



INFLUENCE OF DIETARY FAT ON FECAL MUTAGENICITY IN PREMENOPAUSAL WOMEN

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A dietary intervention study was conducted on 31 premenopausal women (age: 20-40 years) to investigate the relationship between dietary fat and fecal mutagenicity. After a free-living period (baseline) of one menstrual cycle, the subjects were placed on a high-fat diet (40% calories from fat) for 4 menstrual cycles, followed by a low-fat diet (20% calories from fat) for 4 menstrual cycles. One-half of the subjects were randomly assigned throughout the study to a diet with a P:S ratio of 1.0 while the other half was assigned to one with a P:S ratio of 0.3; body weight by group remained constant. Three-day stool samples were collected at the mid-follicular period during the free-living phase and during the 4th menstrual cycle of each of the 2 controlled diet periods. Mutagenicity was assayed by the SOS chromotest. Reduction of dietary fat was accompanied by a significant decrease in fecal mutagenicity in both P:S groups. Combined values, i.e., both P:S groups, were 20.3 units for high-fat diets vs. 8.78 for low-fat diets.

The molecular basis of carcinogenesis involves an understanding of the mechanisms that control cellular proliferation (Barbacid, 1986). In recent years a number of genetic loci have been identified as being capable of promoting phenotypic changes characteristic of tumor cells. These loci are known as oncogenes and their counterparts in normal cells are called proto-oncogenes. The transformation of proto-oncogenes to oncogenes is thought to be the result of a number of mutational events brought about by mutagens in the cellular environment. This forms the basis upon which tests of mutagenicity have been proposed for the assessment of cancer risk.

Diet and diet-related lifestyles are among the factors that appear to contribute to susceptibility to neoplastic diseases (National Research Council, 1982; Nair, 1984). Diets rich in fat and poor in complex carbohydrates have been associated with increased risk of colon cancer in humans. Studies attempting to demonstrate a correlation between dietary fat, fecal mutagenicity and colon cancer risk (Venitt, 1982; Kuhnlein *et al.*, 1983; Wilkins and Van Tassell, 1983; Reddy *et al.*, 1984; Schiffman, 1986; Schiffman *et al.*, 1989) have proved inconclusive. Schiffman *et al.* (1989) have suggested that each class of mutagens must be considered separately since results vary according to the method of extraction and assay for mutagenic activity.

The purpose of the present study is to examine the influence of dietary fat and its degree of unsaturation on fecal mutagenicity in a group of healthy pre-menopausal women, using a quantitative, modified SOS assay for mutagenicity. The results reported here indicate the existence of a quantitative relationship between dietary fat and fecal mutagenicity. There was some suggestive evidence that the degree of unsaturation also contributes to mutagenicity in an unknown manner.

MATERIAL AND METHODS

Subjects

Women, between the ages of 20-40, were recruited from the

greater Beltsville, MD area for an initial interview to determine eligibility for entry into the study. The subjects were screened by means of dietary and medical questionnaires designed to exclude those who had health problems such as metabolic disorders, history of breast and/or thyroid disease, oral contraceptive use during the past year, regular use of prescription medications, menstrual or reproductive problems, pregnancy or breast-feeding during the past year and dietary habits that were non-representative of the general population. Forty women meeting the initial criteria were chosen out of a total of 97 women who responded to our public announcement.

These subjects were examined by a physician from the Georgetown University School of Medicine after having given informed consent according to protocols approved by the Institutional Review Boards of the University, the NCI, DHHS and the Agricultural Research Service, USDA. As part of the medical evaluation prior to entry, a medical history, hematologic profile, fecal occult blood and urinalysis were obtained. Tests for fecal occult blood were negative in all subjects. Those with body weights less than 90% or greater than 120% of the 1983 standards for desirable weights of the Metropolitan Life Insurance Co. were excluded from the study. Of the 37 women who were finally selected, 31 completed the entire study as originally planned. The majority of subjects (24 out of 31) in this study were non-smokers and none had a history of excessive alcohol use.

Diets

Four diets, planned from commonly available foodstuffs, were designed to contribute either 40% or 20% of calories from fat at P:S ratios of either 0.3 or 1.0. The nutrient composition of these diets was calculated using the USDA Lipid Nutrition Laboratory food database, derived from data on food composition from the USDA Handbook 8, the food industry, the Nutrient Coding Center in Minneapolis, and by analysis. A 14-day menu cycle assured variety and acceptability of the food provided. Subjects were initially assigned to 1 of 4 caloric intake levels based upon their estimated need for weight maintenance: 1,600, 2,000, 2,400 and 2,800 kcal. Body weight was maintained by adjusting the caloric intake of the subject. No vitamins, minerals or other supplements or alcohol were permitted during the study. The meals were prepared in the Beltsville Human Nutrition Research Center (BHNRC), where the subjects came for breakfast and dinner. During weekdays a

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carry-out lunch was provided while weekend meals were packaged and taken home on Fridays.

Study design

Subjects, paired on the basis of their Quetelet index (weight in kg/height² in meters) were randomized to 1 of 2 dietary groups (P:S ratios of 0.3 or 1.0) for the entire period of the study. The randomization also ensured that the few smokers in the study population (n = 7) were distributed between the two P:S groups as equally as possible.

After a free-living period (baseline) lasting for one menstrual cycle, all subjects were placed on a high-fat (40% calories from fat) diet with a P:S ratio of either 1.0 or 0.3 for a period of 4 menstrual cycles. They were then transferred to a low-fat diet (20% calories from fat) at the same P:S ratio for 4 more menstrual cycles. Three-day stool samples were collected at the mid-follicular period during the free-living phase and during the 4th menstrual cycle of each of the 2 controlled diet phases (high-fat and low-fat).

Each subject maintained a menstrual cycle chart and a morning basal body temperature chart during the entire period of the study to ensure proper time-frames for stool sampling and to monitor for any anovulatory cycles.

Collection and processing of stools

Stools, passed into clear plastic bags, were enclosed in an outer zip-lock bag and immediately placed on a block of dry ice, which was stored for the 3-day collection period in a styrofoam box. This procedure ensures the preservation of stool samples in a frozen state until they are transported to the laboratory for processing. The stool samples were weighed and homogenized in a paint can with an equal volume of water and a marble, the homogenization being carried out in a paint can shaker for approximately 1 min. Measured aliquots of the homogenate were shell-frozen and lyophilized at -45°C and stored at that temperature until they were analyzed.

Assessment of mutagenicity by the SOS chromotest

Aliquots of the stools (100 mg) were extracted twice with 20-ml aliquots of ethyl acetate, pooled and then flushed with argon. The extracts were evaporated to dryness under partial vacuum in a rotary evaporator and then immediately reconstituted with 1.0 ml of acetone and transferred to a small vial. The acetone extract was evaporated to dryness and the residue was dissolved in 400 µl of pure DMSO. A 3-µl aliquot of the extract was used to make serial dilutions in a 96-well flat-bottomed plate (Falcon) using pure DMSO.

E. coli PQ37 (Quillardet *et al.*, 1982, 1985; Quillardet and Hofnung, 1985) was rehydrated in LB medium (Miller, 1972) and grown overnight at 37°C. The bacterial suspension was diluted with LB medium to a working concentration of 0.04 OD_{600 nm} units. Aliquots (100 µl) of this suspension were added to 96-well test plates containing the serial dilutions of the stool extract. A positive reference consisting of serial dilutions of 4-nitroquinoline oxide (4-NQO) was included in each batch. The test plates were incubated at 37°C on an orbital shaker placed inside the incubator. After 3 hr, 100 µl of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 2.0 mg/ml in 20% DMSO in 0.3 M phosphate buffer, pH 7.5, containing 0.003 M magnesium chloride and 5% methanol) were dispensed into each of the wells, the contents being mixed thoroughly and read immediately in a microplate reader (Titertek Multiscan, Flow, McLean, VA) at 615 nm to establish baseline readings. The plate was then incubated for an additional 3 hr and read again at 615 nm. The net increase in optical density was used to generate a dose-response curve from which a linear regression plot of the linear portion of the graph was constructed. The slopes of the unknown were expressed with re-

spect to that of 4-NQO, a unit of activity being defined as the slope elicited by 1 µg of 4-NQO. The activity of alkaline phosphatase, the constitutive enzyme, was frequently monitored for viability of the test organism as described by Quillardet *et al.* (1982). Furthermore, in each assay, duplicate wells were run at each dose level, along with duplicate controls consisting of the reagents alone. A unit of activity is defined as the SOS inducing potency of 1 µg of 4-NQO under standard conditions.

Statistical analyses (Snedecor and Cochran, 1980)

The data were analyzed by paired *t*-tests and analysis of variance, and an alpha value of 0.05 was considered statistically significant.

RESULTS

The principal characteristics of the study population, excluding dropouts, are shown in Table I. The age, morphometric and biochemical characteristics of the groups randomized by P:S ratio were similar.

The nutrient intake of the subjects during the high-fat and low-fat study periods is tabulated in Table II. In the high-fat diet, 40% of the estimated calories were derived from fat compared to 20% in the low-fat diet.

The fecal stool weights and the moisture content of the stools were similar for the whole group during each of the 3 collection periods (Table III), the slight increase in stool weight during the low-fat period being statistically non-significant.

Table IV presents data on fecal mutagenicity during each of the 3 study periods. Initial fecal mutagenicity in the P:S 0.3 subgroup was slightly lower than in the P:S 1.0 subgroup (19.5 for P:S 0.3 vs. 23.5 for P:S 1.0); the difference was not statistically significant. During the high-fat and low-fat feeding periods, this marginal difference between the 2 P:S groups persisted (16.1 for P:S 0.3 vs. 24.4 for P:S 1.0). Regardless of the P/S ratios of the diets, the mutagenicity observed during the high-fat period was comparable to that during the initial free-living period (combined values were 21.5, free-living vs. 20.3, high-fat). Transition to the low-fat diet resulted in a highly significant drop in fecal mutagenicity in both P:S groups. When the results of the 2 P:S subgroups were combined, the change from a high-fat to a low-fat diet resulted in a 60% decrease in fecal mutagenicity.

DISCUSSION

In this study we have employed a fairly new assay (SOS

TABLE I -- CHARACTERISTICS OF THE RANDOMIZED STUDY POPULATION AT ENTRY

Characteristic	Randomized groups by P:S ratio	
	0.3 n = 15	1.0 n = 16
Age (years) ¹	28.4 ± 1.5	27.1 ± 1.1
Quetelet's index (kg/m ²) ¹	22.32 ± 0.76	21.85 ± 0.72
<i>Plasma lipids</i>		
Plasma cholesterol (mg/dl) ¹	180.4 ± 7.9	195.6 ± 7.1
Plasma triglycerides (mg/dl) ¹	49.9 ± 7.7	49.0 ± 5.7
<i>Nutrient intake/day</i> ^{1,2}		
Energy (kcal/day)	2111 ± 65	2012 ± 63
Protein (% kcal)	14	14
Carbohydrate (% kcal)	45	48
Fat (% kcal)	41	38
P:S ratio	0.5	0.5
Cholesterol (mg/day) ^{1,2}	468 ± 31	392 ± 24

¹Expressed as mean ± standard error. ²Nutrient intake was calculated from an analysis of a 7-day diet record.

TABLE II - DAILY NUTRIENT INTAKE OF STUDY SUBJECTS DURING THE HIGH-FAT AND LOW-FAT CONTROLLED DIET STUDY PERIODS

Nutrient intake (per day)	High fat 40% calories from fat		Low fat 20% calories from fat	
	P:S ratio		P:S ratio	
	0.3	1.0	0.3	1.0
Energy (kcal)	2278 ± 65 ¹	2180 ± 81	2260 ± 97	2208 ± 110
Protein (% kcal)	16	16	17	17
Carbohydrate (% kcal)	45	45	64	64
Fat (% kcal)	39	39	19	19
Cholesterol (mg)	374 ± 11	289 ± 10	230 ± 9	199 ± 9
Saturated fat (g)	44.2 ± 1.3	26.8 ± 1.1	20.9 ± 0.7	12.3 ± 0.6
Oleic acid (g)	30.5 ± 0.9	33.5 ± 1.4	14.9 ± 0.6	17.0 ± 0.8
Linoleic acid (g)	14.6 ± 0.4	26.1 ± 0.9	6.9 ± 0.2	12.9 ± 0.6
Fiber (g) ²	9.5	9.4	12.9	15.6

¹Expressed as mean ± standard error. ²Neutral detergent fiber (analyzed by K. Behall).

TABLE III - FECAL STOOL WEIGHTS AND MOISTURE CONTENT DURING FREE-LIVING AND CONTROLLED DIETARY PERIODS¹

	Initial free-living	Controlled diet periods	
		High fat (40%)	Low fat (20%)
Stool weight (g)	248 ± 26.9	258 ± 28.6	311 ± 36.2
Moisture content (%)	73 ± 1.3	71 ± 1.3	73 ± 1.4

¹Results are expressed as means ± standard error, for stools collected over a 3-day period.

TABLE IV - FECAL MUTAGENICITY DURING FREE-LIVING AND THE CONTROLLED HIGH-FAT AND LOW-FAT STUDY PERIODS¹

P:S ratio	Initial free-living	Controlled diet periods	
		High fat (40%)	Low fat (20%)
0.3	19.5 ± 6.66 (15)	16.1 ± 4.34 (15)	8.19 ± 1.10 ² (15)
1.0	23.5 ± 4.96 (15)	24.4 ± 3.65 (16)	9.33 ± 1.49 ² (16)
Combined	21.5 ± 5.47 (30)	20.3 ± 2.87 (31)	8.78 ± 0.93 ² (31)

¹Results are expressed as mean units/g of lyophilized stools ± standard error. Values within parentheses represent the number of subjects in each group. ²For the comparison between the high-fat and low-fat periods, $p \leq 0.001$. Within any diet period there were no significant differences across the 2 P:S ratio groups.

Chromotest) to demonstrate the effect of dietary fat (caloric contribution) on fecal mutagenicity in pre-menopausal women. The SOS chromotest utilizes *E. coli*, strain PQ37 (Quillardet *et al.*, 1982), in which the β -galactosidase gene, *lac Z*, is placed under the control of *Sul A* (formerly called *Sfi A*), one of the SOS genes. In addition to the *Sul A::lac Z* operon fusion, there is a deletion in the *lac* region so that the activity of β -galactosidase is entirely dependent upon *Sul A* expression (Quillardet *et al.*, 1982). Mutagenicity is indirectly inferred from *Sul A* expression which is directly proportional to the concentration of genotoxic agents in the environment. Since the assay is based upon the activity of induced β -galactosidase, one has to guard against endogenous activity contributed by aqueous fecal extracts, as pointed out by Bosworth and Venitt (1986). The assay as designed in this study uses an ethyl acetate extract of lyophilized stool samples to eliminate this possible source of error.

In a previous report, samples from this study were analyzed for the fecapentaenes (Taylor *et al.*, 1988), a class of fecal mutagens reported in North American populations (Schiffman, 1986). We found that fecapentaene levels were slightly increased on the low-fat diet. Since fecapentaenes are highly

active in the SOS assay (Nair *et al.*, 1990) we would have expected a concomitant rise in SOS mutagenicity during the low-fat period. However, mutagenicity decreased during the low-fat period and in fact a direct comparison of fecapentaene levels and mutagenicity in samples from the low-fat period revealed a significant inverse relationship. One possible explanation for the apparent inconsistency between the fecapentaene levels and mutagenicity is that the fecapentaene assay during the low-fat period gave spuriously high values due to interference from non-mutagenic non-fecapentaene pigments of plant origin absorbing in the same spectral region as the fecapentaenes or due to their inherent anti-mutagenicity (Ames, 1989).

Most of the studies on diet and fecal mutagenicity have used test systems involving reverse mutation of auxotrophic microorganisms. When applied to fecal extracts, these methods are highly susceptible to interference from growth promoters in the extract (Venitt *et al.*, 1986). Recognizing these problems, some investigators had compared the reverse mutation assays with the SOS chromotest as applied to human stools (Venitt and Bosworth, 1986).

In the present study we have taken these limitations into consideration in adapting the SOS chromotest to fecal extracts, and the results suggest that dietary fat, regardless of the degree of unsaturation, contributes to fecal mutagenicity. However, the relationship between dietary fat intake and fecal mutagenicity appeared to be somewhat pronounced in the P:S 1.0 group. The role of dietary polyunsaturated fatty acids in this case is not clear and we will have to wait until further studies clarify this aspect. We hope the approach described in this paper using the SOS chromotest will be useful for the generation of mutagenicity data that could be treated as a continuous variable in numerical and statistical analyses.

Several correlational studies have suggested that populations consuming high-fat diets are more likely to exhibit fecal mutagenicity than those consuming low-fat diets (Ehrich *et al.*, 1979; Mower *et al.*, 1982; Reddy *et al.*, 1980). Thus, fecal mutagenicity has been postulated as an endpoint associating high-fat diet and the risk of colorectal cancer. Only a few dietary intervention trials have attempted to test this hypothesis on a small number of subjects, with mixed results (Kuhnlein *et al.*, 1983; Bruce and Dion, 1980). The results of the present investigation, conducted among a larger group of subjects than previous dietary intervention studies, strongly support the hypothesis that low-fat diets contribute to a lowering of fecal mutagenicity as measured by the SOS chromotest. This effect apparently cannot be explained by an effect on fecapentaenes, the most prevalent fecal mutagen identified in North Americans. Consequently, the search should continue for non-fecapentaene fecal mutagens that could mediate an effect of high-fat diets on the risk of colorectal cancer.

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