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The association of folate, zinc and antioxidant intake with sperm aneuploidy in healthy non-smoking men

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BACKGROUND: Little is known about the effect of paternal nutrition on aneuploidy in sperm. We investigated the association of normal dietary and supplement intake of folate, zinc and antioxidants (vitamin C, vitamin E and β-carotene) with the frequency of aneuploidy in human sperm. METHODS: Sperm samples from 89 healthy, non-smoking men from a non-clinical setting were analysed for aneuploidy using fluorescent *in situ* hybridization with probes for chromosomes X, Y and 21. Daily total intake (diet and supplements) for zinc, folate, vitamin C, vitamin E and β-carotene was derived from a food frequency questionnaire. Potential confounders were obtained from a self-administered questionnaire. RESULTS: After adjusting for covariates, men with high folate intake (>75th percentile) had lower frequencies of sperm with disomies X, 21, sex nullisomy, and a lower aggregate measure of sperm aneuploidy ($P \leq 0.04$) compared with men with lower intake. In adjusted continuous analyses, total folate intake was inversely associated with aggregate sperm aneuploidy (−3.6% change/100 μg folate; 95% CI: −6.3, −0. 8) and results were similar for disomies X, 21 and sex nullisomy. No consistent associations were found between antioxidant or zinc intakes and sperm aneuploidy. CONCLUSIONS: Men with high folate intake had lower overall frequencies of several types of aneuploid sperm.

Keywords: aneuploidy; antioxidants; sperm chromosomes; folate; zinc

Introduction

Aneuploidy in humans occurs in at least 5% of all clinically recognized pregnancies and may account for over one-third of all spontaneous abortions (Hassold and Hunt, 2001). There is a high paternal contribution for sex chromosome aneuploidies, which are the most frequent numerical chromosomal abnormalities in humans at birth (Hecht and Hecht, 1987; Hassold, 1998). The probability of having a child with paternally transmitted aneuploidy may be related to the frequency of abnormal sperm in the father (Eskenazi *et al*., 2002). Based on fluorescent *in situ* hybridization (FISH) methods of sperm analysis, it is estimated that 1–4% of a healthy male's sperm have some type of aneuploidy, but there is high inter-individual variation (Downie *et al*., 1997; Guttenbach *et al*., 1997; Rubes *et al*., 2005).

The mechanism for aneuploidy in human sperm cells remains poorly understood. Chemotherapy treatment and exposure to certain pesticides, including organophosphates, carbaryl and fenvalerate, have been associated with higher frequency of aneuploidy in human sperm (Robbins *et al*., 1997a; Martin *et al*., 1999; Padungtod *et al*., 1999; Perreault *et al*., 2000; Wyrobek *et al*., 2000; Baumgartner *et al*., 2001; Recio *et al*., 2001; Xu *et al*., 2003; Xia *et al*., 2004; Wyrobek *et al*., 2005a,b; Xia *et al*., 2005), but little is known about the underlying host factors that affect sperm aneuploidy (Morel *et al*., 1997; Hassold and Hunt, 2001; Robbins *et al*., 2005; Sherman *et al*., 2005). There are inconsistent associations of human sperm aneuploidy with paternal age (Griffin *et al*., 1995; Martin *et al*., 1995; Robbins *et al*., 1995; Kinakin *et al*., 1997; Guttenbach *et al*., 2000; Lowe *et al*., 2001; Luetjens *et al*., 2002; Rives *et al*., 2002; Bosch *et al*., 2003; Dakouane *et al*., 2005) and with lifestyle factors such as cigarette, alcohol and caffeine use (Robbins *et al*., 1997b; Rubes *et al*., 1998; Shi *et al*., 2001; Robbins *et al*., 2005). In 2006, we reported no evidence of a paternal age effect on aneuploidy in human sperm in a non-clinical study population (Wyrobek *et al*., 2006).

The role of nutrition, specifically micronutrient intake, remains unexamined as a factor in sperm aneuploidy; however, there is substantial evidence of the role of micronutrients, such as zinc, folate and antioxidants, in the maintenance of normal spermatogenesis and sperm maturation as well as in DNA metabolism, synthesis, repair and transcription (Ames *et al*., 1993; Institute of Medicine, 2000a; Eggert-Kruse

et al., 2002; Fenech, 2002; Wong *et al*., 2002; Wang *et al*., 2004; Ebisch *et al*., 2007). Additionally, dietary supplementation of vitamin C and vitamin E has been associated with reduced aneuploidy in oocytes from mice (Tarin *et al*., 1998). Zinc deficiency has been associated with increased DNA strand damage in sperm of rats (Evenson *et al*., 1993). Folate deficiency has been shown to increase DNA strand breaks (Pogribny *et al*., 1995), DNA hypomethylation (Pogribny *et al*., 1997), chromosomal instability and aneuploidy (Wang *et al*., 2004; Beetstra *et al*., 2005) in lymphocytes. Decreased folate metabolism in mothers has also been associated with increased risk of having an infant with trisomy 21 (Down syndrome) (James *et al*., 1999; Hobbs *et al*., 2000; O'Leary *et al*., 2002; Bosco *et al*., 2003; Takamura *et al*., 2004; da Silva *et al*., 2005); however, the effects of folate and other micronutrients on paternally transmitted aneuploidies remain virtually unknown.

The purpose of the present analysis was to determine whether normal dietary and supplemental intake of zinc, folate, vitamin C, vitamin E and β-carotene is associated with the frequency of sperm aneuploidy in a population of healthy non-smoking men (Wyrobek *et al*., 2006). In this population, we previously reported positive associations between antioxidant intake (vitamin C, vitamin E and β-carotene) and semen quality, especially for motility (Eskenazi *et al*., 2005), but no association between antioxidant intake and DNA fragmentation, as measured by the sperm chromatin structure assay (Silver *et al*., 2005).

Materials and Methods

Our study population consisted of 97 healthy male volunteers, employed or retired from a university research laboratory, who were recruited as part of the Age and Genetic Damage in Sperm (AGES) study (Eskenazi *et al*., 2003; Wyrobek *et al*., 2006). To ensure a broad age distribution, at least 15 men were enrolled from each age decade from 20 to 70 years. In addition, we enrolled eight men over age 70 years. Exclusion criteria included: cigarette smoking in the last 6 months; current reproductive or fertility problems; history of vasectomy, undescended testicle or prostate cancer; previous semen analysis with zero sperm count; or chemotherapy or radiation treatments for any type of cancer. Information on volunteers who were excluded or refused to participate has been published previously (Eskenazi *et al*., 2003; Schmid *et al*., 2007). Study procedures received Institutional Review Board approval at the University of California, Berkeley, and at Lawrence Livermore National Laboratory, and all participants gave written informed consent.

Each participant was mailed a study questionnaire along with semen collection instructions, a sterile container and a protective thermos (Eskenazi *et al*., 2005; Silver *et al*., 2005). The self-administered questionnaire asked about sociodemographic characteristics, reproductive history, medical history and lifestyle habits. Also included was a 100-item Modified Block Food Frequency Questionnaire (FFQ) (Huang *et al*., 2002) to estimate average daily dietary and supplemental intake. The FFQ assessed the frequency and serving size of major food types and vitamin supplements over the previous year, including specific intake information for: multivitamins (i.e. Regular One-a-Day, Centrum or therapeutic type); antioxidant combination vitamins; vitamins A, C, and E; β-carotene; calcium; iron; zinc; and selenium. 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.

Participants were instructed to provide a semen sample by masturbation after abstaining from ejaculation for 2 to 7 days. Semen containers were placed into an insulated thermos and delivered anonymously to a drop-box at the analysis laboratory within 2 h of collection. Conventional semen quality analyses were performed immediately upon receipt and aliquots of semen were transferred into eppendorf vials and stored frozen at $-80^{\circ}C$ for later analyses by sperm FISH (Wyrobek *et al*., 2006).

Sperm FISH aneuploidy

Sperm aneuploidy and diploidy were measured by multicolor FISH as previously described (Wyrobek *et al*., 2005a,2006). Briefly, frozen semen aliquots were thawed at room temperature and 5 μl of semen was smeared onto ethanol-cleaned glass microscope slides and allowed to air-dry overnight. Decondensation of sperm nuclei was achieved using the dithiothreitol/3,5-diiodosalicylic acid pretreatment protocol as previously described (Robbins *et al*., 1995). The following mix of labeled probes was used for detection of chromosomes X, Y and 21: a mix of SpectrumGreen and SpectrumOrange labeled CEP X for chromosome X (Vysis, Downers Grove, IL, USA), a FITC-labeled probe for the DYZ1 locus of chromosome Y (Oncor, Gaithersburg, MD, USA) and a locus-specific probe that hybridizes to chromosome 21, q22.13–q22.2 (LSI^{TM}) labeled with Spectrum Orange^{TM} (Vysis). Using our probe combinations, normal sperm were scored as 21-X (i.e. containing one red and one yellow domain) or 21-Y (i.e. containing one red and one green domain). Strict microscope scoring criteria were applied to differentiate between normal and abnormal sperm (Robbins *et al*., 1997b; Baumgartner *et al*., 1999).

FISH slides were analysed for aneuploidy using 5000 cells from 2 different regions of the hybridization area, for a total of 10 000 sperm per donor. The aneuploidy counts of the two scoring regions had to match statistically by chi-square analysis to be acceptable. Two technicians scored the entire study population, analysing a total of 951 049 sperm nuclei. Only one technician usually scored each slide; although for quality control purposes, six slides were scored by both technicians. No substantial differences were found in scoring criteria between technicians.

The sperm FISH aneuploidy results for this study population have previously been published (Wyrobek *et al*., 2006). We examined five types of sperm aneuploidy in these analyses: XY sperm (sperm FISH genotype: X-Y-21), disomy X (X-X-21), disomy Y (Y-Y-21), sex nullisomy (21-0) and disomy 21 (X-21-21 or Y-21-21), which are associated with the risk of fathering offspring with Klinefelter, triple X, XYY, Turner and Down syndromes, respectively. We also calculated a summary measure of these aneuploidies: aggregate aneuploidy (sum of XY sperm, 21 nullisomy, sex nullisomy and disomies for chromosomes X, Y and 21). The aneuploidy variables are expressed as frequencies per 10 000 sperm.

Statistical analysis

Of the 97 AGES study participants, we excluded 7 participants with azoospermia or low sperm counts insufficient for analysis by sperm FISH and 1 subject with implausible FFQ data (<700 calories/day), leaving a total of 89 men. Calculations were performed using STATA release 8.0 (STATA Corporation, College Station, TX, USA).

Total micronutrient intake, i.e. dietary intake plus supplement intake, was examined both categorically and continuously for vitamin C, vitamin E, β-carotene, folate and zinc. Intake was divided into three categories: low intake (<25th percentile), moderate intake (25–75th percentile) and high intake (>75th percentile). The intake categories were created using all 96 AGES participants with complete FFQ data so that each participant remained in the same intake category for all subsequent analyses (Eskenazi *et al*., 2005; Silver *et al*., 2005). An antioxidant summary variable was created *a priori* as described previously based on intake categories for vitamin C, vitamin E and β-carotene. The antioxidant summary variable consists of three categories: low (low intake for at least two of the antioxidants), high (high intake for at least two of the antioxidants) and moderate (all other combinations of intake) (Eskenazi *et al*., 2005; Silver *et al*., 2005).

The relationship between micronutrient intake categories and each type of sperm aneuploidy was examined using negative binomial regression modeling. Negative binomial regression is used for sperm aneuploidy because it is count data with a greater variance, or dispersion, than a traditional Poisson distribution. For each type of sperm aneuploidy, we constructed negative binomial regression models with the continuous aneuploidy outcome as the dependent variable and the micronutrient categories and potential confounders as the independent variables. Micronutrient categories were included as dummy variables in the regression models, with low intake as the reference group. The following covariates were evaluated as potential confounders: age; duration of abstinence (days); body mass index (BMI); ever use of tobacco, alcohol, tea or caffeine (yes/no); total daily kilocalorie intake; history of chronic diseases (includes high blood pressure, heart disease, diabetes, thyroid problems, ulcers, stomach problems, hepatitis, liver disease, epilepsy and neurological problems), urogenital infections, mumps or prostate enlargement (yes/no); season of semen collection (fall, winter and spring/summer); scoring technician (scorer 1 or 2); history of working with occupational chemicals, radiation or radioisotopes (yes/no); and occupational exposures (as measured from LLNL dosimetry records). Covariates that were significant at $P < 0.2$ in non-parametric univariate analyses (Mann–Whitney two-sample statistic and the Kruskal–Wallis rank test) or that were of biologic interest (i.e. duration of abstinence) were investigated in the multivariate models.

Final regression models were constructed using a stepwise backward elimination process. Covariates remained in the final models if they changed the micronutrient coefficients by more than 10% or had a *P*-value ≤0.1. We checked the fit of the final models using residual versus fitted plots and quantile–quantile plots. Men who were outliers for any aneuploidy category (>3.5 SD in adjusted models) were dropped from the final models if their inclusion significantly affected the regression results and model fits. Results are expressed as adjusted means and standard errors (SEs) for each intake category. Covariates were set at their mean value when calculating the adjusted means. After the final models were completed, we used ordered nutrient intake variables (i.e. 1, low intake; 2, moderate; 3, high) to obtain *P*-values for trend. All *P*-values presented are two-sided.

In addition, we also used negative binomial regression modeling to evaluate the relationship between aneuploidy and micronutrient intake using continuous intake variables. These analyses were performed for total intake (diet + supplements) of vitamin C, vitamin E, β-carotene, zinc and folate, as well as for dietary intake only of the same micronutrients in a subset of non-supplement users ($n = 38$). Continuous regression models were adjusted for the same covariates that were used in the corresponding categorical model. Results from continuous models are expressed as a percent change in aneuploidy per daily micronutrient intake calculated from the antilog of the regression coefficient [(antilog $\beta - 1) \times 100$)]. The adjusted-regression graph has covariates set to their mean values.

Results

Sociodemographic and other characteristics of the men in our study are presented in Table I. The median age was 42 years (range 22–80) and the median duration of abstinence was 4 days (range 2–20 days). The men were predominantly white (91%), highly educated (53% with post-graduate education), vitamin supplement users (57%) and had never used tobacco (73%).

As shown in Table II, the median daily total intake (diet + supplements) was 162 mg for vitamin C, 23.7 mg α-tocopherol equivalents (αTE) for vitamin E, 2586 μg for β-carotene, 475 μg for folate and 12.3 mg for zinc. The median daily total nutrient values in our study are slightly higher than values from men ages 20–70 years in a nationally representative sample measured about the same time period (1997–1998) with a similar FFQ (Subar *et al*., 2001), especially for vitamins C and E (162 mg versus 101 mg, 24 mg versus 9.0 mg, respectively). Dietary-only values were similar to men of all ages from the National Health and Nutrition Examination Survey (NHANES), 1999–2000 (Wright *et al*., 2003; Ervin *et al*., 2004). The percent of men consuming less than the recommended dietary allowance (RDA) (Institute of Medicine,

Table I. Characteristics of the AGES study population ($n = 89$).

Age of subject (years)	
Mean ± SD	44.7 ± 15.1
Abstinence (days)	
Mean ± SD	4.9 ± 3.1
Daily kilocalorie intake	
Mean ± SD	1733 ± 507.4
Body mass index $(kg/m)^2$	
Mean ± SD	25.0 ± 3.2
Vitamin supplement use in last year, *n* (%)	
No	38 (43)
Yes	51 (57)
Ever tobacco use[a], *n* (%)	
No	65 (73)
Yes	24 (27)
Alcohol use in the last 3 months, *n* (%)	
No	30 (34)
Yes	59 (66)
Season of sample collection, *n* (%)	
Fall	39 (44)
Winter	29 (32)
Spring/summer	21 (24)
Prior infections of urinary tract or reproductive system[b], *n* (%)	
No	66 (72)
Yes	23 (28)
Work hazard in the last 3 months[c], *n* (%)	
No	73 (80)
Yes	16 (20)

[a]Individuals who have ever used cigarettes, cigars, pipes, chew or snuff. All participants were current non-cigarette smokers; two participants were current cigar or pipe smokers. [b]Individuals who have ever had infections of the kidney, bladder, urinary tract, prostate or seminal vesicles; sexually transmitted diseases, herpes, abnormal dishcarge from penis, urethritis, painful or swollen testes. [c]Individuals who worked with any toxic chemical or radioactive agent during the last 3 months.

Table II. Self-reported micronutrient intake from diet and supplements measured from the FFQ ($n = 89$).

Micronutrient	Median	Minimum	Maximum	US median[a]	RDA[b]	% of men below RDA
Dietary intake only						
Vitamin C (mg)	99	26	291	70	90	39%
Vitamin E[c] (mg)	8.2	2.2	55.0	7.9	15	84%
β-Carotene (μg)	1634	215	15 581	N/A[d]	—	—
Folate (μg)	349	115	1150	356	400	64%
Zinc (mg)	8.8	3.8	28.8	11.5	11	79%
Total intake (diet + supplements)						
Vitamin C (mg)	162	26	3394	101	90	20%
Vitamin E[c] (mg)	23.7	2.2	833	9.0	15	43%
β-Carotene (μg)	2586	401	33 444	N/A[d]	—	—
Folate (μg)	475	115	1150	N/A[d]	400	42%
Zinc (mg)	12.3	3.8	74.0	11.4	11	47%

[a]Median values taken from nationally representative samples. For dietary intake only, data come from the National Health and Nutrition Examination Survey (NHANES, 1999–2000), $n = 4206$ men of all ages. Diet measured using one 24-h recall (Wright *et al*., 2003; Ervin *et al*., 2004). For total intake, data come from the Eating at America's Table study (EATS, 1998–1999), $n = 226$ men ages 20–70 years. Diet measured using the 1995 Block FFQ (Subar *et al*., 2001). [b]Recommended dietary allowance (RDA) values from Dietary Reference Intakes developed by the The Institute of Medicine (Institute of Medicine 2000a,b, 2002). No RDA is available for β-carotene. [c]Vitamin E presented as alpha tocopherol equivalents (αTE). [d]β-Carotene and folate were not reported for the EATS study. NHANES reported carotenes, which included β-carotene and other provitamin-A carotenoids.

2000a–2002) for each micronutrient ranged from 20% (vitamin C) to 47% (zinc) for total intake and from 39% (vitamin C) to 84% (vitamin E) for dietary intake only (Table II). Total daily intake for vitamin C, vitamin E, β-carotene, zinc and folate were significantly inter-correlated ($r > 0.41$, $P < 0.001$ for all). Dietary and supplement intake of a micronutrient were not correlated except for vitamin C ($r = 0.30$, $P = 0.003$). Vitamin supplement use was significantly related to higher levels of total intake for each micronutrient ($P < 0.001$ for all, Mann–Whitney test), as expected. For example, the median total intake of vitamin C for supplement users was 331 mg and 94 mg for non-users. More than 90% of the men in the high intake groups for each micronutrient were supplement users, compared with less than 40% in the low intake groups.

Overall, total intake for each micronutrient was unrelated to age, lifestyle or medical conditions with a few notable exceptions. Higher total β-carotene intake was related to lower BMI, never having used tobacco and not consuming caffeine or alcohol in the last 3 months; higher total vitamin E intake was associated with lower BMI, current tea use and increased duration of abstinence; increased total folate intake was associated with current tea use and never having used tobacco ($P < 0.10$ for all; Supplemental Table I).

The unadjusted mean frequency and standard deviation (SD) of sperm aneuploidy per 10 000 sperm among the men in our study was 8.9 (7.4) for XY sperm, 3.0 (2.1) for disomy X, 5.0 (3.3) for disomy Y, 17.0 (11.3) for sex nullisomy, 9.5 (7.1) for disomy 21 (X or Y) and 52.7 (21.7) for aggregate aneuploidy. Inter-donor variation within categories of sperm aneuploidy ranged from 39 to 74%. There were significant inter-correlations among the aneuploidy types as previously reported (Wyrobek *et al*., 2006).

Table III summarizes the relationships of total micronutrient intake (diet + supplements) and sperm aneuploidy adjusting for potential confounders. High total folate intake was related to 18–30% lower frequencies of several types of aneuploidy compared with men with lower intake. Specifically, men with high folate intake had 18% lower frequency (albeit non-significantly) of sperm with sex nullisomy compared with men with moderate intake ($P = 0.12$) and 26% lower frequencies of sperm with sex nullisomy compared with men with low intake ($P = 0.04$) with a significant trend across intake categories ($P = 0.03$). Men with high folate intake also had ~30% lower frequencies of disomy X and disomy 21 compared with men with moderate intake ($P = 0.04$ and 0.003, respectively) with no significant test for trend ($P > 0.2$ for both) due to the low intake group having lower aneuploidy frequencies than the moderate intake group. Overall for the summary measure of aggregate aneuploidy, men with high folate intake had 19% lower sperm aneuploidy compared with men with moderate intake (45.6 versus 56.0, $P = 0.01$) and 20% lower aneuploidy compared with men with low intake (45.6 versus 57.0, $P = 0.02$), with a significant decreasing trend across intake categories ($P = 0.04$).

Men with high total zinc intake had 50% lower frequencies of disomy X than the moderate intake group ($P < 0.001$) and 39% lower frequencies than the low intake group ($P = 0.02$). There was a significant trend across the intake categories for disomy X ($P = 0.04$). Total zinc intake was not inversely related to any other type of sperm aneuploidy; although men with low total zinc intake had lower frequencies of sex nullisomy compared with the moderate intake group ($P = 0.03$).

There were no associations between total intake for antioxidants vitamin C, vitamin E or the antioxidant composite variable and any type of measured sperm aneuploidy; however, men who consumed high levels of total β-carotene had lower frequencies of disomy Y compared with men who consumed moderate (4.0 versus 5.5, $P = 0.03$) or low (4.0 versus 5.6, $P = 0.04$) levels, with a significant test for trend ($P = 0.04$).

Results from adjusted regression models using continuous total intake showed similar results for total folate intake as the categorical models, including significant or borderline significant results for aggregate aneuploidy, sex nullisomy and disomies X and 21. Figure 1 shows the adjusted negative binomial regression line and data points for the relationship of total

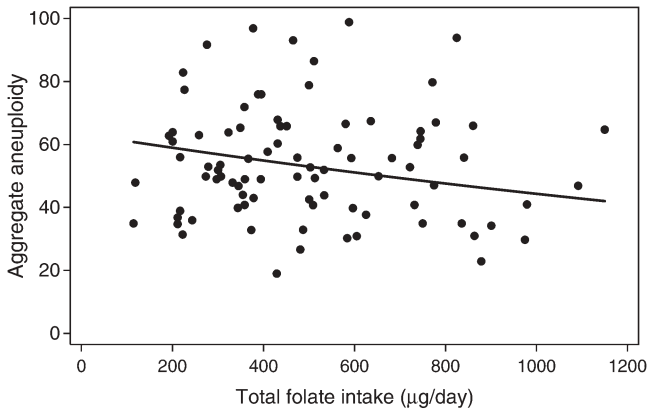

Figure 1: Individual data points are shown along with adjusted negative binomial regression lines for total folate intake and aggregate aneuploidy ($\beta = -0.004$, $P = 0.01$).
Covariates are held constant at their mean values. See Table III for covariates adjusted for in the final model. Aggregate aneuploidy is expressed as frequency per 10 000 sperm

folate intake and aggregate aneuploidy. After adjusting for covariates, we calculated a −3.6% decrease in aggregate aneuploidy per 100 μg increase in daily total folate intake (95% CI: −6.3, −0.8). Results were similar for total folate and disomy X (−6.2%/100 μg total folate, $P = 0.05$), disomy 21 (−4.6%/100 μg total folate, $P = 0.08$) and sex nullisomy (−4.2%/100 μg total folate, $P = 0.06$), and are not shown.

There was also a significant inverse association between continuous total β-carotene intake and disomy Y, similar to categorical models. After adjusting for covariates, there was a −2.8% decrease in disomy Y per 1000 μg increase in daily total β-carotene intake (95% CI: −4.8, −0.7). When five men who reported very high total β-carotene intake (>25 000 μg/day; >3.5 SD over the mean) were dropped from the analysis, the results become non-significant. We found no additional significant associations between any type of sperm aneuploidy and continuous total β-carotene, zinc, vitamin C or vitamin E intake (data not shown).

Limiting the continuous regression analysis to dietary intake only in non-supplement users ($n = 38$), there were suggestive inverse relationships ($P < 0.2$) for vitamin E intake and XY sperm (−1.6%/mg vitamin E, $P = 0.14$), disomy Y (−2.0%/mg vitamin E, $P = 0.12$), disomy 21 (−2.2%/mg vitamin E, $P = 0.06$), sex nullisomy (−1.6%/mg vitamin E, $P = 0.13$) and aggregate aneuploidy (−1.4%/mg vitamin E, $P = 0.05$). These relationships were driven by two men who consumed more than 30 mg of αTE each day (>2.5 SD over the mean, data not shown). When the two men were dropped from the analyses, none of the findings with dietary vitamin E had *P*-values <0.3. There were no significant associations for dietary folate, zinc, vitamin C or β-carotene intake and sperm aneuploidy in the subgroup of non-supplement users.

Discussion

This is the first study to examine the associations between micronutrient intake (as measured by an FFQ) and human sperm aneuploidy. We found evidence of a statistically significant inverse relationship between total daily folate intake (diet + supplements) and the frequencies of sperm aneuploidy in a population of healthy men across a wide age range. We found no consistent relationships between intakes of zinc or the antioxidant vitamin C, vitamin E or β-carotene on the frequencies of aneuploid sperm.

In our study population, men with daily total folate intake over 722 μg or 1.8 times the RDA had between 20 and 30% lower frequencies of disomy X, disomy 21, sex nullisomy and aggregate aneuploidy compared with men with lower intake (Table III). The consistent associations with folate intake across several types of sperm aneuploidy and between categorical and continuous regression models argue that the finding is not simply due to chance.

Our findings with folate are consistent with *in vitro* studies of human lymphocytes that reported decreased aneuploidy frequencies across increasing physiologic concentrations of folate (Wang *et al*., 2004; Beetstra *et al*., 2005). Our findings are also consistent with evidence in human females that errors in the folate metabolism pathway may lead to abnormal chromosomal segregation. Several case–control studies have shown that abnormal maternal folate metabolism (e.g. folate enzyme gene polymorphisms) is associated with an increased risk of having a child with Down syndrome; however, results have been conflicting (James *et al*., 1999; Hobbs *et al*., 2000; Hassold *et al*., 2001; O'Leary *et al*., 2002; Bosco *et al*., 2003; Chango *et al*., 2005; da Silva *et al*., 2005), possibly due to the range of folate enzyme gene polymorphisms studied, the limited sample size in some studies, and the genetic and dietary differences among geographical populations (Gueant *et al*., 2003; James, 2004). Botto *et al*. (2004) found no significant relationship between maternal periconceptional multivitamin intake (including folic acid) and trisomies 13, 18 and 21 in live born or stillborn infants, although the power of this case–control study was limited. The effect of paternal folate gene polymorphisms or paternal periconceptional vitamin use on the incidence of having a child with aneuploidy is unknown.

Folate metabolism is critical for proper cell function. Its main roles are to provide carbon groups for purine and pyrimidine synthesis and to create the vital methyl donors methionine and *S*-adenoslymethionine (Eskes, 2006). The exact mechanism by which folate metabolism can affect meiotic nondisjunction is unclear; however, errors in the folate metabolism pathway, caused by folate deficiency or decreased enzyme activity due to genetic polymorphisms, can increase the incorporation of uracil into DNA and DNA hypomethylation, as well as increase the concentration of cellular homocysteine in somatic cells (Cantoni, 1985; Blount *et al*., 1997; Pogribny *et al*., 1997; Chen *et al*., 2001). There is also evidence that DNA hypomethylation may cause abnormal chromosomal segregation and chromosomal instability in somatic cells (Harrison *et al*., 1983; Leyton *et al*., 1995; Sciandrello *et al*., 2004; Gisselsson *et al*., 2005).

Our study also found significant inverse associations between total zinc intake and disomy X, and between total β-carotene intake and disomy Y, although the results were

Table III. Micronutrient intake and sperm aneuploidy. Adjusted aneuploidy mean frequencies per 10 000 sperm and standard errors (SE) by level of micronutrient intake (low, <25th percentile; moderate, 25–75th percentile; high, >75th percentile) ($n = 89$).

Micronutrient category	Intake range	Disomy X[a]	XY sperm[b]	Disomy Y[c]	Sex nullisomy[d]	Disomy 21[e]	Aggregate aneuploidy[f]
Folate							
Low	114–333 μg	3.2 (0.13)	8.7 (0.11)	5.0 (0.11)	22.3 (0.10)	9.0 (0.11)	57.0 (0.06)
Moderate	343–683 μg	3.5 (0.09)	8.9 (0.08)	5.4 (0.07)	20.0 (0.07)	10.3 (0.07)	56.0 (0.04)
High	722–1150 μg	2.4 (0.19)[i]	10.0 (0.12)	4.7 (0.12)	16.4 (0.11)[h,j]	7.6 (0.12)[i]	45.6 (0.07)[h,i,j]
Zinc							
Low	3–8 mg	3.1 (0.13)	9.7 (0.11)	5.3 (0.11)	16.0 (0.10)	9.6 (0.11)	54.3 (0.07)
Moderate	8–22 mg	3.8 (0.08)	8.4 (0.08)	5.1 (0.08)	20.7 (0.06) [g]	9.2 (0.07)	54.8 (0.04)
High	22–74 mg	1.9 (0.16) [h,i,j]	10.1 (0.11)	5.0 (0.12)	17.8 (0.10)	9.4 (0.11)	51.3 (0.06)
Vitamin C							
Low	26–99 mg	3.4 (0.13)	8.6 (0.11)	4.8 (0.11)	20.4 (0.09)	9.5 (0.11)	54.5 (0.06)
Moderate	106–400 mg	3.1 (0.10)	9.0 (0.08)	5.2 (0.08)	17.8 (0.07)	10.4 (0.08)	53.7 (0.04)
High	436–3394 mg	3.1 (0.14)	9.8 (0.11)	5.2 (0.12)	19.2 (0.10)	8.4 (0.12)	53.4 (0.07)
Vitamin E							
Low	2–9.6 mg	3.4 (0.14)	8.0 (0.11)	5.1 (0.11)	21.8 (0.11)	10.4 (0.12)	54.6 (0.07)
Moderate	9.9–118 mg	3.2 (0.10)	9.8 (0.08)	5.5 (0.07)	19.3 (0.07)	9.5 (0.08)	55.7 (0.05)
High	142–833 mg	2.9 (0.15)	8.9 (0.12)	4.4 (0.12)	18.6 (0.11)	9.2 (0.12)	50.5 (0.07)
β-Carotene							
Low	400–1168 μg	3.0 (0.14)	8.4 (0.11)	5.6 (0.10)	17.5 (0.11)	8.6 (0.11)	51.9 (0.07)
Moderate	1263–3943 μg	3.5 (0.09)	9.4 (0.08)	5.5 (0.08)	20.1 (0.07)	10.3 (0.08)	56.2 (0.05)
High	3972–33 444 μg	2.7 (0.15)	9.0 (0.11)	4.0 (0.12) [h,i,j,*]	21.7 (0.10)	8.7 (0.11)	52.1 (0.07)
Antioxidant composite							
Low		3.1 (0.16)	8.0 (0.12)	5.0 (0.12)	19.3 (0.11)	9.7 (0.12)	54.0 (0.07)
Moderate		3.2 (0.09)	9.4 (0.07)	5.4 (0.07)	18.7 (0.06)	9.2 (0.07)	55.4 (0.04)
High		3.1 (0.16)	9.4 (0.12)	4.5 (0.13)	18.8 (0.10)	9.5 (0.12)	50.6 (0.08)

Vitamin E presented as alpha tocopherol equivalents (αTE).
[a]Disomy X and vitamin C model adjusted for BMI and infection of the urinary tract or reproductive system; vitamin E model adjusted for tea consumption and high blood pressure; β-carotene model adjusted for BMI; antioxidant summary model adjusted for BMI, high blood pressure and infection of urinary tract or reproductive system; zinc model adjusted for age, tea consumption and high blood pressure, folate model adjusted for tea consumption. [b]$n = 88$, -1 outlier; All XY sperm models adjusted for history of mumps and scorer; in addition, vitamin C, vitamin E, β-carotene, antioxidant summary and folate models adjusted for work hazards; β-carotene, antioxidant summary and zinc models adjusted for infection of the urinary tract or reproductive system; vitamin C, zinc and folate models adjusted for season of collection; and folate model adjusted for tea consumption. [c]All Disomy Y models adjusted for scorer, dosimetry and season of collection. In addition, vitamin C, antioxidant summary, zinc and folate models adjusted for mumps; vitamin C, antioxidant summary and folate models adjusted for alcohol use; and vitamin C and vitamin E models adjusted for duration of abstinence. [d]All 21–0 models adjusted for infection of urinary tract or reproductive system; in addition; vitamin C model ($n = 87$, -2 outliers) adjusted for age and history of chronic disease; vitamin E model adjusted for age, caffeine use, prostate enlargement and history of chronic disease; β-carotene model adjusted for mumps and chronic disease; antioxidant summary model ($n = 87$, -2 outliers) adjusted for chronic disease; zinc model ($n = 87$, -2 outliers) adjusted for age; and folate model adjusted for age, caffeine use and high blood pressure. [e]All disomy 21 models adjusted for work hazards and scorer; in addition; vitamin C model ($n = 88$, -1 outlier) adjusted for age; vitamin E model ($n = 88$) adjusted for age and tea consumption; β-carotene model ($n = 87$, -2 outliers) adjusted for age; antioxidant summary model ($n = 87$, -2 outliers) adjusted for age, dosimetry, BMI and infection of urinary tract or reproductive system; zinc model ($n = 87$, -2 outliers) and folate model ($n = 87$, -2 outliers) adjusted for age, tea consumption and dosimetry. [f]Aggregate aneuploidy = sum of disomies X, Y, 21, sex nullisomy, 21 nullisomy and XY sperm. $n = 88$, -1 outlier; All aggregate aneuploidy models adjusted for scorer. In addition, vitamin C model adjusted for infection of urinary tract or reproductive system, season of collection, abstinence and smoking; vitamin E model adjusted for mumps and tea consumption; β-carotene model adjusted for infection of urinary tract or reproductive system and mumps; antioxidant summary model adjusted for mumps, high blood pressure and work hazards; zinc model adjusted for infection of urinary tract or reproductive system, season of collection, abstinence and high blood pressure; and folate model adjusted for infection of urinary tract or reproductive system, season of collection, age and tea consumption. [g]Moderate versus low intake $P < 0.05$. [h]High versus l*ow intake* $P < 0.05$. [i]High versus moderate intake $P < 0.05$. [j]Adjusted test for trend $P < 0.05$. *P-value ≤ 0.01

not consistent across the other sperm aneuploidy types. In addition, there was one significant positive association between zinc intake and sex nullisomy. We cannot rule out the possibility that these findings are due to chance, given the number of analyses performed; however, further studies are required. Of specific interest would be very high intake of β-carotene given that men with very high intake drove the significant inverse relationship with disomy Y.

Among men who did not use vitamin supplements ($n = 38$), we found suggestive evidence of an inverse relationship between dietary intake of vitamin E and four sperm aneuploidy types; however, two men with high daily vitamin E intake drove these findings. A larger sample size of non-supplement users is required to better evaluate the relationship between dietary vitamin E intake and sperm aneuploidy. No significant associations were found between folate intake and sperm aneuploidy in the non-supplement users, likely due to the lower levels of intake among the non-supplement users.

Although we found no consistent evidence of an association between antioxidant intake and the frequency of aneuploid sperm, it has been hypothesized that antioxidants may be particularly important in protecting human sperm from peroxidative damage due to the high amount of poly-unsaturated fatty acids in sperm (Wong *et al*., 2000). Low dietary intake of antioxidants and high levels of oxidizing agents in semen have been associated with decreased motility, count, viability and abnormal morphology in both human and animal sperm (Chinoy *et al*., 1986; Dawson *et al*., 1990; Kessopoulou *et al*., 1995; Vezina *et al*.,

1996; Lewis *et al*., 1997). In this study population, we previously reported significant associations between vitamin C intake and sperm numbers (count, concentration and total progressively motile sperm count), between vitamin E intake and progressive motility and total progressively motile sperm, and between β-carotene and sperm concentration and progressive motility (Eskenazi *et al*., 2005). The null finding between antioxidant intake and sperm aneuploidy is consistent with our recently reported result that sperm aneuploidy is not correlated with semen quality parameters in this sample (Wyrobek *et al*., 2006).

Our study had several methodological strengths. It is the largest sample size to date of sperm aneuploidy using the triple probe FISH methodology. In addition, detailed questionnaire information on demographic, medical, occupational and lifestyle risk factors allowed for control of confounding in the statistical models. Also, the relative homogeneity of study participants (educated, white and non-smokers) helped reduce the chance that our findings resulted from unmeasured health or behavioral factors. This homogeneity increases the internal validity of our study, but limits the generalization of study findings to clinical groups and more diverse populations (Jha *et al*., 1995).

The FFQ is considered the most effective and practical method for assessing usual dietary and supplemental intakes in large epidemiologic studies (Willett, 1998). The FFQ used in this study has been validated against dietary records in numerous other populations, although not in our population (Block *et al*., 1992; Mares-Perlman *et al*., 1993; Subar *et al*., 2001). We employed the FFQ to determine typical daily intake for the year prior to the semen analyses. Although the sensitive period for sperm aneuploidy is the 3-month window prior to sample collection (Robbins *et al*., 1997a), report of dietary intake over the previous year likely reflects this immediate time period since diet is fairly consistent over time and participants tend to bias their responses toward more recent intake (Willett, 1998).

As with any instrument that assesses dietary intake, there are also limitations associated with using an FFQ. Although an FFQ is good at estimating 'usual' intake over a period of time, less detailed information is collected with an FFQ on foods consumed, portion sizes and cooking methods compared with other dietary assessment measures. Thus, we decided to use quantiles of intake in our main statistical analyses to roughly rank the men's nutrient intakes and avoid misclassification of exposure. Also, responses on an FFQ may not reflect the biological concentrations in the blood or in various germ-cell tissue compartments. Although an abbreviated version of the FFQ used in this study has been shown to correlate significantly with erythrocyte folate levels in women (Clifford *et al*., 2005), no validation studies with biological nutrient values were performed in this study population. Thus, it is unknown whether study participants' responses on the FFQ correlate with biochemical indicators. Additional studies comparing sperm aneuploidy frequencies with micronutrient levels in seminal fluid and blood are required, but even these may not fully reflect the concentration of micronutrients in the correct biological compartment during the stages of spermatogenesis that are sensitive for aneuploidy induction.

Our study had several other methodological limitations. The cross-sectional design could not determine whether folate intake and aneuploidy in sperm are causally related; a randomized clinical trial would provide more definitive results. In addition, because of the correlation among the intake of different micronutrients, we could not definitively determine whether the results derived specifically from folate intake. For example, in our sample, total folate intake was related to total intake for vitamin C, vitamin E, β-carotene and zinc. Also, the high number of vitamin supplement users in the study population limited our ability to characterize the effects of nutrient intake from diet only, excluding supplements. As this is the first study to analyse the relation of micronutrient intake and the frequency of sperm aneuploidy, our findings merit further studies. In particular, a randomized controlled trial testing the effects of total folate intake on sperm aneuploidy is required to confirm the findings and establish causality.

In summary, we found a statistically significant negative association between total folate intake and disomy X, disomy 21, sex nullisomy and aggregate aneuploidy. The current RDA for folate in men age 19 years and older is 400 μg. In our study, we observe lower rates of sperm aneuploidy in men consuming >700 μg of total folate per day, which is still below the tolerable upper intake level of 1000 μg/day from supplements and fortified food (Institute of Medicine, 2000b). If other studies confirm our findings between folate intake and sperm aneuploidy, a possible public health intervention would be to increase the RDA for men considering fatherhood to reduce the risk of chromosomal anomalies in their offspring.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/

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