94.0)	14 (77.8)
Yes	13 (14.9)	6 (31.6)	3 (6.0)	4 (22.2)
History of cancer, N (%)				
No	83 (95.4)	18 (94.7)	47 (94.0)	18 (100.0)
Yes	4 (4.6)	1 (5.3)	3 (6.0)	0 (0.0)
Body mass index, N (%)				
≤25	44 (50.6)	10 (52.6)	21 (42.0)	13 (72.2)
>25	43 (49.4)	9 (47.4)	29 (58.0)	5 (27.8)
Abstinence, N (%)				
≤5 days	67 (77.0)	12 (63.2)	40 (80.0)	15 (83.3)
>5 days	20 (23.0)	7 (36.8)	10 (20.0)	3 (16.7)
Ever fathered any pregnancy, N (%)				
No	31 (35.6)	7 (36.8)	19 (38.0)	5 (27.8)
Yes	56 (64.4)	12 (63.2)	31 (62.0)	13 (72.2)
Regular single or multivitamin use, N (%)†				
No	38 (43.7)	18 (94.7)	20 (40.0)	0 (0.0)
Yes	49 (56.3)	1 (5.3)	30 (60.0)	18 (100.0)
Kilocalorie intake per day				
Mean (SD)	1745 (505)	1598 (432)	1803 (519)	1740 (532)

† *P* value for characteristic and antioxidant intake < .05.
* Low antioxidant composite intake was defined as at least 2 low-intake rankings of the 3 antioxidants; high antioxidant composite intake was defined as at least 2 high-intake rankings of the 3 antioxidants; and moderate intake was defined as all other combinations of intake.

levels in the range associated with a lower risk of chronic diseases (NIH, 2001). Fifty-six percent (49 men) consumed less than 3 mg beta-carotene per day.

Mean DFI, SD DFI, and %DFI are highly correlated (r = .86 to .97). HDS is not well correlated with the other 3 DFI outcomes (r = −.15 to .14). More than 25% of the study population had mean DFI, SD DFI, and %DFI values at levels that have been correlated with decreased fertility: 26 men (30%) had mean DFI greater than or equal 300 fluorescence channel units, 42 men (48%) had SD DFI greater than or equal to 200 fluorescence channel units, 30 men (35%) had greater than or equal 30% of %DFI. Only seven men (8%) had greater than or equal 15% HDS.

History of cancer, history of smoking, older age, and longer duration of abstinence were significantly (P < .1)

Table 2. *Means (95% confidence interval) of various measures of sperm DNA fragmentation by level of antioxidant intake, adjusted for age and abstinence (N = 87). The Age and Genetic Effects in Sperm study, Livermore, Calif, 1997–1998*

	Intake Range*	Mean DFI	SD DFI	%DFI	High DNA Stainability
Beta-carotene					
Low	401–1167 mcg	250.7 (227.9, 275.9)	180.5 (160.9, 200.0)	20.0 (16.0, 24.9)	5.0 (3.9, 6.4)
Moderate	1263–3942 mcg	264.9 (247.6, 283.4)	†206.7 (192.9, 220.5)	21.2 (18.2, 24.8)	†6.9 (5.8, 8.2)
High	3973–33444 mcg	253.8 (230.8, 279.1)	190.6 (171.2, 210.0)	19.8 (15.9, 24.7)	5.0 (3.9, 6.4)
P for trend		0.87	0.49	0.96	0.98
Vitamin C					
Low	26–99 mg	259.6 (236.7, 284.7)	190.9 (171.7, 210.1)	22.3 (18.0, 27.5)	5.1 (4.0, 6.5)
Moderate	107–400 mg	254.0 (237.4, 271.7)	193.0 (179.0, 207.0)	18.8 (16.1, 21.9)	6.5 (5.4, 7.8)
High	460–3369 mg	267.3 (241.8, 295.4)	208.5 (187.7, 229.3)	22.9 (18.2, 28.8)	5.6 (4.3, 7.3)
P for trend		0.70	0.23	0.94	0.53
Vitamin E					
Low	2–10 αTE	253.4 (230.5, 278.6)	189.0 (169.2, 208.8)	20.4 (16.4, 25.5)	5.8 (4.5, 7.4)
Moderate	10–118 αTE	263.2 (246.2, 281.5)	199.4 (185.3, 213.4)	20.9 (17.9, 24.4)	6.1 (5.1, 7.3)
High	142–833 αTE	253.9 (230.0, 280.2)	196.3 (175.6, 216.9)	20.0 (15.9, 25.1)	5.6 (4.3, 7.3)
P for trend		0.95	0.60	0.89	0.90
Antioxidant composite					
Low		253.4 (228.9, 280.6)	184.4 (163.2, 205.6)	21.4 (16.9, 27.1)	5.1 (3.9, 6.7)
Moderate		263.3 (247.2, 280.4)	201.4 (188.4, 214.5)	20.6 (17.8, 23.8)	6.4 (5.4, 7.6)
High		250.6 (225.2, 278.9)	193.1 (170.9, 215.4)	19.7 (15.4, 25.2)	5.4 (4.1, 7.2)
P for trend		0.92	0.54	0.63	0.72

* mg αTE, milligrams alpha-Tocopherol equivalents (see ''Materials and Methods'' for definitions); DFI, DNA fragmentation index. %DFI, percentage of cells with DFI greater than 247 channels of fluorescence.

† $P < .05$ for moderate versus low intake.

associated with increased mean DFI, SD DFI, and %DFI; however, history of cancer and smoking were highly correlated with age and were not associated with increased DFI after adjusting for age. Increased abstinence was associated with decreased HDS ($P < .1$).

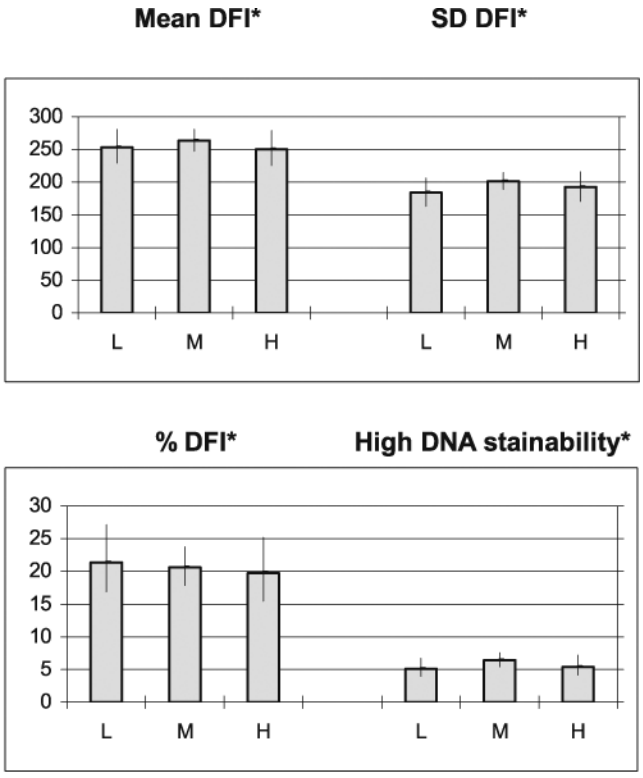
Crude and adjusted linear regression of DFI outcomes on antioxidant intake revealed no statistically significant dose-response association between any antioxidant intake measure and any DFI outcome (adjusted results shown in Table 2 and Figure). The only significant difference was that those participants with moderate intake of beta-carotene (1263–3942 μg) had an increase in SD DFI compared with participants with low intake of beta-carotene (adjusted means = 206.7 and 180.5, respectively, $P = .03$). In addition, after controlling for covariates, moderate beta-carotene intake was associated with increase in the percent of immature sperm (as measured by HDS) compared with low beta-carotene intake (adjusted means = 6.9% and 5.0%, respectively, $P = .04$). No significant difference in SD DFI or HDS was found between participants with high beta-carotene intake and participants with either moderate or low intake. The partial R^2 for nutrient intake variables in all adjusted models remained close to zero, indicating that antioxidant intake was not a strong predictor of damage to chromatin structure.

Logistic regression examining the association of antioxidant intake and DFI values above and below those related to poorer fertility revealed results similar to those obtained by linear regression (data not shown). There was no statistically significant association between any antioxidant intake and any DFI outcome, except for an increased risk of SD DFI greater than 200 channels of fluorescence for participants with moderate beta-carotene intake as compared with participants with low beta-carotene intake (odds ratio adjusted for age and duration of abstinence = 6.4, 95% CI = 1.3, 32.3).

Investigation of antioxidant intake based on diet alone (without supplements) or of supplement use alone revealed no significant associations with DFI outcomes.

Discussion

Our study did not detect any association between increased antioxidant intake (vitamin C, vitamin E, beta-carotene, or their composite) and improved sperm chromatin integrity as measured by the DNA fragmentation index. There is a suggestion of a non-dose–related association between beta-carotene and two DFI measurements (SD DFI and HDS), with the group with moderate intake having the poorest sperm chromatin integrity. We cannot rule out the possibility that this finding was due to chance. Although it has been suggested (Omenn et al, 1996; Murata and Kawanishi, 2000) that high levels of beta-carotene



Adjusted means and 95% confidence intervals of DNA Fragmentation Index (DFI) outcomes by antioxidant composite intake (L indicates low; M, moderate; H, high), holding age and abstinence constant at their means (N = 87). The AGES study, Livermore, Calif, 1997–1998. *Mean DFI and SD DFI are expressed in fluorescence channel units. %DFI and high DNA stainability are percentages.

could induce oxidative DNA damage, this is unlikely to explain our results because serum beta-carotene levels from human intervention trials may not be high enough to induce damage (Kleinjans et al, 2004). Furthermore, it would not explain why the moderate intake group showed the poorest sperm chromatin integrity.

The lack of a dose association between antioxidant intake and sperm chromatin integrity differs from the results of studies of sperm treated in vitro (Hughes et al, 1998; Lopes et al, 1998) with the antioxidants cysteine, glutathione, and urate. This discrepancy may be because the antioxidants used in the previous studies had a stronger antioxidant activity than vitamins C and E and beta-carotene or because they were added directly to the semen samples.

We assessed antioxidant intake from a FFQ. However, responses on a FFQ may not reflect the concentration of antioxidants in testicular, epididymal, or seminal fluids. If levels in biological fluid more accurately reflect the relevant biological dose, then a direct measure of antioxidants in seminal fluid may be more appropriate. However, a single measurement may not accurately reflect the relevant antioxidant exposures during the entire period of spermatogenesis, and these measures may reflect factors other than dietary intake, such as individual differences in nutrient absorption and metabolism and other sources of physiologic variation (Willett, 1998). The advantage of the FFQ is that it can ascertain usual intake over a period of time. In this study, men were asked to report usual intake in the past year. Although dietary intake in the prior 3 months would more accurately reflect antioxidant exposure during the period of spermatogenesis, diet is fairly consistent over time and reports usually reflect the most recent diet (Willett, 1998).

This study had some limitations. First, the FFQ questionnaire used had not been validated in men, but used previously only in studies of women's health (Huang et al, 2002). Second, only one semen sample was taken from each man. While several sperm samples collected over 1–2 weeks may have been preferable, it may have reduced the number of men who would have participated and thus reduced the power of our study. Even so, the sample size obtained may have been insufficient to detect small changes among the antioxidant intake groups.

AGES is the first study to investigate the effects of antioxidant intake on sperm chromatin integrity. Although antioxidant intake in this study was not related to improved sperm chromatin structure, we recently reported in the same study population that higher antioxidant consumption was associated with better semen quality, including greater sperm numbers and motility (Eskenazi et al, 2005). The AGES study population was composed of healthy volunteers from a homogeneous setting and excluded smokers and men with major reproductive problems. Although findings in healthy volunteers may be generalizable to the general population of healthy men, they may not be generalizeable to clinic-based infertile populations. Thus, the results of this study do not preclude possible beneficial effects of high antioxidant supplementation on sperm chromatin integrity for men with fertility problems.

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