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Effects of Dietary Fat, Calcium, and Vitamin D on Growth and Mammary Tumorigenesis Induced by 7,12-Dimethylbenz(*a*)anthracene in Female Sprague-Dawley Rats[1]

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ABSTRACT

This study was designed to test the influence of dietary calcium and vitamin D levels on the promotional effect of high-fat diets on chemically induced mammary carcinogenesis. In a small preliminary experiment (Experiment A), 40 female Sprague-Dawley rats, 43 days old, were randomly divided into 5 groups (8 rats/group) and fed a semipurified diet containing 3% sunflower seed oil (SF) by weight, 1.5 mg of calcium/kcal and 0.5 IU vitamin D/kcal of diet. After 1 week, each rat was given 2.5 mg of dimethylbenz(*a*)anthracene by gastric gavage. One week later, the animals were switched to 1 of 4 diets varying in fat (3 or 20% SF by weight), calcium (1.5 or 0.25 mg/kcal), vitamin D (0.5 or 0.05 IU/kcal), and phosphate or to a fifth diet containing 3% SF by weight, 0.1 mg of calcium/kcal and 0.05 IU of vitamin D/kcal. In all 5 diets, calcium:phosphate weight ratios were maintained at 1.2:1. In animals fed the high-fat diet, reduction of dietary calcium (1.5 to 0.25 mg/kcal) and vitamin D (0.5 to 0.05 IU/kcal) increased the incidence of mammary lesions from 37 to 75% and the total number of lesions from 4 to 16. A trend toward an increase in lesion weight and total lesion burden was also seen.

To confirm these results, the experiment was repeated using the same protocol; 126 rats were divided into 6 groups, treated with dimethylbenz(*a*)anthracene, and fed the diets as described. A sixth diet was included that contained 20% SF by weight, 0.01 mg of calcium/kcal, and 0.05 IU of vitamin D/kcal. As for Experiment A, in animals fed the high-fat diet, reduction of dietary calcium (1.5 to 0.25 mg/kcal) and vitamin D (0.5 to 0.05 IU/kcal) resulted in an increase in total mammary lesions from 31 to 55, a significant increase in average lesion burden/rat with lesions (1.6 ± 0.6 to 12 ± 3 g), and a trend toward increasing weight of lesions. The effect was less obvious in animals fed the low-fat diet where, in both experiments, an increase in the incidence of mammary lesions was observed only when the dietary calcium was reduced from 1.5 to 0.1 mg/kcal. These data suggest that decreasing calcium and vitamin D increase the promoting effects of a high-fat diet on mammary tumorigenesis in the rat.

INTRODUCTION

Many experiments have shown that rats and mice fed high-fat diets develop mammary tumors more readily than those fed low-fat diets (1, 2). Dietary fat appears to exert its main effects at the promotional stage of carcinogenesis by providing a more favorable environment for growth and proliferation of tumor cells (1, 3). A level of 4–5% *n*-6-polyunsaturated fatty acid (linoleic acid) is apparently required for maximum promotion (4). When this requirement is met, the enhancement of carcinogenesis seems to be related to the total amount rather than the type of dietary fat (5). This agrees with epidemiological data which show that breast cancer incidence and mortality correlate better with total dietary fat than with any particular type of fat (6).

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There are many parallels in studies of colon cancer and mammary cancer. Epidemiological studies show similar correlations of geographical distributions and dietary fat intakes with rates of both colon and mammary cancer (7, 8). Experiments on animals have demonstrated that, as in breast cancer, dietary fat acts as a promoter rather than an initiator of chemically induced colon cancer (9). Direct instillation of bile acids (10) or fatty acids (11) intrarectally into rodents was irritating and toxic to the colon resulting in an increase in the proliferation of colonic epithelial cells; these damaging effects were reduced by p.o. administration of calcium salts (10, 11).

It has been proposed that dietary calcium may reduce the promotional effects of dietary fat in the colon (12). The use of dietary calcium carbonate supplements reduced colonic epithelial proliferation rates in human subjects at high risk for colon cancer to the lower level normally seen in subjects at low risk for developing colon cancer (13). Studies in two different animal models suggest that increased dietary calcium or vitamin D can reduce the promotional effect of high dietary fat or increased bile acids on colon carcinogenesis (14, 15).

Because of the similar epidemiological correlations of colon and mammary cancer with dietary fat intake, this study was designed to test the influence of dietary calcium and vitamin D levels on the promotional effect of high-fat diets in chemically induced mammary cancer. The dietary calcium levels used were similar to the common calcium intakes of North American women on a nutrient density basis in comparison to the high levels found in the standard laboratory rat diet (16, 17). North American young women consume 1687 ± 769 (SD) kcal and 673 ± 496 mg calcium per day (18) giving a ratio of means of 0.4 ± 0.5 mg of calcium/kcal. A group of elderly women were found to consume 576 ± 24 mg of calcium daily (19).

Three levels of dietary calcium were used in this study: 1.5 mg/kcal, the standard recommended level of calcium intake for rats as used in laboratory animal feeds; and two reduced levels, 0.25 and 0.10 mg/kcal, both of which fall within the observed range of calcium intakes for North American women (18, 19). Because unbalanced dietary calcium:phosphate ratios can affect growth and mineralization in rats (20), phosphate was also reduced to maintain a 1.2:1 weight ratio (equivalent to a 1:1 molar ratio) in all cases.

The nutritional requirements and *in vivo* metabolism of calcium are intimately related to those of vitamin D. The human daily dietary intake of vitamin D of healthy young women is estimated at about 159 ± 172 IU compared with a recommended daily allowance of 300 IU (7.5 μg) for this group (18), while in elderly women, an estimated daily dietary intake of 81 ± 9 IU was less than one-half of the 200 IU (5 μg) of the recommended daily allowance for that group (19).

These levels of vitamin D would be in the range of 0.05 to 0.1 IU/kcal in the human diet. Two levels of vitamin D were used in this study: 0.5 IU/kcal, in the range of the recommended level of vitamin D intake suitable for rats in laboratory feeds; and a reduced level of 0.05 IU/kcal, falling within the actual

Table 1 *Diet groups*

Group	n	Sunflower seed oil		Calcium		Vitamin D	
		% of kcal	% by wt	mg/kcal	% by wt	IU/kcal	IU/100 g
A	29	38.5	20	1.5	0.70	0.50	233
B	29	38.5	20	0.25	0.12	0.05	24
C	21	38.5	20	0.10	0.05	0.05	24
D	29	7.0	3	1.5	0.58	0.50	196
E	29	7.0	3	0.25	0.10	0.05	20
F	29	7.0	3	0.10	0.04	0.05	20

Table 2 *Composition of semipurified diets*

Ingredient	3% fat		20% fat	
	Wt	kcal	Wt	kcal
Casein[a]	19.4	77.6	23.4	93.4
Dextrose[b]	69.0	276.0	46.2	184.9
Alphacel[c]	4.1		5.0	
Salt mix[d]	2.6		3.2	
Vitamin mix[c, e]	1.8	7.4	2.2	8.8
Sunflower seed oil[f]	3.0	27.0	20.0	180.0
Total	99.9	388	100	467.1

[a] U.S. Biochemical Corp., Cleveland, OH.
[b] Canadian Starch Company, Toronto, Ontario, Canada.
[c] ICN, Cleveland, OH.
[d] See Table 3.
[e] Vitamin Diet Fortification Mix. The vitamin D_2 content was varied by using a mix lacking vitamin D_2 and adding the desired amount of vitamin D_2.
[f] Procter & Gamble, Cincinnati, OH.

Table 3 *Mineral mix*

Compound	g/100 g of mineral mix providing following calcium, in final diet		
	1.5 mg/kcal	0.25 mg/kcal	0.10 mg/kcal
Calcium carbonate[a]	2.1	3.65	3.65
Calcium hydrogen orthophosphate	73.5	7.42	0
Citric acid	0.227	0.227	0.227
Cupric citrate·2.5H_2O	0.046	0.046	0.046
Ferric citrate·5H_2O	0.558	0.558	0.558
Magnesium oxide	2.50	2.50	2.50
Manganese citrate	0.835	0.835	0.835
Potassium iodide	0.001	0.001	0.001
Dipotassium hydrogen orthophosphate	8.1	4.83	4.83
Potassium sulfate	6.8	6.8	6.8
Sodium chloride	3.06	3.06	3.06
Disodium hydrogen orthophosphate·2H_2O	2.14	2.14	2.14
Zinc citrate·2H_2O	0.133	0.133	0.133
Dextrose[b]	0	75.22	67.8
Calcium:phosphate (g:g)	1.2:1	1.2:1	1.2:1
Calcium:magnesium (g:g)	15 :1	2.5:1	1 :1

[a] All chemicals listed are analytical grade.
[b] Canadian Starch Co., Toronto, Ontario, Canada.

range of dietary intake of North American women. It has been suggested that nondietary sources of vitamin D (*i.e.*, endogenously produced by sunlight on exposed skin) are minor in northern climates, contributing to vitamin D inadequacy and increased risk of colon cancer (21, 22).

MATERIALS AND METHODS

Two separate experiments were conducted. The first preliminary experiment (Experiment A) involved 8 rats in 5 dietary groups (no Group C) as described below. The experiment was then repeated with 21 rats in each of the 6 dietary groups referred to as Experiment B. The same protocols and conditions were used for both experiments.

Female Sprague-Dawley rats (43 days old), obtained from Charles River Laboratories (St. Constant, Quebec, Canada), were randomly divided into 6 groups and housed 3 animals to each stainless steel hanging cage with a wire mesh bottom, in a temperature-controlled room with a light-dark cycle of 12 h. They were fed a semipurified diet

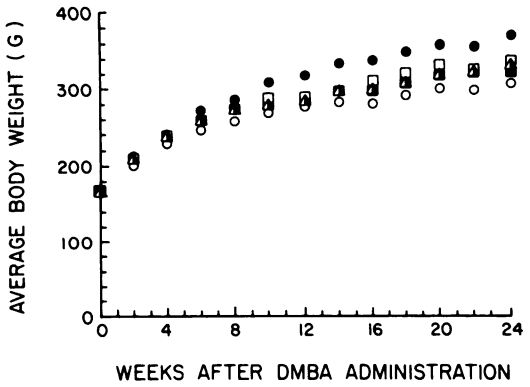

Fig. 1. Effect of feeding diets differing in fat content on average body weight of rats. (□) Diet A (20% sunflower seed oil, 1.5 mg of calcium/kcal); (●) Diet B (20% sunflower seed oil, 0.25 mg of calcium/kcal); (■) Diet C (20% sunflower seed oil, 0.10 mg of calcium/kcal); (△) Diet D (3% sunflower seed oil, 1.5 mg of calcium/kcal); (○) Diet E (3% sunflower seed oil, 0.25 mg of calcium/kcal); (▲) Diet F (3% sunflower seed oil, 0.10 mg of calcium/kcal).

containing 3% sunflower seed oil by weight and 1.5 mg of calcium/kcal of diet. After 1 week, all the rats were given 2.5 mg of 7,12-dimethylbenz(*a*)anthracene (Eastman Kodak, Rochester, NY) by gastric gavage in 0.25 ml of olive oil. One week later, the animals were switched to 1 of 6 diets varying in fat, calcium, phosphate, and vitamin D content (Table 1).

The composition of the diets and the sources of dietary ingredients are listed in Table 2 and the composition of the mineral mixes in Table 3. The amounts of fiber, protein, vitamins, and minerals were constant with regard to energy. Food and water were provided *ad libitum*. Feed consumption was measured in 7 cages of 3 animals per diet during Experiment B. Preweighed feed containers were placed in cages lined with absorbent paper and 48 h later the containers were removed and weighed. Spilled feed collected on the filter paper was weighed and subtracted from the total. Average feed consumption was calculated on the basis of kcal of total energy/rat/day.

All animals were weighed weekly. Each animal was palpated for the presence of mammary nodules and their location was noted every 2 weeks for 25–27 weeks. During this period, animals with tumors of the ear canal or with large or necrotic tumors were killed and necropsied for mammary lesions. The remaining animals were killed either by decapitation prior to collecting blood or by ether inhalation and examined for nodules after Week 27. Samples of all nodules were fixed in neutral, buffered formalin; embedded in paraffin; stained with hematoxylin, phloxin, and saffron; and examined histologically in a blinded and randomized fashion. The lesions were classified as described by Medina (23). Serum samples were analyzed for calcium (24) and phosphate (25) in the Clinical Biochemistry Laboratory, University Hospital, London, Ontario, Canada, using Technicon Methods No. SD4-0003 FM9 and SD4-0004 FM9, respectively. The data shown are the results of two experiments run under similar conditions. The first preliminary experiment involved 8 animals in 5 dietary groups (no Group C). The experiment was then repeated with 21 rats in each of the 6 dietary groups. The data were analyzed by χ^2 analysis, Wilcoxon rank test, and analysis of variance (26).

The experiment was terminated when the incidence of animals with large necrotic tumors began increasing in Group B, to prevent an imbalance in numbers of animals.

Table 4 *Effect of diet on body weight, feed consumption, and serum calcium and phosphate levels: Experiment B*

Diet	Fat (% by wt)	Calcium (mg/kcal/day)	Final body wt (g)	Av. feed consumption (kcal/rat)	Serum calcium[a] (mM)	Serum phosphate[a] (mM)
A	20	1.5	344 ± 11[b]	75 ± 3[c]	2.28 ± 0.08[d]	1.71 ± 0.05[e]
B	20	0.25	375 ± 16	83 ± 6	2.37 ± 0.03	1.66 ± 0.06[f]
C	20	0.10	322 ± 14	90 ± 3[c]	ND[g]	ND
D	3	1.5	320 ± 9	74 ± 2	2.52 ± 0.05[d,h]	1.89 ± 0.06[e]
E	3	0.25	296 ± 16	63 ± 2	2.2 ± 0.1[h]	2.0 ± 0.1[f]
F	3	0.10	332 ± 11	69 ± 3	ND	ND

[a] $n = 6$.
[b] Mean ± SEM, $n = 21$.
[c-f, h] All values with common superscripts are significantly different.
[c-e] $P < 0.05$.
[f] $P < 0.1$.
[g] ND, not determined.
[h] $P < 0.01$.

Table 5 *Influence of dietary calcium and fat on mammary lesions*

Diet	Fat (% by wt)	Calcium (mg/kcal)	n	% incidence	% of adenocarcinomas	Total mammary lesions	Av. wt. of lesions (g)	Av. no. of lesions/rat with lesions	Av. lesion burden/rat with lesions (g)
					Experiment A				
A	20	1.5	8	37.5	100	4	1 ± 1[a]	1.3 ± 0.3	2 ± 1
B	20	0.25	8	75	100	16	2 ± 1	3.2 ± 0.7	7 ± 4
D	3	1.5	8	50	83	6	0.3 ± 0.1	1.5 ± 0.6	0.4 ± 0.2
E	3	0.25	8	62.5	71	17	0.8 ± 0.4	3 ± 1	2 ± 1
F	3	0.10	8	100	62.5	16	2.5 ± 2	2 ± 1	5 ± 4
					Experiment B				
A	20	1.5	21	52	100	31	0.6 ± 0.1[b]	2.6 ± 0.5	1.6 ± 0.6[d]
B	20	0.25	21	57	96	55	2.7 ± 0.6[b,c]	5 ± 1	12 ± 4[d,e]
C	20	0.10	21	52	89	38	1.0 ± 0.3	3.4 ± 0.6	4 ± 1
D	3	1.5	21	48	92	25	2 ± 1	2.4 ± 0.5	4 ± 3
E	3	0.25	21	43	88	26	1.4 ± 0.5	2.8 ± 0.8	4 ± 1
F	3	0.10	21	62	85	26	0.4 ± 0.1[c]	2.2 ± 0.5	0.9 ± 0.3[e]

[a] Mean ± SEM.
[b-e] Values with common superscripts are significantly different, at $P < 0.05$.

RESULTS

As seen in Fig. 1 and Table 4, the animals on the diets containing a high level of fat tended to be heavier than those on the low-fat diet. This is consistent with the increased caloric intake as measured by feed consumption. The calcium content of the diet appeared to have no significant effect on feed consumption or growth of animals consuming the low-fat diets; however, animals consuming diets low in calcium but high in fat tended to consume more kcal than those animals on the high-fat diet containing the standard level of calcium. The group on Diet B gained the most weight in the study. The animals eating Diet C, which was the highest in fat and the lowest in calcium, were small, visibly sickly, and excitable, despite a higher caloric intake than in the other animals, possibly due to the known effect of higher levels of dietary fat reducing the availability for absorption of dietary calcium (27, 28).

The serum levels of calcium were highest in the animals consuming Diet D, suggesting that fat had as significant an effect as dietary calcium on calcium absorption. The phosphate levels were highest in the rats consuming either Diet D or Diet E ($P < 0.01$) (Table 4). Calcium intake appeared to have an effect on serum calcium levels only in diets low in fat ($P < 0.05$), while serum phosphate levels appeared to be affected only by fat levels ($P < 0.05$).

Animals fed the high-fat Diet B had a higher number of mammary lesions than animals fed the high-fat Diet A containing the higher levels of calcium and vitamin D (Table 5). The mammary lesions found in animals on both high-fat Diets A and B were primarily adenocarcinomas, which were significantly larger (370%) ($P < 0.05$) and more abundant in the group consuming less calcium and vitamin D. The total weight of tumors was significantly greater in these animals ($P < 0.05$).

The total number of mammary lesions increased with reduction of calcium to the 0.25-mg/kcal level in the high-fat diet, but a marked increase in incidence of mammary tumors was observed only when the level of calcium was reduced to the lowest level of 0.1 mg/kcal in the animals fed the low-fat diets in Experiment A. It should be noted, however, that the percentage of adenocarcinomas usually decreased as the calcium level was reduced at either level of fat.

High dietary fat, as compared to low dietary fat, in Experiment B increased the number of mammary lesions in the presence of high dietary calcium and vitamin D (Group A *versus* Group D in Table 5). Dietary fat showed a marked promotional effect when dietary calcium and vitamin D were reduced (Group B *versus* Group E in Table 5) as indicated by total mammary lesions and average lesion burden per rat with lesions ($P < 0.05$).

DISCUSSION

These studies indicate that an inadequate intake of dietary calcium and vitamin D on a high-fat diet may enhance mammary tumor development. This effect appears to be less pronounced in animals fed low-fat diet. This is similar to the effect seen in colon carcinogenesis (14, 15).

The increase both in incidence of rats developing mammary lesions and in total numbers of mammary tumors seen in animals consuming diets low in calcium is consistent with the increase in proliferation rates of mammary epithelial cells seen in studies on mice fed diets varying in fat and calcium content (29). Preliminary studies in our laboratory suggest a similar

effect of dietary fat and calcium on proliferation rates of epithelial cells in rat mammary tissue as measured by labeling indices of [^{3}H]thymidine incorporation into DNA (30).

These data suggest the possibility that dietary calcium and vitamin D affect the promotion of mammary carcinogenesis by high dietary fat. The mechanism may involve some aspects of calcium metabolism in tissues (31). The increased susceptibility of the animals on the high-fat diets to growth and health problems caused by the lowest level of calcium intake is consistent with a decreased absorption of dietary calcium due to binding of the calcium to fat in the diet and subsequent excretion (27). Animal nutrition studies have regularly demonstrated an increased dietary requirement for calcium with increased fat content of animal feed (28).

The recommended daily allowance of calcium for adult women is 800 mg of elemental calcium per day with a recommended energy intake in the range of 2000 kcal/day (32). This yields a calcium:energy intake ratio of 0.4 mg/kcal, a figure significantly lower than the minimum of 1.5 mg/kcal recommended for laboratory animals (33), and is lower than the suggested optimum for adult women (34). It is found that the majority of women do not even take this requirement, and the problem increases with age (18, 19, 35). Similarly, the recommended daily intake for adults of 200 IU of vitamin D or 0.1 IU/kcal is not fully met in young women (18) and is less than one-half (or below 0.05 IU/kcal) in older women (19), as compared with 0.5 IU/kcal in some rodent diets with a minimum of 0.3 IU/kcal (33).

The mechanism by which dietary calcium and vitamin D protect against the promoting effect of high-fat diets on mammary tumorigenesis is unknown and may be different from the direct binding suggested in order to mediate the effect of calcium in the colon (12). There are several possibilities. Carcinogenesis is associated with a decrease in immunosurveillance which can be mediated by natural killer cells. Natural killer cell function is depressed by 7,12-dimethylbenz(*a*)anthracene (36) and shows a requirement for calcium (37); therefore dietary calcium may influence carcinogenesis by altering the activity of natural killer cells. Decreased serum calcium can also cause intracellular increases in calcium and consequent alteration of cellular functions (38).

The long-term modification of dietary patterns to reduce the intake of fat is difficult and expensive. The maintenance of an adequate intake of dietary calcium and vitamin D could be more acceptable to most individuals as a means of reducing the risk of breast cancer.

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