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Levels of 5-hydroxymethyl-2′-deoxyuridine in DNA from women participating in an intervention trial of low-fat and low-energy diets

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Oxidative DNA damage in blood appears to be useful as a marker of systemic oxidative stress levels. Dietary factors such as fat and energy intakes have been indicated to affect oxidative stress levels, and this may be an important mechanism by which diet can modulate cancer risk. The primary objective of this study was to investigate the effects of dietary intervention in premenopausal women on the levels of one type of oxidative DNA damage: 5-hydroxymethyl-2′-deoxyuridine. The trial randomly assigned women to control, low-fat, low-energy or combination low-fat/low-energy diets for 12 weeks. Blood samples were obtained every 2 weeks, and DNA was analysed for the levels of 5-hydroxymethyl-2′-deoxyuridine. Levels of DNA damage declined with time in each diet arm, including the control arm. The decreases were greater in the two arms with low-energy intake, but not significantly so. The numbers of women who exhibited decreased 5-hydroxymethyl-2′-deoxyuridine levels at 12 weeks versus baseline levels, however, was significantly greater in women assigned to any intervention diet (79%) than in the control arm (50%). Low-fat and low-energy diets therefore had a small effect on changes in oxidative DNA damage levels. The women participating in this study were not selected on the basis of increased cancer risk; therefore, they may have had low baseline levels of damage that were not amenable to further reduction by dietary change.

Keywords: oxidative DNA damage, low-fat diet, energy restriction, oxidative stress, clinical trial.

Introduction

Increased systemic oxidative stress levels, as evidenced by DNA and lipid oxidation products in blood and urine, are associated with increased risks of various cancers, including breast cancer (Frenkel *et al.* 1998, Ambrosone 2000, Djuric *et al.* 2001a, Kang 2002, Halliwell 2002). This oxidative stress may occur early in the carcinogenic process. In one prospective study, Frenkel *et al.* (1998) found that oxidative DNA damage was elevated in blood from persons who were diagnosed with cancers of the breast and colon 0.5–6 years later.

A number of dietary factors, such as intake of antioxidants (Moller and Loft 2002), can contribute to oxidative stress levels. Energy restriction, however, appears to be a particularly potent way to decrease oxidative damage levels and increase endogenous antioxidant defences in rodent models. This has been one mechanistic explanation for the increase in life span associated with energy restriction (Feuers *et al.* 1989, Hart *et al.* 1999). In primates, however, energy

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restriction did not reverse age-related changes in gene expression, as it did in mice, although oxidative damage in skeletal muscle was decreased (Zainal *et al.* 2000, Kayo *et al.* 2001). The composition of the diet also may be important. In the rodent model, there was little or no effect of energy restriction on 5-hydroxymethyl-2′-deoxyuridine (5-OHmdU) levels using the newer AIN-93G rodent diets that contain higher levels of certain vitamins and minerals than the AIN-76 diet (Djuric *et al.* 2002). In humans, the effects of energy restriction on levels of oxidative damage have been investigated to a more limited extent, with both negative and positive findings. In one study, 20% caloric restriction for 10 weeks resulted in no change in urinary levels of 8-hydroxy-2′-deoxyguanosine (Loft *et al.* 1995). In obese subjects, however, weight loss has been shown to result in decreased lipid and protein oxidation (Dandona *et al.* 2001, Davi *et al.* 2002). Energy restriction in humans and animals also may increase DNA repair ability (Lunec *et al.* 2002, Cabelof *et al.* 2003).

Another factor that may affect the levels of oxidative stress is fat intake. In a study that included women at increased risk for breast cancer, we have shown previously that a lower fat intake was associated with lower oxidative DNA damage levels (Djuric *et al.* 1991a). Reduction in fat intake in healthy men and women has also been shown to reduce the susceptibility of low density lipoprotein to oxidation (Yu-Poth *et al.* 2000). In a rodent study, dietary fat intake affected oxidative DNA damage levels in short-lived blood cells linearly while in mammary gland epithelium there was a plateau effect (Djuric *et al.* 2001b). Low-fat diets can affect oxidative stress levels directly by decreasing exposure to lipid hydroperoxides, or indirectly by changing metabolic pathways from lipogenesis to glycolysis with an accompanying increase in reducing equivalents in the cell (Hietanen *et al.* 1991). In particular, the intake of polyunsaturated fats is important since these fatty acids are more readily peroxidized than monounsaturated and saturated fatty acids. An increased degree of unsaturation of dietary fatty acids has been correlated with increased oxidative damage in the rat mammary gland (Haegele *et al.* 1994).

The relative effects of low-fat and low-energy diets are difficult to separate in human studies since these intakes are typically linked with each other. The Women's Diet Study was an intervention study designed to address this issue. The study was conducted using a 2×2 factorial design; the four diets were control, low-fat, low-energy, and combination low-fat/low-energy. The dietary counselling used for the low-fat diets stressed maintenance of energy intake while decreasing fat to 15% of the energy intake. With either the low-energy or combination low-fat/low-energy diets, a 25% reduction in energy intake was required relative to the reported baseline intake. Compliance to the diets, determined from monthly 4-day food records, appeared to be excellent (Djuric *et al.* 1999). Here we report on levels of one type of oxidative DNA damage, 5-OHmdU, in peripheral blood cell DNA obtained from participants in the Women's Diet Study.

Materials and methods

Subjects

The Women's Diet Study was approved by the Human Investigation Committee of Wayne State University, Detroit, USA, and subjects gave signed, informed consent to participate. The details of the

study have been described previously (Djuric *et al.* 1999). Briefly, premenopausal women, 25–50 years of age, who were at least 5.5 kg (12 lb) but not more than 50% over their optimal weight and who were not taking oral contraceptives were eligible. The mean body mass index (BMI) of subjects was 27.8 kg m^{-2} (range 22.3–33.9 kg m^{-2}). The women all indicated that they had stable body weight (within 1.82 kg [4 lb]) for at least 2 months prior to randomization. Each eligible participant had a fat intake above 25% of total energy intake, and an energy intake above 1600 kcal day^{-1}, as determined from 4-day food records. A baseline questionnaire was used to obtain demographic information, health status and physical activity patterns.

After stratification by race (Caucasian or other), subjects were randomized to one of four diets: control, low-fat (15% of energy from fat with maintenance of energy intake), low-energy (25% reduction from reported baseline intake with maintenance of percentage energy from fat), and a combination of low-fat and low-energy. After intervention, the reported fat intakes were 24, 17, 32 and 18% of energy intake in the control, low-fat, low-energy and combination arms, respectively. The respective decreases in energy intakes were 3, 7, 23 and 29% (Djuric *et al.* 1999). Blood was drawn every 2 weeks after an overnight fast into 10 ml heparinized tubes. Participants were paid $20 for each venipuncture as an incentive.

Preparation of DNA samples

Each blood sample was coded by number, and the diet arm assignment was blinded until after the analyses were completed. Nuclei were prepared from 8 ml of freshly drawn blood by adding 5 volumes of sucrose buffer (320 mM sucrose, 10 mM TRIS, pH 7.4, 5 mM $MgCl_2$, 10 ml Triton, 50 mM mannitol), according to the method of Ciulla *et al.* (1988). Centrifugation was used to isolate the nuclei. Nuclei were suspended in 1 ml of 1% sodium dodecyl sulphate (SDS), 1 mM ethylene diamine tetra-acetic acid (EDTA), 20 mM TRIS, pH 7.4, and 50 mM mannitol, and stored at -70°C.

Samples were analysed in batches of about 48; all the samples collected from each individual were analysed in the same batch. The nuclei in SDS solution were treated with 60 units of heat-treated RNase T1 and 200 μg RNase A for 30 min at 37°C. Then 2 mg of protease K was added and the incubation continued for 2 h at 37°C. After incubation, saturated NaCl was added (1:10 volume) and one extraction with chloroform/isoamyl alcohol (48:2) was performed, leaving the precipitated proteins at the interface. After one more extraction with *n*-butanol, the DNA in the aqueous phase was precipitated by the addition of two volumes of ice-cold ethanol.

The DNA was recovered by centrifugation and dissolved in 200 μl of water. Typically 100–300 μg DNA was obtained from 10 ml of blood. To check the equivalency of 5-OHmdU levels from batch to batch, two aliquots of calf-thymus DNA were analysed with each batch. These 'standard' DNA solutions were stored frozen in 10 mM mannitol at -35°C until use. This included one unoxidized sample and one sample that had been oxidized with 200 μM iron, 200 μM EDTA and 100 μM hydrogen peroxide in 50 mM phosphate buffer, pH 7.4, for 30 min. The DNA samples were hydrolysed enzymatically at 37°C using sequential enzyme addition: 50 units DNAase I for 3 h, 3 units nuclease P1 for 1 h, 2.5 units alkaline phosphatase for 30 min and a cocktail of DNase I, 0.015 units phosphodiesterase I and 1 unit phosphodiesterase II, 0.1 unit acid phosphatase and 1 unit alkaline phosphatase overnight.

5-OHmdU analysis

Nucleosides were isolated from the enzymatic hydrolysate using Sep-Paks and derivatized prior to analysis by gas chromatography–mass spectrometry (GC-MS) using isotopically labelled internal standards, as previously described (Djuric *et al.* 1991b). Briefly, samples were derivatized with 70 μl *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane:acetonitrile 2:1 at 120°C for 20 min. GC/MS was conducted using a Ultra 2 column (Hewlett-Packard, Palo Alto, California, USA) (25 m × 0.2 mm × 0.011 μm film thickness), with helium as the carrier gas, and a model 5971A mass-selective detector. The levels of 5-OHmdU and thymidine in each sample were calculated from standard curves. For 14 control women with four to seven samples per woman, the coefficient of variation for intra-individual variation was an average of 52% (the coefficient of variation for the assay ranged from 4–11%).

Statistical methods

The study used a 2 × 2 factorial design to explore the independent and interactive effects of low-fat (yes/no) and low-energy (yes/no) intakes on the levels of 5-OHmdU over time. Random permuted blocks were used to help balance accrual across the four diet arms within each race stratum. The planned sample size was 120 women. The 60 versus 60 women available from the factorial design to test the main effect of either dietary intervention gave a power of 0.83 to detect a difference as small as 13% in mean 5-OHmdU levels at any time point (two-sided $\alpha = 0.05$), based on pilot data. The actual sample size achieved was lower, with 81 women completing 12 weeks of the study, resulting in a power of 0.64.

After generating descriptive and graphical statistics, several of the 5-OHmdU values obtained appeared to be spuriously high. Grubb's test (Grubbs 1950) was applied to identify statistical outliers, and this resulted in deletion of 3% of the 594 data points. In order to normalize the data, natural logarithmic transformation was necessary. Potential covariates were explored for their relationships to 5-OHmdU levels. The effect of categorical variables was examined by t-tests at each time point: current illness such as colds (yes/no when blood taken), education (college graduate or not), family history of cancer in first-degree relatives (yes/no) and race (Caucasian or not). None of these showed an appreciable influence on 5-OHmdU levels (no more than one significant difference at any of the seven time points). Other potential covariates with continuous distribution were examined using univariate regression models, including age, weight, days since last menstrual period, BMI, and dietary intakes of alcohol, caffeine, β-carotene, iron, vitamin C and α-tocopherol. None of these variables were significantly correlated with 5-OHmdU levels either at baseline or at 12 weeks ($p > 0.15$ and Pearson $r < 0.15$ for each case). Based on these findings, no covariates were included in the statistical models described below.

All available longitudinal 5-OHmdU data was analysed, consistent with the intention-to-treat principle. To deal with occasional missing data at any of the seven time points, incomplete mixed models repeated measures analysis of variance (ANOVA) was conducted using the PROC MIXED program of SAS version 6.12 (SAS 1994, SAS Institute Inc. 1995, Littell *et al.* 1996). The initial model included 15 terms: each binary diet intervention effect; their interaction; linear, quadratic and cubic time effects; and the interactions of all time effects with all diet intervention effects. Eventually, the most parsimonious (i.e. reduced) model was determined. If a time effect was detected, the Tukey–Kramer multiple comparisons method (Littell *et al.* 1996) was used to determine at which specific time points the mean 5-OHmdU levels differed significantly. The best of 14 possible covariance structures for the repeated measures data was selected based on smallest value of Aikake's information criterion. In an attempt to smooth the pattern of 5-OHmdU means, we again applied this modelling approach after combining various time points.

The second analytic approach was an unbalanced two-way ANOVA of the ratios of 12 week to baseline 5-OHmdU levels (Littell *et al.* 1996). Square-root transformation of the 5-OHmdU ratios was required to achieve normality.

The third and final analysis method was to compare the proportion of women who showed a $\geq 5\%$ decrease (week 12 versus baseline) in 5-OHmdU level across the diet arms. This last approach utilized Fisher's exact test to compare these proportions.

Results

Mean levels of 5-OHmdU over time are shown in figure 1. All mixed models repeated measures ANOVA were performed on the log-transformed data. There was no significant interaction effect between the binary diet intervention variables,

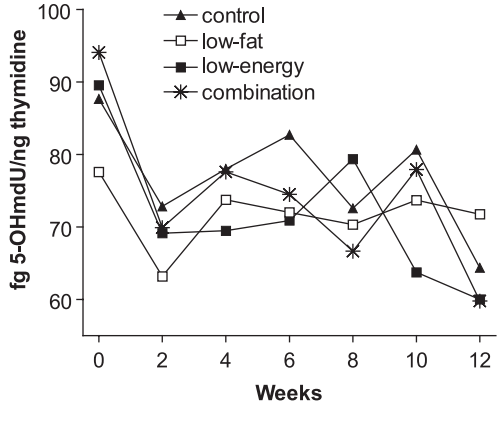

Figure 1. Mean levels of 5-OHmdU in DNA from nucleated blood cells by diet arm. Standard error (SE) bars are not shown for clarity, but the SE ranged from 4.39–11.24 fg 5-OHmdU ng^{-1} thymidine across all time points and diet arms. The data represent 104 women at week 0 and 81 women at week 12. The five intermediate time points represent data from between 72 and 85 women.

and there was no significant main effect of either variable on the levels of 5-OHmdU over time. This indicated no significant influence of the low-fat (yes/no) and/or the low-energy (yes/no) intervention. However, the overall linear effect of time (for all women combined) was significant ($p = 0.001$), with significant decreases relative to baseline observed at 2 and 12 weeks ($p = 0.024$ and $p < 0.001$, respectively). Inclusion of all outlier 5-OHmdU values did not change these results.

Similar modelling using individual diet arms (i.e. a four-category predictor variable) did not change the findings. When women on any intervention arm were combined (three diet arms versus the control arm), there was still no statistically significant effect of diet on 5-OHmdU levels (data not shown). The overall linear effect of time was again significant ($p = 0.001$), with significant decreases relative to baseline being observed at 2 and 12 weeks ($p = 0.024$ and $p < 0.001$, respectively). Combining the time points in two different ways (in attempt to smooth the patterns in figure 1) did not change the findings described above.

Analysis of the ratio of 5-OHmdU levels at week 12 to those at week 0 may have practical significance. The ratio minimizes the effects of day-to-day variation in the analytical technique, since all samples from a given woman were analysed on the same day. The mean ratios of 5-OHmdU levels at week 12 to those at week 0 were 0.86 in the control, 0.86 in the low-fat, 0.75 in the low-energy and 0.80 in the combination arms. Two-way ANOVA analyses indicated no significant difference in the mean ratios for either or both diet interventions. Various combinations of the diet arms did not change this finding regarding the relative change in 5-OHmdU levels over 12 weeks.

Finally, the proportions of women who exhibited DNA damage levels that had decreased by at least 5% from week 0 to week 12 were compared. Similar to using ratios, this should de-emphasize the importance of absolute levels of 5-OHmdU since it is a measure of change in levels. These proportions were 12 out of 24 (50%) for the control arm, and 75–81% for the three diet arms (see table 1). Although the proportion of women with decreased 5-OHmdU levels was greater in each intervention arm versus control, the sample sizes were small and the difference versus control was statistically significant only when women in all three intervention arms were combined ($p = 0.0092$). From this we conclude that low-fat and/or low-energy diets may have beneficial effects on levels of 5-OHmdU in blood, but since it was not evident in the analysis of pre- to post-intervention ratios or in the repeated

Table 1. Decreases of 5% or more in the levels of 5-OHmdU after 12 weeks of dietary intervention in 81 women.

		Decreased levels of 5-OHmdU	
Diet arm	Total no. in group	*n*	%
Control	24	12	50
Low fat	16	12	75
Low energy	21	17	81
Combination (low fat/low energy)	20	16	80
Any intervention	57	45*	79

* Significantly different from control; $p = 0.0092$ using Fisher's exact test, two-tailed.

measures ANOVA model, the effect of diet on 5-OHmdU levels in this study was small.

Discussion

There was a statistically significant linear trend for mean DNA damage levels to decrease with time for all diet arms combined (including the control), which was unexpected. Such a time-dependent phenomenon was also observed in a feeding study by Chen *et al.* (1999) measuring 8-oxodeoxyguanosine, but in that case the control subjects were fed a pre-formulated liquid diet that may have increased vitamin intakes in controls who were not energy restricted. In the present study, it is possible that the subjects on the control arm changed their eating patterns; they did report an increase of 0.46 servings/day of fruits and vegetables (Djuric *et al.* 2003). In addition, it is well documented that individuals do tend to eat less when they are recording their intakes (Mela and Aaron 1997). Control subjects in this study therefore may have been restricting their intakes at all time points, even at baseline, resulting in no significant changes in reported energy intake relative to baseline, although actual energy intake may have been decreased compared with usual intake. This illustrates the importance of using a control group in studies with oxidative stress as the endpoint. In a review of studies on the effects of antioxidant supplementation, it was concluded that most of the positive studies did not include a placebo control arm (Moller and Loft 2002).

The present study does have several limitations. In particular, only one type of oxidative DNA damage was examined, only blood was available, and DNA repair was not assessed. There are numerous kinds of oxidative DNA damage that have been identified in DNA, and it is possible that some of these other forms of DNA damage may be relatively more responsive to dietary changes. Levels may also be different in DNA from blood and that from other tissues. For example, nickel exposure of rats resulted in differential increases in various oxidative lesions, and rates of repair were slower in kidney than in liver (Kasprzak *et al.* 1997). The effects of dietary energy restriction on DNA repair have also been reported to be tissue specific (Um *et al.* 2003). Although many studies have shown an enhancing effect of energy restriction on DNA repair, this has not always been the case in human studies (Raffoul *et al.* 1999, Gackowski *et al.* 2001). Interestingly, pro-oxidant states, such as that induced by a high-fat diet, can cause stimulation of DNA repair pathways to offset increased formation of oxidized lesions (Martinet *et al.*, 2001, Lunec *et al.* 2002, Sreekumar *et al.* 2002). With regard to 5-OHmdU, human cells have an efficient repair glycosylase for this lesion, and any increases in formation may be short-lived due to repair (Rusmintratip and Sowers 2000) (Boorstein *et al.* 2001).

In our previous study, which showed beneficial effects of a low-fat diet on levels of 5-OHmdU, the subjects were all women at increased risk for breast cancer due either to a family history of breast cancer or mammography patterns (Djuric *et al.* 1991a). Although it is not possible to compare baseline levels in the two studies due to differences in the analytical techniques, women at increased risk for breast cancer are expected to have relatively higher levels of oxidative DNA damage in their blood

(Djuric *et al.* 2001a). Dietary intervention therefore may be relatively more effective in persons who have initially elevated levels of damage. For example, the antioxidant effects of a high-fruit and vegetable, moderately reduced-fat diet (the Dietary Approaches to Stop Hypertension [DASH] diet) were detected in plasma from obese, but not lean, subjects (Lopes *et al.* 2003). Thompson *et al.* (1999) showed decreases in the levels of oxidative stress markers after a 14 day high-fruit and vegetable intervention in both urine and blood, with the biggest decreases in women who had low baseline levels of α-carotene. When oxidative stress levels are low at baseline, there may be biological limitations to how much more levels can be reduced. There may also be technical problems in quantifying very low levels of damage due to the sensitivity of the method used. The results shown here nevertheless suggest that there is little effect of energy and/or fat restriction on 5-OHmdU in DNA from healthy, premenopausal women during 12 weeks of dietary change.

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