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Effect of a Calcium-enriched Diet on the Colonic Epithelial Hyperproliferation Induced by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine in Rats on a Low Calcium and Fat Diet[1]

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ABSTRACT

We examined whether hyperproliferation of colonic crypt epithelium during cancer induction by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), in rats on a low fat and calcium diet could be reduced by added calcium p.o. From the age of 4 weeks, 104 male Sprague-Dawley rats received a low fat (3.5%), low calcium (0.05% calcium ion), and low vitamin D (0.4 IU/g) diet. Sixty-four also had calcium salts, derived from either calcium lactate or solubilized calcium carbonate, added to their drinking water; therefore their total calcium intake was about 1% of daily diet. At age 12 weeks the rats were divided into 4 treatment groups: 8 rats, not receiving added calcium, had rectal saline instillations weekly (saline control group) and were sacrificed after a further 28 weeks; 3 groups of 32 rats each received intrarectal MNNG (1.5 mg) weekly. One group, not receiving added calcium, was the MNNG control group; while the second group also received added calcium lactate, and the third group received calcium carbonate. Groups of 24 were sacrificed periodically until 28 weeks of treatment. Rats were sacrificed and epithelial proliferation was estimated, 1 week after the last intrarectal instillation, by *in vivo* labeling with tritiated thymidine and measuring the ratio of labeled to total colonic crypt epithelial cells. The mean labeling index of the MNNG treated and added calcium groups were significantly higher (8.7–9.5%) than that of the saline controls (2.8%) only at week 28; however, it was then still significantly less than that of the MNNG controls not having added calcium (17.9%). Hyperproliferation, during induction of colonic cancer by MNNG in rats on a low calcium diet, can be reduced by a calcium enriched diet even in the presence of a low fat intake.

INTRODUCTION

Increased proliferative activity is among the earliest changes that occur in large bowel crypt epithelium in diseases predisposing to colorectal cancer and in experimental cancer induced in animals (1–3). It is believed that this increased DNA synthesis is a phase that could lead to malignancy (4, 5).

Nutritional and to a lesser extent familial factors are apparently of paramount importance in the etiology of colorectal cancer (6, 7). Based, in part, on epidemiological studies (8, 9) it was hypothesized that the western style diet is calcium deficient relative to the fat intake and that the calcium would form insoluble and less toxic complexes with the fat (10). Following on this, the effect of added dietary calcium on colonic epithelial proliferation was examined in subjects at high risk for familial colon cancer or recurrent adenomas and it was shown that calcium supplementation reduced this proliferation (11, 12). Thus, it was suggested that dietary calcium supplements might be effective in diminishing the risk for colorectal malignancy (5, 10).

These short term human studies were preceded by animal experiments demonstrating that epithelial damage, following colonic instillation of fatty and bile acids, resulted in a compensatory increased proliferation of the colonic epithelium. This could be reduced by the simultaneous administration of calcium salts (13, 14). In addition to the hypothesized formation of calcium-fat complexes, suggested alternative mechanisms of proliferation inhibition have been based on studies relating to the dietary fat, vitamin D, and calcium in experimental models. These include an intraluminal mucosal stabilization effect or a systemic intracellular mechanism (15–21).

The carcinogen MNNG,[3] given to rats by i.r. instillation, causes progressive changes in the colonic mucosa which lead to invasive adenocarcinoma (22, 23). We repeated this experiment in an attempt to answer whether a diet adequate in calcium, as compared to a low calcium diet, could reduce the progression to hyperproliferation, which was expected in the induction phase, before dysplasia and invasive carcinoma. In addition, we examined whether this phenomenon could occur even in the presence of a low fat-vitamin D but high residue diet (24). This diet was chosen in an attempt to minimize the intraluminal calcium-fat binding and/or systemic effects of calcium.

MATERIALS AND METHODS

Subjects. One hundred four male Sprague-Dawley rats, 4 weeks old, with a mean weight of 80 g, were obtained from the Tel-Aviv University. Groups of 4 animals were housed in plastic cages, 47 x 26 x 20 cm, with wire tops and coarse sawdust bedding. The rodents were kept in a temperature (24°C) and humidity controlled clean room with 12-h light and dark cycles. Each animal had access to water and chow *ad libitum*.

Diet and Calcium Treatment. The diet (Miluoth, Western Galilee) was based on a modified AIN-76 semisynthetic formula feed (14, 16, 25) (Table 1) and was given from the age of 4 weeks. This was calculated to give 3.6 kcal/g diet and included 3.5% fat, 0.05% calcium ion, 0.4% phosphate, 0.4 IU/g vitamin D, and 16.5% cellulose. In comparison to the original AIN-76 diet (5% fat, 0.5% calcium ion, 0.4% phosphate, 1 IU/g vitamin D, and 5% cellulose) this diet is low in fat, calcium, and vitamin D but high in cellulose. The latter was added in order to increase dietary bulk without increasing caloric intake and yet have a minimal effect on colonic mucosal proliferation (24). During this study period each cage of rats consumed an average of 80 g food/day, giving a mean intake of 9 mg calcium ion/animal/day which we believe to be still adequate for this period of rat growth.

Except for 2 control groups (Table 2, treatment groups 1 and 2), an additional amount of calcium was added to the drinking water, and provided *ad libitum*. Calcium containing drinking water was prepared by dissolving 6.24 g of calcium carbonate or 12.47 g of anhydrous calcium lactate (BDH) in 1000 ml of tap water. The calcium carbonate was dissolved first in 10 ml of 5% acetic acid and the solution brought to pH 6.5 by titration with sodium hydroxide (0.1 mol), the final product being a calcium acetate with traces of sodium bicarbonate and acetate.

Each cage of rats drank a mean of 120 ml water/day; thus the study cages receiving added calcium (Table 2, treatment groups 3 and 4) imbibed a mean of 748 mg calcium salts derived from $CaCO_3$ or 1496 mg from calcium lactate. We calculated that each animal, except the controls, consumed an average of 74 mg calcium ion/day when drinking

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[3] The abbreviations used are: MNNG, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; LI, ratio of labeled to total colonic crypt epithelial cells; i.r., intrarectal.

Table 1 *Composition of modified AIN-76 diet*[a]

Ingredient	% in diet
Casein	20.0
DL-Methionine	0.3
Corn starch	50.0
Dextrose	4.0
Cellulose	16.5
Soy bean oil	3.5
Chlorine bitartrate	0.2
Vitamin mix[a,b]	2.0
Mineral mix[a,c]	3.5

[a] Prepared by Miluoth, Ltd., Western Galilee, Israel; including vitamins from Teva Pharmaceuticals, Israel.

[b] Vitamin mix/kg diet: nicotinamine, 30.0 mg; pyridoxine HCl, 7.0 mg; thiamine HCl, 6.0 mg; riboflavin, 6.0 mg; folic acid, 2.0 mg; D-biotin, 0.4 mg; vitamin B_{12}, 0.001 mg; vitamin D_3, 400 IU; vitamin A, 4000 IU; vitamin E, 50 IU; vitamin K, 0.005 mg.

[c] Mineral mix providing the following elements/kg diet: phosphate, 4000 mg (from sodium phosphate-monobasic and potassium phosphate-monobasic); potassium, 3600 mg; chloride, 1560 mg; sodium, 1000 mg; sulfate, 1000 mg; magnesium, 500 mg; calcium, 500 mg (from calcium carbonate); manganese, 54 mg; iron, 35 mg; zinc, 30 mg; copper, 6 mg; chromium, 0.4 mg; iodine, 0.2 mg; selenium, 0.1 mg.

Table 2 *Treatment groups and changes in LI (mean ± SD) during study diet,*[a] *without or with MNNG and added calcium*

	LI × 100 at following times (wk) of i.r. saline or MNNG[b]			
Treatment groups[a,b]	8	16	20	28
1. Diet,[a] i.r. saline[b] ($N = 8$)				2.80 ± 0.8[c]
2. Diet,[a] i.r. MNNG[b] ($N = 32$)	2.30 ± 0.6[c]	8.90 ± 3.8[d]	7.30 ± 1.0[d]	17.90 ± 3.4[e]
3. Diet,[a] $CaCO_3$, i.r. MNNG[b] ($N = 32$)	3.00 ± 1.0[c]	4.10 ± 1.2[c]	3.40 ± 0.7[c]	9.50 ± 4.0[d]
4. Diet,[a] calcium lactate, i.r. MNNG[b] ($N = 32$)	2.90 ± 0.5[c]	3.20 ± 0.8[c]	3.20 ± 0.7[c]	8.70 ± 1.6[d]

[a] Study diet, with or without added calcium, started at age 4 weeks and continued until sacrifice.

[b] Treatment with weekly i.r. saline or MNNG initiated at age 12 weeks. Proliferation determined 1 week after last i.r. instillation.

[c] Not significantly different.

[d] Significantly different from *c* or *e* ($P < 0.001$).

[e] Significantly different from *c* or *d* ($P < 0.001$).

water prepared from calcium carbonate (which contains 40% calcium ion) or 69 mg calcium ion/day when drinking water with calcium lactate (which contains 18.4% calcium ion).

Thus, the overall amount of calcium in diet and drinking water of treatment groups 3 and 4 did not exceed 1% by weight of consumed food and drinking water. This total amount of calcium (twice that in the standard AIN-76 diet) was chosen so as to be different enough from that in the study diet of treatment groups 1 and 2 (one-tenth of that in the AIN-76 diet) that an effect might be evident, yet physiological and well below the level that could cause toxicity from hypercalcemia. The ratio of dietary calcium to phosphate was 1:8 in the study diet but fell to 1:0.4 in the diet of rats receiving added calcium.

MNNG Treatment. The MNNG experiments started at 12 weeks of age, after the rats had been on the study diet (with or without added calcium) for 2 months. A stock solution of MNNG was prepared by dissolving 15 mg MNNG in 10 μl of dimethyl sulfoxide and then in 2 ml of 0.9% NaCl solution. The final solution was brought to pH 6.5 and passed through a 0.45-μm Millipore filter. Two hundred μl of the solution, which contained 1.5 mg of MNNG, were instilled weekly into all animals except the saline controls (Table 2, treatment group 1). These control rats were given weekly i.r. instillations of equivalent volumes of 0.9% NaCl solution (22). The instillation of MNNG or saline was performed with the use of a 7.5-cm-long 18-gauge cannula needle (Becton Dickinson) with a special ball tip.

Study Protocol. All animals had the above described modified AIN-76 study diet from age 4 weeks with (treatment groups 3 and 4) or without (treatment groups 1 and 2) added calcium, and they developed normally. At 12 weeks of age there were no significant differences between those receiving or not receiving added dietary calcium and their mean weight was 350 g. They were then divided into 4 treatment groups (Table 2). A control group of 8 rats had the study diet but tap water to drink and received weekly i.r. saline instillations (treatment group 1). The remaining 96 rats received weekly rectal instillations of MNNG and were divided equally into 3 groups: a further control group of 32 animals who received only tap water to drink (treatment group 2); and 2 groups of 32 rats each who had received calcium salts derived from calcium carbonate (treatment group 3) or lactate (treatment group 4) added to their drinking water.

Eight animals each, from treatment groups 2, 3, and 4, were sacrificed after 8, 16, 20, and 28 weeks of MNNG instillation, 1 week after the last MNNG treatment. The control group of 8 rats, without MNNG treatment but receiving 28 weekly i.r. instillations of saline, was sacrificed 1 week after the last saline treatment (Table 2).

One h prior to being killed the rats were transferred to individual cages and received an i.p. injection of 250 μCi of tritiated thymidine (Nuclear Research Center, Negev; specific activity, 20 Ci/mmol) in sterile water. When sacrificed by neck dislocation the animals underwent a macroscopic examination of their internal organs to detect tumors. The colons were excised and samples of colon, 8 cm from the anal verge, were removed. This site for colon sampling was chosen because it represented the location of maximum exposure to MNNG. In a prior study where rats received MNNG instillations for 48 weeks, 85% of the animals bore adenocarcinomas in this target area of MNNG instillation (23). The removed pieces of colon were placed in buffered formalin.

Kinetic Studies of Colonic Mucosa. The samples prepared for autoradiography were dehydrated and embedded in paraffin blocks and sectioned, and slides were prepared as described elsewhere (12). The slides were coated with NTB_2 (Eastman Kodak Co., Rochester, NY) by the dipping method and then placed in a light-tight box at 4°C for 3 weeks. The slides were then dipped in Kodak D-19 solution at 18°C to stop further development and then in Kodak fixative F-10, washed, and stained lightly with hematoxylin and eosin.

Scoring of Slides and Statistical Analysis. Ten coded slides were prepared from each animal and read by the same technician. Twenty-five longitudinally sectioned well oriented colon crypts were counted for each animal, taking care not to recount the same crypt. Only straight portions of crypts were counted and budding or branching parts were ignored in order to obtain the most standardized results.

The parameters scored were the number of epithelial cells in a hemicrypt (one side of a crypt) and the number of [^{3}H]thymidine-labeled cells (those with 4 or more silver grains) in the same hemicrypt. The labeling index represents the ratio of total number of labeled cells to the total number of crypt cells counted.

Statistical analysis was based on mean values of the study parameters at completion of each treatment. The analysis was performed using both the Kruskal-Wallis and Mann-Whitney test statistic (two-tail approximation), the former, for overall testing of differences between treatments or time periods, and the latter, for examination of significance between subgroups, when overall significant differences were found.

RESULTS

The 104 animals completed the study, their eating habits and general behavior remained stable during the rectal instillation experiments. They continued to develop normally and at the same time periods there were no differences in body weight and appearance of fur between any of the study groups.

In this paper we will not deal with the macroscopic or microscopic changes in the colon of the animals. In two rats (treatment group 2, receiving MNNG without additional dietary calcium) in which a tumor was macroscopically detected, tissue samples for scoring were taken 2 cm distal to the tumor burden.

The LI (%) of the saline control rats (group 1), on the study diet but without MNNG or added calcium, was 2.8 ± 0.8 (SD) after 28 weeks of rectal instillations (Table 2). This value was similar to that of the rats receiving MNNG and additional

calcium (groups 3 and 4) until the 28th week, when there was a significant rise in LI.

The control rats receiving MNNG without additional calcium (group 2) had a significant rise in their LI noted at weeks 16 and 20 (Table 2). At week 28 the LI rose further, significantly more so than in the rats receiving MNNG and added calcium (groups 3 and 4).

No significant differences were found, during the whole experiment, between animals treated with added calcium salts derived from either $CaCO_3$ or calcium lactate (treatment groups 3 and 4) (Table 2). These rats, also receiving MNNG, had a rise in their LI only at week 28, which was then significantly greater than the LI of the saline controls without MNNG or added calcium (group 1), but significantly less so than the LI of group 2 receiving MNNG without added calcium.

DISCUSSION

This report demonstrates the effect of a calcium enriched diet on reducing the colonic epithelial hyperproliferation of rats during the initiation phase of colon cancer induction by MNNG, even when receiving a low fat diet. This direct acting carcinogen is a suitable agent because in our prior studies carcinoma was observed to occur in over 85% of animals at 48 weeks of treatment (23). We stopped the MNNG instillations after 28 weeks in order to exclude misleading proliferation due to tumor induction; in fact two cases of macroscopic neoplasia were then found.

The animals were given a low calcium diet, 0.05% calcium ion, which corresponds to 300–400 mg calcium ion daily in a human diet of 2000–2500 kcal, while the low dose of vitamin D provided to the rats is equivalent to 400 IU in a similar human diet, and is the recommended daily allowance for young adults. For this experiment we did not change the phosphate in the study diet to correspond with the amount of calcium provided. Excess phosphate could have bound to some of the dietary calcium making it less available for local release in the colon (14, 26). We based the altered AIN-76 diet on low fat (3.5%), but high residue, in order to minimize tumor promoters other than MNNG. The dietary cellulose content, even though relatively high, is believed to be poorly fermented within the rat colon and has, by itself, minimal effects on mucosal proliferation (24). However, we did not measure stool or colonic pH, nor do we know about its calcium binding effects (24, 26). This diet did not disturb the general well being of the rats and appeared adequate for the duration of the study, nor did the MNNG have an adverse nutritional effect over this time period. This is also reflected by the similar LIs of the saline control group without added calcium and the basal LI values of those having added calcium.

We also studied the effects, on this proliferative activity, of providing an adequate calcium intake by adding calcium salts to the drinking water. It was believed that calcium lactate, as a source of calcium, avoids the effects of pH damage in the upper gastrointestinal tract and is better absorbed than the carbonate salt. We therefore gave calcium in the form of calcium lactate and compared the results with those obtained with $CaCO_3$, solubilized with acetic acid. We, as others, did not notice physiological or proliferative differences between the groups receiving either of these calcium salts (12, 27).

This study shows that MNNG application increasingly alters the colonic crypt epithelial proliferation. A significant ($P = 0.008$) 5.7-fold increase in LI was found, on a low calcium diet, after 28 weeks of MNNG administration, when two rats were detected to have macroscopic tumors. This change in LI was mainly due to a progressive and significant increase in the number of labeled cells, while at the same time there was a significant increase in the crypt length of the MNNG treated animals, as compared to the saline control group.

The results demonstrate that the proliferation of crypt epithelial cells in calcium treated animals initially was similar to that of controls that did not receive added calcium. Thus, this low calcium diet *per se* did not affect proliferation. This was true until 20 weeks of MNNG treatment; at 28 weeks their LI rose to a level intermediate to that of the saline controls and to the control group receiving MNNG. These modulating effects of calcium are similar to those reported in humans at potentially high risk for colorectal neoplasia and treated with dietary calcium supplements (11, 12). However, our studies have the advantage of being in a homogeneous population of rats in which the diet can be controlled exactly.

There is an increasing interest in understanding the role of calcium in the molecular events associated with the process of cellular proliferation and its function in or prevention of carcinogenesis. Its possible modes of action on reducing proliferation are considered to be intraluminal (especially in the presence of a relatively high fat diet by forming insoluble calcium-fat complexes) (2, 10, 19), locally on the mucosa (especially when having a fermentable high residue diet which produces an acid pH, thereby releasing ionic calcium salts) (9, 17), and/or systemic (10, 18, 19, 21). Interestingly, we obtained suppression by an adequate calcium intake even while the rats received a low fat and vitamin D but high cellulose diet. As stated, cellulose is believed to be relatively inert in the rat colon and not significantly influence proliferation. It could be postulated that the paramount effects in this model are, by having calcium available (systemically or locally) for stabilization, inducing differentiation and thereby reducing the need for cell replacement (15). This will require confirmation by using other models with added vitamin D and/or high calcium or fat diets (18, 19). It would also be of interest to continue studying the role of calcium in the later stages of tumor promotion by MNNG and/or by a systemic carcinogen.

In conclusion, our studies have demonstrated that it is possible to partially but significantly reduce the colonic crypt epithelial hyperproliferation that occurs during the induction phase of carcinogenesis by administration of a calcium enriched diet, as compared to a low calcium diet, even in the presence of a low fat intake.

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