Effect of calcium supplementation on mucosal cell proliferation in high risk patients for colon cancer

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SUMMARY **Recent findings suggest that supplemental calcium could lower the abnormally high proliferation rate found in the colonic mucosa of subjects at high risk for colon cancer. In this double blind controlled study, this effect in volunteers previously operated upon for a colorectal adenocarcinoma was tested. Thirty subjects were randomised to receive either elemental calcium 1200 mg/day or a placebo. Mucosal proliferation was measured with tritiated thymidine labelling before and after the 30 day intervention period. Diets, faecal pH and the concentration of calcium and bile acids in the aqueous phase of feaces were also measured. Labelling index did not differ significantly in the two groups before intervention (placebo 4·0(2·4) v calcium 4·9(2·9), but the difference approached significance afterwards (4·4(2·4) v 6·5(3·4), p=0·06). Individual changes occurring with intervention were tabulated and comparison of the means for the groups was not significant ($\triangle$=0·3 vs $\blacktriangle$=1·8, p=0·11). Calcium concentration, faecal pH and deoxycholic acid concentration increased in the calcium group (p=0·02, 0·005 and 0·004 respectively). Calcium does not show any effect in decreasing colonic mucosal proliferation in this high risk group for colon cancer; it may increase faecal pH and the production of deoxycholic acid in the colon.**

Colorectal cancer remains a leading cause of cancer related deaths in North America and no major change in incidence has been reported over the last decade. Environmental factors are suspected of playing an aetiological role in this disease; in particular, high fat consumption has been under scrutiny.[1-5] One proposed mechanism explaining the effect of fat is an increase in bile acid secretion[6] which subsequently leads to a rise in concentration of potentially toxic secondary bile acids in the colonic lumen.[7] This hypothesis is supported by animal experiments showing that these compounds can induce mucosal cell damage, increase proliferation rates[8 9] and enhance carcinogenesis by acting as tumour promoters[10] and by field studies showing that high risk populations for colon cancer have higher concentrations of bile acids in their stools.[3 11 12]

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In animals, calcium was shown to inhibit colonic mucosal damage induced by bile acids under various experimental conditions[13-17] presumably by reacting with the acidic lipids to form insoluble and biologically inactive calcium soaps.[14 17-19]. These observations have led some to speculate that calcium could affect the human colon in a similar manner and, possibly, decrease the risk for colon cancer.[18] Some investigators showed a protective effect against the disease[20] while others observed no association.[21] Two recent studies, however using human colonic biopsies from high risk individuals suggested a decrease in mucosal cell proliferation or labelling index (LI) – a proposed measure for assessing cancer risk[22] – after the administration of calcium supplements.[23 24]

In this randomised double blind controlled study, we investigated the effect of supplemental calcium on mucosal proliferation assessed by tritiated thymidine labelling in patients previously operated upon for colorectal cancer. This group was selected because individuals with colon cancer have been found to

have higher proliferation rates than controls[22] and because they are at a high risk of developing a metachronous colonic neoplasm.[25-28] Because acidic lipids are thought to affect mucosal damage and proliferation, we measured bile acid concentration in the aqueous phase of stools as well as calcium concentration. Faecal pH was also assessed to determine whether there was a relationship with bile acids solubility. Dietary intakes of the subjects were recorded to ensure comparability between the groups and to explore any correlation with the afore-mentioned parameters.

Methods

STUDY DESIGN

Thirty subjects previously operated upon for a colorectal adenocarcinoma who had a curative resection within the last three to 60 months and were considered free of disease upon entry were recruited. Exclusion criteria included a history of hyperpara-thyroidism, hypercalcaemia, renal calculus or lactose intolerance. The volunteers were randomly allocated to one of two groups. Group 1 received placebo capsules containing lactose and cellulose (Novopharm Ltd, Scarborough, Ontario, Canada) while group 2 took calcium carbonate capsules containing 200 mg elemental calcium each (Calcium carbonate 500 mg, Novopharm Ltd., Scarborough, Ontario, Canada). Each volunteer was instructed to take two capsules three times a day at mealtime so the total amount of calcium added in the calcium group

Table 1 Comparison on sex, age, height, weight, body mass index, Duke's stage at operation between the two treatment groups

Variable*	Placebo	Calcium	p^5
Sex (M/F)	11/4	10/5	
Age	63·0	65·0	NS
	(9·9)	(8·1)	
Height (cm)	169·1	161·7	0·03
	(7·2)	(7·2)	
Weight (kg)	78·9	72·1	NS
	(14·8)	(10·8)	
Body mass index†	0·27	0·28	NS
	(0·04)	(0·05)	
Dukes'‡			
stage A	1	3	NS
B1	5	3	
B2	5	9	
C1	2	3	
C2	2		

*Group means are given for continuous variables, standard deviation in parentheses; †body mass index = $\frac{weight}{(height)^2}$; ‡number of patients in each stage are given; §significance level of test for difference between two groups from t-test for continuous variables, from χ^2 test for categoric variables.

Table 2 Comparison on dietary patterns between the two groups before and after intervention

Daily intake*	Before intervention			After intervention		
	Placebo	Calcium	$p^†$	Placebo	Calcium	$p^†$
Total calories	2260	1771	0·04	2443	2001	NS
(kcal)	(797)	(350)		(709)	(680)	
% Fat	36·7	33·1	NS	36·6	33·7	NS
	(6·4)	(5·7)		(6·0)	(5·7)	
% Protein	18·3	18·6	NS	16·9	18·3	NS
	(4·5)	(2·8)		(3·2)	(3·7)	
% CHO	45·0	48·3	NS	46·8	47·4	NS
	(8·6)	(8·0)		(8·0)	(8·1)	
Calcium (mg)	919·5	669·4	NS	904	851	NS
	(417·5)	(425·5)		(367)	(578)	
Fibre (g)	19·2	18·9	NS	21·3	18·2	NS
	(9·2)	(7·9)		(8·8)	(9·2)	
Starch (g)	126·1	92·9	NS	131·8	100·6	NS
	(70·1)	(28·4)		(60·7)	(37·5)	

*Group means are given, standard deviation in parentheses; †significance level of test for difference between two groups from t-test.

was 1200 mg/day. The intervention period lasted 30 days.

All volunteers were asked to stay on their regular diet. Before and after intervention (day 0 and 30), they underwent a sigmoidoscopic examination with-out preparation during which three rectal biopsies were taken 10-15 cm from the anal verge; they also provided a stool sample which was immediately frozen. One exception was a colostomy patient in whom the biopsies were taken 10-15 cm from the colostomy opening. Each volunteer was provided with capsules for 30 days with instructions for taking them. Also, sets of three-day food records were taken before intervention and on the last three days of supplementation. Those few who could not attend the scheduled return visit were rescheduled for the earliest available day and were provided with additional capsules. Compliance to the assigned regimen was assessed by pill count and measurement of calcium concentration in the aqueous phase of stools (see Laboratory analysis). The study was approved by the Human Subject Committee of the University of Toronto.

LABORATORY ANALYSIS
Labelling index technique and measurement
The biopsies were immersed in 15 ml medium (alpha-minimal essential medium minus RNA DNA + 10% fetal calf serum + penicillin-streptomycin (Gibco 600-5070)dil 1/100 in medium). They were cleaned under a dissecting microscope and transferred into 2 ml sterile septum fitted vials (Varian, no. 96-0000 99-00) containing 1·2 ml medium. No more than two biopsies were placed into any one vial. Six microlitres

of 1 mCi/ml tritiated thymidine (5 µCi/ml final concentration) (Amersham, Oakville, Ontario, Canada) was added and, after sealing, 2·4 ml pure oxygen was injected through the septum.[29 30] The vials were incubated on a rocking stage at 37°C for 90 minutes. After incubation, oxygen was released by inserting a needle attached to a 3 ml syringe into the septum. The specimens were washed with six changes of Krebs-Ringer solution at room temperature. For purposes of orientation, they were placed mucosa side up on pieces of Gelfoam (Upjohn Co, Don Mills, Ontario, Canada), moistened with Krebs-Ringer, fixed in 10% phosphate buffered formalin, sectioned and processed for autoradiography.[31]

The biopsies were examined under the microscope by a technologist who was blinded as to the origin of the specimen. Data on crypt and cell number were recorded on a microcomputer for analysis and labelling index (LI) in percentage was computed for each specimen and subject according to the following formula:

$$LI = \frac{\text{number of labelled cells counted}}{\text{number of total cells counted}}$$

A cell was considered labelled when five or more grains were overlying the nucleus. Because of differences in size, the number of crypts and cells scored varied greatly among the different specimens but at least 2000 cells were counted for each of them. Seven sets of biopsies could not be evaluated because the crypts were not cut along a longitudinal plane.

Biochemical analysis of faeces
The aqueous phase of faeces was extracted from the stool by a method previously described.[6] Briefly, the thawed samples were diluted 1:1 by weight with deionised water and homogenised. pH was then measured using a special spear tipped electrode (8163 Ross TM, Union Research Inc, Cambridge, MA, USA). Aliquots of the homogenate were centrifuged at 20000 g for two hours at 18-20°C. The supernatant water was carefully removed and assayed for bile acids by gas chromatography after derivatization.[32] Results are given for the average of two determinations. Calcium concentrations was measured in the supernatant by standard calorimetric methods.[33]

NUTRIENT ANALYSIS
Three day food records were evaluated by the Nutrition Coordinating Center at the University of Minnesota, Minneapolis. Daily intakes of major macronutrients and vitamins were assessed by computerised nutrient data base.

STATISTICAL ANALYSIS
Pertinent demographic and dietary information was

Table 3 Comparison of values for LI, faecal pH, total bile acids and deoxycholic acid (DOC) pre and postintervention

Variable	Group*	Before†	After†	Change‡	p§
LI (%)	PL	4·0 (2·4)	4·4 (2·4)	0·3 (1·7)	
	CA	4·9 (2·9)	6·5 (3·4)	1·8 (2.3)	0·11
Faecal pH	PL	6·8 (0·5)	6·6 (0·5)	−0·3 (0·5)	
	CA	6·8 (0·8)	7·1 (0·7)	0·3 (0·5)	0·005
Bile acids (µg/ml)	PL	58 (12-395)	59 (7-639)	2 (62)	
	CA	62 (7-398)	50 (21-619)	18 (162)	0·76
DOC acid (µg/ml)	PL	33 (12-94)	19 (3-78)	−16 (23)	
	CA	23 (0-71)	37 (0-126)	12 (24)	0·004

*For group, PL represents placebo and CA represents calcium; †means and standard deviation (in parentheses) are given for LI and faecal pH; Medians and range (in parentheses) are given for bile acids and DOC acid; †change (Δ) represents mean (standard deviation) of changes observed for each individual in whom there is a before and after value. § p values from *t*-test given for differences in change with intervention (Δ) between the two groups.

obtained from subjects to evaluate comparability of the two groups. For those variables, Student's *t* test was used for analysis because distributions were symmetric. Student's *t* test was also used for calcium, faecal pH and labelling index. For most bile acids, the Wilcoxon's rank-sum test was used because the distribution was non-symmetric.

To assess whether significant changes occurred as a result of intervention, paired *t*-tests were used for dietary variables, calcium, faecal pH and labelling index. Wilcoxon's rank-sum test was used to assess changes in the bile acids.

In order to decide whether or not the changes could be attributed to calcium supplementation, changes in labelling index, calcium, faecal pH and bile acids for the two groups were compared. Student's *t* test was used because the values for all these changes were symmetrically distributed. Correlation analyses (Pearson's and Spearman's) were used to access the associations between dietary variables, LI and faecal biochemistry.

Results

Both groups were similar with respect to sex distribution, age, weight, body mass index (weight/height[2]) and stage of disease at surgery. The placebo group was taller, however (Table 1). The groups were not compared for the type of operation performed. Mean daily food intakes before intervention were comparable in terms of percentages of total calories derived from fat, protein, carbohydrate and on the amounts of fibre and starch consumed (Table 2). Overall, the placebo group consumed more

calories and calcium but only the former showed a statistically significant difference. After intervention, the groups remained essentially similar in their dietary intakes according to those same variables.

Compliance was assessed by pill count on the return visit. On the average 98% of the pills were taken in the placebo group while 95% were taken in the calcium group with a mean intervention time of 32.3 days (range 27-38). We also measured calcium concentration in the aqueous phase of faeces. As expected, there was a marked increase in the calcium group after intervention (from 4·77 mmol/l pre-intervention to 7·94 mmol/l postintervention, p=0·01) while no difference was apparent in the placebo group (from 5·04 mmol/l to 5·36 mmol/l) and comparison of the changes (△) was significant (p= 0·02).

Labelling index for the two groups was comparable before intervention (Table 3). After supplementation, however, LI was higher in the calcium group (p=0·06). Thus, cell proliferation was unaffected by intervention in the placebo group while there was a significant increase in the calcium group (p=0·05) (Fig. 1). Nevertheless, when these changes (△ and ▲) with intervention were compared, they were found not to be significantly different (p=0·11); therefore we cannot ascribe the increase in the calcium group to calcium supplementation. Changes
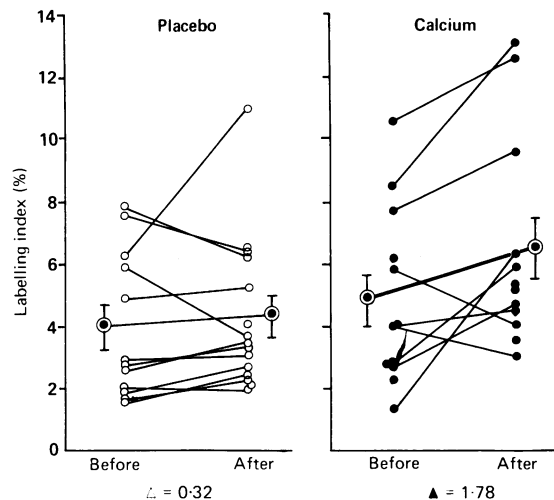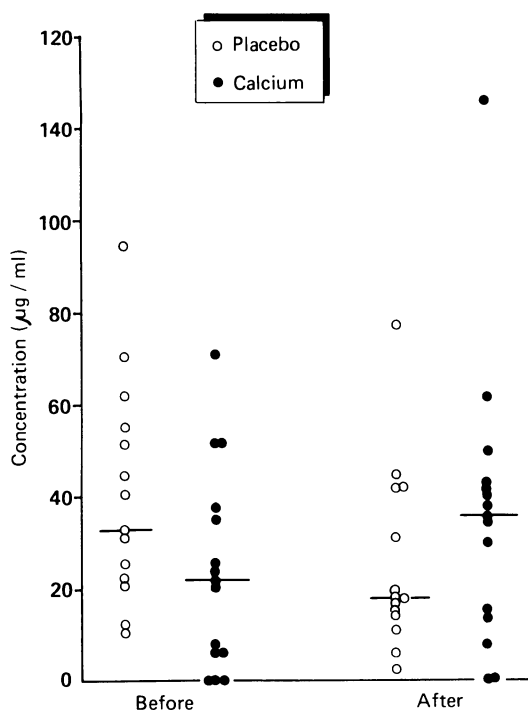

Fig. 2 *Deoxycholic acid (DOC) concentration in the aqueous phase of faeces before and after intervention for placebo (○) and calcium (●) groups. (–) represents group medians. Change with intervention significant at p=0·02 in placebo group (△), not significant at p=0·07 in calcium group (▲). Comparison of these two changes (△ and ▲) statistically significant at p=0·004.*

in LI were not compared for the various types of colonic resection – that is, right hemicolectomy, etc, because the small number of individuals in each subgroup would have made the results difficult to interpret. For faecal pH, there was no difference between the two groups before intervention but a significant increase was noted in the calcium group after supplementation (p=0·03) whereas there was a decrease in the placebo (p=0·07). Comparing these changes, the increase in the calcium group was significantly larger than in the placebo group (p=0·005) (Table 3).

The aqueous phase of stool samples was analysed for bile acids. Total bile acid concentrations were similar for both groups before and after intervention. For deoxycholic acid, there was an increase in concentration in the calcium group after intervention (p=0·07) while there was a decrease in the placebo group (p=0·02). Comparison of those changes showed a significant difference at p=0·004. Figure 2 shows the distribution of values for this compound. No significant correlation could be found between

Fig. 1 *Effect of intervention on labelling index (LI) for placebo and calcium group. Lines connect LI from the same individual; ⊙ represents group means. Comparison of mean values before and after intervention not significant for placebo group, significant at p=0·05 for calcium group. △ and ▲ represent means of change in LI with intervention within each group. 95% confidence intervals for △ is (−0·78, 1·42) and for ▲ is (0·01, 3·55), p=0·11.*

labelling index, faecal pH, bile acid concentrations and calcium concentration in the aqueous phase of faeces. No dietary variable was associated with LI, faecal pH or bile acids.

Discussion

Calcium has become a nutrient of major interest in today's nutrition oriented society. Supplementation with up to 1500 mg/day has been recommended for postmenopausal women to prevent osteoporosis[34] although not everyone agrees on its efficacy.[35] Two papers from the same research group recently showed a reduction in mucosal cell proliferation of subjects at high risk for colon cancer after calcium supplementation[23 24] suggesting another possible beneficial effect. In the present study, we tested the effect of supplemental calcium (1200 mg/day) in a different high risk group that is patients previously operated upon for the disease. Before treatment individuals with this malignancy have been shown to have higher cell proliferation in the colonic mucosa using tritiated thymidine labelling (LI)[22] than normal subjects. Our goal was thus to use this method to determine whether or not supplementing calcium could lower cell replication after curative surgery. We used previously operated cancer patients receiving a placebo as controls because we wanted to study only the effect of calcium and because we wanted to make the groups as comparable as possible with regards to other possibly confounding variables – that is, resection. Indeed, they were similar on most variables studied except for the pre-intervention caloric consumption. This difference could be the result of failure of some of the volunteers to record their whole daily intake.

Our results were different from those of the previous trials.[23 24] We showed no lowering in LI after calcium supplementation in the calcium group compared with the placebo group. There were major differences between these two studies and ours which could account for the discrepancy in results. First, in Lipkin's study on individuals at high risk for familial colonic cancer,[24] patients were selected by virtue of their high LI and there was no control group; therefore, the decrease in LI observed after intervention could merely reflect a natural change over time. Alternatively, the decrease could have resulted from a change in diet during the study period, although the subjects were asked to continue on their conventional diets, but there were no dietary records to exclude this possibility. This last point is important as a high fat intake was shown to increase LI.[36] Second, in the earlier study, the subjects were given calcium for three months, while we intervened for one month. The effect of calcium on cell prolifer-

ation may require the longer period to manifest itself. This seems unlikely, however, as the animal experiments on which both trials were based showed an effect within a few hours. Third, in our study LI was lower than that reported by other investigators[22 37 38] and that seen in one of our previous studies[39] for patients with colon cancer. One has to remember that none of the previous work was done with patients who had had their disease resected; in other words, the subjects still had cancer when the biopsies were taken. Colon resection could reduce LI or the tumour could induce changes in cell proliferation in the entire organ which disappear when it is removed. Our values are closer to previously reported normal values – that is,4·9.[22] Alternatively, this could result from differences in diet of the different groups studied, the scoring criteria, or the timing of biopsies.

Buset reported similar findings to those of Lipkin's by showing a decrease in proliferation after calcium supplementation.[23] He suggested, however, that this was seen only in patients with high initial labelling index. Again, because this study did not include controls, one can speculate as to whether this decrease is secondary to calcium or merely a return to more normal values which happens over time. Also, no attempt to control for other potentially confounding parameters was made – that is, fat content, fibre content or other high calcium foods in the diet, casting some doubt on the real effect of calcium *in vivo*. After calcium supplementation, we observed an increase in stool pH, calcium concentration and deoxycholic acid concentration in the aqueous phase of faeces. All these changes ($\triangle$) when compared with those in the control group were significant (0·005, 0·02, 0·004 respectively). There was, however, no correlation between LI and deoxycholic acid or total bile acids.

The strength of our findings lies in the consistency of all the parameters we measured. LI, faecal pH and bile acid concentrations all point to the conclusion that calcium supplementation offers no benefit as measured through these potential markers of colon cancer risk. Furthermore, two animal studies from this Institute produced similar results; in those studies, high calcium was found to increase colonic tumour incidence, faecal pH and bile acids.[40]

The hypotheses underlying this study was that calcium binds with bile acids and takes them out of solution thus lowering their toxicity for the colonic epithelium. This binding process, however, is a complex reaction influenced by many interrelated factors. It may in fact interfere with bile acids solubility and reabsorption in the terminal ileum and thus increase the amounts delivered to the colon. The supplements may also affect faecal pH which is a

strong determinant of bile acid solubility in faeces and transformation of primary bile acids into their secondary counterparts.[41][42] In this trial, we found no lowering of colonic cell proliferation in colon cancer patients supplemented with calcium. Reported studies need to be carefully evaluated and more trials need to be done before we can reject or accept calcium as part of the chemoprevention armamentarium.

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