Dietary calcium does not reduce experimental colorectal carcinogenesis after small bowel resection despite reducing cellular proliferation

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Abstract

It has been proposed that colorectal carcinogenesis is accompanied by increased mucosal cell proliferation and that the converse may also apply. To examine this thesis, the crypt cell production rate (CCPR) was measured in eight groups of rats ($n=187$) that had received 1,2 dimethylhydrazine, 70% small bowel resection, supplemental dietary calcium, or a combination of these. Analysis of variance showed the following: (1) the CCPR decreased between the ileum and distal colon; (2) the CCPR decreased between 16 and 32 weeks; (3) 1,2 dimethylhydrazine and small bowel resection increased the CCPR and calcium decreased the CCPR independently of one another; (4) the CCPR interacted with 1,2 dimethylhydrazine x small bowel resection, calcium x 1,2 dimethylhydrazine and interacted between the site of bowel and calcium, 1,2 dimethylhydrazine, small bowel resection, and 1,2 dimethylhydrazine x small bowel resection ($p=0.014$ to $p<0.001$). The tumour yield was reduced by calcium in 1,2 dimethylhydrazine treated animals ($\chi^2=14.1$, $df=3$, $p<0.01$) but was unaffected by calcium in 1,2 dimethylhydrazine and small bowel resection treated animals despite significant differences in the CCPR. An increase of the CCPR both preceded and accompanied colorectal carcinogenesis but reduction of the CCPR was not invariably accompanied by reduced carcinogenesis.

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Figure 1: Experimental design. DMH=1,2 dimethylhydrazine; SBR=small bowel resection; CA=calcium.

diet, particularly one that is high in animal fat, seems to have a major influence on the development of colorectal cancer.^{1,2} Yet a population with a high dietary intake of fat, protein, and meat can also be at very low risk of colorectal cancer.³ One of the factors that may be protective is calcium,⁴ but the epidemiological data are contentious.⁵ Therefore, some investigators have examined the role of dietary calcium in influencing colonic crypt cell kinetics, since the latter may be a marker of neoplastic predisposition.⁶⁻¹⁰

Williamson and Rainey (among others) have proposed that conditions which increase intestinal cell proliferation also potentiate intestinal carcinogenesis.¹¹ Under experimental conditions, hyperplasia follows 20-80% of small bowel resection,^{12,13} jejunioileal bypass,^{14,15} subtotal colectomy,¹⁶ colostomy closure,¹⁷ pancreaticobiliary diversion,¹³ and topical instillation of bile acids.¹⁸ These manoeuvres are all associated with increased colonic tumourigenesis.^{11,19-22} In man, there seems to be an expanded colonic crypt cell proliferative compartment in a number of situations associated with an increased risk of colorectal cancer, including in subjects with a family history of colorectal cancer²³ and in patients with ulcerative colitis,²⁴ as well as those with sporadic adenoma or cancer^{10,25} and those with familial adenomatous polyposis.²³

It is of interest that supplementary calcium is able to abolish the increased tumour yield after enterectomy²⁶ and will also reduce crypt cell production.²⁷ While the evidence linking increased cell proliferation and colorectal cancer is good, the converse is less clear cut. Moreover, one experimental study showed increased tumour formation with dietary calcium.²⁸ To help clarify some of these issues, a single experimental study was undertaken to compare the effects of dietary calcium and 70% small bowel resection on experimental colonic tumourigenesis and crypt cell proliferation in the terminal ileum, caecum, and distal colon, thereby permitting multivariate statistical analysis to determine independently significant and interacting factors influencing mucosal proliferation.

Methods

STUDY DESIGN (FIG 1)

(a) Effect of dietary calcium on normal small and large bowel mucosa proliferation

Forty animals were randomly divided into two equal groups of which one group received calcium supplementation. Eight animals from

each group were killed at 16 weeks and the remainder at 32 weeks.

(b) Effect of dietary calcium and 1,2 dimethylhydrazine on small and large bowel mucosa proliferation

Sixty animals received 1,2 dimethylhydrazine and were divided into two equal groups, of which one group received calcium supplementation. Eight animals from each group were killed at 16 weeks and the remainder (22 per group) were sacrificed at 32 weeks.

(c) Effect of dietary calcium, 1,2 dimethylhydrazine, and 70% small bowel resection on small and large bowel mucosa proliferation

Eighty seven animals underwent 70% small bowel resection and were divided into two groups. In the first group (n=40), half received calcium supplementation (n=20); 16 animals (eight receiving calcium) were sacrificed at 16 weeks; the remainder were sacrificed at 32 weeks. The second group (n=47) received 1,2 dimethylhydrazine, and 24 randomly chosen animals were given calcium supplementation. Four of these animals were lost for technical reasons and four because of intestinal obstruction; the remainder (n=39) were killed at 32 weeks.

The crypt cell production rates (CCPR) were determined at each point of sacrifice. For 24 hours before sacrifice at 32 weeks, animals were placed in metabolism cages and a 24 hour stool collection was performed for faecal fat measurement. At the time of sacrifice, blood was obtained for measurement of serum calcium, phosphate, and albumin. Finally, the intestinal tract was excised, fixed in formalin, stained with haematoxylin and eosin, then coded.

ANIMALS, CALCIUM INTAKE, AND CALCIUM SUPPLEMENTATION

Female Wister rats (Charles Rivers, UK, Ltd) weighing 100–150 g were housed in the Biomedical Services Unit (University of Birmingham) with a 12 hour regular light-dark cycle. The animals were weighed weekly and given food (diet 86A, Pilsbury Ltd, UK, containing 19% protein, 3.4% fibre, and 0.8% calcium) and water (6 mg calcium carbonate/l, Severn Trent Water Authority) ad libitum, which was recorded daily. The normal average dietary calcium intake was 140 mg/24 hours which was doubled to 280 mg/24 hours in the above specified groups by the addition of calcium lactate to the drinking water. Calcium supplementation was begun in all the relevant groups at six weeks.

CARCINOGENESIS

This was induced with subcutaneous 1,2 dimethylhydrazine (Aldrich Chemical Co Ltd) 40 mg/kg body weight, weekly for five weeks. Controls received carrier solution (saline/EDTA).

SMALL BOWEL RESECTION

A 70% small bowel resection was performed under general anaesthesia after measuring the small bowel and preserving the distal 10 cm of ileum. An end to end anastomosis was formed using a single layer of interrupted 6/0 silk.

CCPR

A previously in vivo stathmokinetic method was employed.^{29 30} Under halothane anaesthesia, six to eight animals were killed serially at 15–30 minute intervals for 135–180 minutes, after intraperitoneal administration of vincristine (Oncovin, Eli-Lilly, Basingstoke, UK) 1 mg/kg body weight. Mucosa was obtained from the caecum, distal colon 2 cm from the anus, and the terminal ileum 5 cm from the ileocaecal valve. The number of metaphase arrest figures were counted from 30 crypts at each site in the large bowel and from 10 crypts in the small bowel. The CCPR (cells/crypt/hour) was determined by the slope of the regression line comparing the number of mitotic figures with time.

SERUM CALCIUM, PHOSPHATE, AND ALBUMIN

This was determined by automated analysis in the Department of Chemical Pathology using a SMAC machine.

FAECAL FAT ANALYSIS

The fats were saponified with 5% alcoholic potassium hydroxide, extracted with HCl and ether, then measured calorimetrically.³¹

HISTOLOGICAL ANALYSIS

The slides were examined by a consultant pathologist (HT) who was unaware of the group origin of the tissue. Tumours were assessed as benign or malignant and were graded by degree of dysplasia (benign) or differentiation (malignant).

ETHICAL CONSIDERATIONS

The studies were undertaken under Home Office and University of Birmingham regulations ensuring the humane treatment of animals at all times.

TABLE 1 Mean (SD) animal weights at 16 and 32 weeks (the starting weights were all similar – mean 133 g (17) g, range 100–150 g)

Group	Weight (g) at 16 wks	Weight (g) at 32 wks
Control	282 (39)	336 (31)
Control+calcium	283 (29)	349 (27)
1,2 dimethylhydrazine	293 (20)	330 (28)
1,2 dimethylhydrazine+calcium	293 (21)	313 (32)
Small bowel resection	278 (34)	325 (36)
Small bowel resection+calcium	285 (38)	327 (27)
1,2 dimethylhydrazine+small bowel resection	277 (23)	323 (37)
1,2 dimethylhydrazine+small bowel resection+calcium	290 (25)	336 (42)

None of the groups was significantly different from the others at each of the time points.

TABLE II Summary of the number and site of tumours in the different groups receiving 1,2 dimethylhydrazine

Group	No of animals	No with tumours	Total no of GI tumours	No of tumours at each site			
				Colon	Duodenum	Anastomosis	Other
1,2 dimethylhydrazine	22	13	26 (11)*	24 (11)*	2	0	1†
1,2 dimethylhydrazine+calcium	22	5	11 (3)	8 (3)	2	0	1†
1,2 dimethylhydrazine+small bowel resection	21	11	17 (3)	15 (3)	1	1	1‡ (1)§
1,2 dimethylhydrazine+small bowel resection+calcium	22	15	25 (5)	18 (4)	4 (1)	3	0

*Numbers in parentheses are benign tumours; †renal carcinoma; ‡middle ear carcinoma; §mammary adenoma.

STATISTICAL ANALYSIS

Categorical data were analysed by the χ^2 test, and non-parametric continuous data by the Kruskal-Wallis and Mann-Whitney U (two tailed) tests. The data relating to CCPR were analysed using the *Statistical Package for the Social Sciences* (SPSS). Independently significant effects were investigated using a repeated measures analysis of variance (ANOVA). The within subjects repeated measures on crypt cell production were the three different sites (ileum, caecum, and distal colon) and the between subject factors were calcium, 1,2 dimethylhydrazine, and small bowel resection. All possible interactions were also tested for independent significance. For descriptive purposes the CCPR is given as the slope ($\pm$ SEM) of each regression line; the R-sq% is also given, which represents the extent to which each data set is described by a straight line. Unless stated otherwise, significance was set at $p<0.05$.

Results

ANIMAL WEIGHTS

There were no significant differences between

any of the animal groups with respect to weight (Table I).

TUMOUR YIELD

There was a significant reduction of tumours in the group receiving calcium supplementation and 1,2 dimethylhydrazine compared with the group receiving 1,2 dimethylhydrazine alone (Fig 2). There were no significant differences with regard to the proportions of benign or malignant tumours or their distribution (Table II). There were no significant differences in respect of the degree of dysplasia or the histological grades or stages (data not shown).

CCPR AT 16 WEEKS

The results are shown in Table III and the statistical analysis in Table IV. The bowel site had the greatest effect on crypt cell production (ileum>caecum>distal colon). Calcium, 1,2 dimethylhydrazine, and small bowel resection all had independently significant effects on crypt cell production at this time and there was a significant interaction between the site of the gut and small bowel resection. Both 1,2 dimethylhydrazine and small bowel resection individually increased CCPR while calcium generally reduced this, in a simple arithmetic manner (that is, there was no interaction).

CCPR AT 32 WEEKS

The results are shown in Table V, and the statistical analysis in Table VI. The independently significant effects found at 16 weeks were maintained at 32 weeks. Moreover, the additional groups at 32 weeks allowed testing of interactions between calcium, 1,2 dimethylhydrazine, and small bowel resection, revealing two way but not three way interactions. There was also a highly significant reduction in CCPRs at all sites from 16 weeks to 32 weeks ($p<0.001$) upon which the above changes were superimposed.

SERUM CALCIUM, PHOSPHATE, AND ALBUMIN

None of the results were significantly different between the groups (Table VII; p set at <0.02 because of multiple testing).

FAECAL FAT EXCRETION

Calcium increased faecal fat excretion in two of the three pairs of groups in which this was measured (Table VIII; p set at <0.02 because of multiple testing).

Figure 2: Tumour yield at 32 weeks of sacrifice.

TABLE III The mean (SEM) crypt cell production rate in units of cells/crypt/h (R-sq%) in the different groups at different sites of the bowel, sacrificed at 16 weeks

Group	Ileum	Caecum	Distal colon
Control	20.9 (1.5) (97.2%)	11.2 (0.6) (98.1%)	5.2 (0.4) (95.8%)
Control+calcium	22.1 (2.0) (95.5%)	7.8 (0.4) (98.2%)	5.3 (0.2) (99.5%)
1,2 dimethylhydrazine	25.9 (2.9) (94.0%)	12.4 (0.7) (98.1%)	8.7 (0.8) (91.5%)
1,2 dimethylhydrazine+calcium	25.4 (2.0) (97.1%)	6.1 (0.8) (96.0%)	6.4 (0.6) (95.5%)
Small bowel resection	39.3 (3.9) (95.4%)	12.8 (0.5) (99.3%)	9.9 (1.2) (89.1%)
Small bowel resection+calcium	23.7 (2.8) (91.0%)	9.1 (0.6) (95.5%)	8.9 (0.6) (95.3%)

Discussion

Our results are consistent with observations in man that mucosal proliferation decreases with distance from the ileocaecal valve to the rectum.²⁵⁻³² Two previous experimental studies have shown an opposite effect,³³⁻³⁴ which may be due to the different species or sex of animals used and the methods of assessing cellular proliferation (stathmokinetic technique *v* labelling index using tritiated thymidine). In agreement with

earlier studies, both carcinogen administration²⁶⁻³³ and 80% small bowel resection²⁷ increased the colonic CCPR, whereas supplementary calcium had the opposite effect.²⁷ Between 16 and 32 weeks, the CCPR became significantly decreased at all three intestinal sites. Time had a greater effect on reducing the CCPR than did dietary calcium. Two other experimental studies¹⁵⁻¹³ have shown an increase in the CCPR with age, but studies in man are variable.³⁵⁻³⁶ Our data are also consistent with observations in man that mucosal proliferation is increased in patients with large adenomas or cancer.¹⁰⁻²⁵ Since colorectal cancer is distributed predominantly in the distal large bowel and rectum where the CCPR is least, additional factors to those which cause cell proliferation may be important for tumourigenesis.

The latter notion is further supported by the tumour yields in the different groups, which did not always correlate with the mucosal proliferation rates (compare Fig 2 and Table V). For example, the tumour yields in the 1,2 dimethylhydrazine group and the 1,2 dimethylhydrazine plus small bowel resection plus calcium group were very similar but the distal colonic CCPR differed by a factor of $\times 2$. Of three previous studies involving 30–85% enterectomy, two showed an increase in tumour yield²¹⁻²⁶ and one, no difference.¹⁶ Hart *et al*²¹ administered 1,2 dimethylhydrazine (20 mg/kg) weekly for a total of 16 weeks (12 weeks after enterectomy) and at sacrifice at 50 weeks, the increase in tumours was at the anastomosis and not in the colon. Using Fischer rats given a total dose of 90 mg/kg azoxymethane, Williamson *et al*¹⁶ found no increase in tumours in the group that had 85% enterectomy and attributed this to poor weight gain, since body mass influences carcinogenesis. In the present study there were no significant differences in body weight at the time of sacrifice so this is not the explanation for the discrepancy in tumour yield between this and the two other studies.²¹⁻²⁶ Williamson and Rainey have made the important observation that ileal resection reduces the latent period for tumour formation but not the overall tumour yield.¹¹

Appleton *et al* have indicated that the mucosal proliferative changes brought about in experimental carcinogenesis by small bowel resection or calcium supplementation, or both, are closely linked to tumour yield.²⁶ This conclusion was based on sophisticated but completely separate experiments.²⁶⁻²⁷ In the first study, CCPR was measured in 58 male Sprague-Dawley rats (mean (SD) starting weight 358 (25) g) sacrificed seven weeks after an 80% small bowel resection or

TABLE IV Tests (ANOVA) for between and within subjects effects using unique SS on the production of crypt cells at 16 weeks sacrifice

Source	df	F ratio	p value
Between subjects:			
Calcium	1	9.3	0.011
1,2 dimethylhydrazine x site	1	58.2	<0.001
Small bowel resection	1	119.3	<0.001
Calcium x 1,2 dimethylhydrazine	1	0.1	NS
Calcium x small bowel resection	1	2.1	NS
Within subjects:			
Site	2	524.7	<0.001
Calcium x site	2	1.2	NS†
1,2 Dimethylhydrazine x site	2	1.8	NS
Small bowel resection x site	2	28.4	<0.001
Calcium x 1,2 dimethylhydrazine x site	2	1.6	NS
Calcium x small bowel resection x site	2	2.7	NS

Site refers to ileum, caecum, and distal colon; 'x' denotes interaction; †significant using pooled MSE (F=4.3, p=0.019).

TABLE V The mean (SEM) crypt cell production rate in units of cells/crypt/h (R-sq%) in the different groups at different sites of the bowel, sacrificed at 32 weeks

Group	Ileum	Caecum	Distal colon
Control	9.1 (0.6) (99.7%)	4.7 (0.5) (97.8%)	3.9 (0.6) (91.5%)
Control+calcium	7.0 (1.1) (91.5%)	3.9 (0.5) (93.5%)	2.1 (0.4) (85.8%)
1,2 dimethylhydrazine	16.6 (1.8) (97.1%)	9.7 (1.6) (90.6%)	10.0 (1.5) (91.2%)
1,2 dimethylhydrazine+calcium	10.3 (0.9) (97.2%)	3.6 (0.5) (88.9%)	3.9 (0.3) (96.4%)
Small bowel resection	17.3 (1.3) (97.5%)	6.7 (1.1) (90.5%)	11.2 (1.9) (89.4%)
Small bowel resection+calcium	12.6 (2.3) (88.3%)	4.4 (0.7) (91.3%)	4.9 (0.3) (98.1%)
1,2 dimethylhydrazine+small bowel resection	21.2 (1.3) (98.4%)	12.0 (1.0) (98.1%)	9.5 (0.9) (95.6%)
1,2 dimethylhydrazine+small bowel resection+calcium	11.2 (1.1) (97.1%)	4.2 (0.3) (97.0%)	4.9 (0.6) (91.5%)

TABLE VI Tests (ANOVA) for between and within subjects effects using unique SS on the production of crypt cells at 32 weeks sacrifice

Source	df	F ratio	p value
Between subjects:			
Calcium	1	64.6	<0.011
1,2 dimethylhydrazine	1	14.8	0.002
Small bowel resection	1	54.5	<0.001
1,2 dimethylhydrazine x small bowel resection	1	12.4	0.003
Calcium x 1,2 dimethylhydrazine	1	7.8	0.014
Calcium x small bowel resection	1	3.8	NS
Calcium x 1,2 dimethylhydrazine x small bowel resection	1	0.7	NS
Within subjects:			
Site	2	2019.4	<0.001
Calcium x site	2	28.3	<0.001
1,2 dimethylhydrazine x site	2	11.4	<0.001
Small bowel resection x site	2	86.5	<0.001
1,2 dimethylhydrazine x small bowel resection x site	2	41.2	<0.001
Calcium x 1,2 dimethylhydrazine x site	2	15.5	<0.001
Calcium x small bowel resection x site	2	1.8	NS
Calcium x 1,2 dimethylhydrazine x small bowel resection x site	2	1.2	NS

Site refers to ileum, caecum, and distal colon; 'x' denotes interaction.

TABLE VII Results of mean (SD) serum calcium, phosphate, and albumin at 16 weeks

Group	Calcium (mmol/l)	Albumin (g/l)	Phosphate (mmol/l)
Control	2.66 (0.16)	36.5 (3.9)	2.04 (0.27)
Control+calcium	2.75 (0.16)	35.7 (3.0)	2.20 (0.30)
1,2 dimethylhydrazine	2.76 (0.08)	39.7 (3.2)	1.73 (0.23)
1,2 dimethylhydrazine+calcium	2.84 (0.16)	39.0 (3.4)	1.91 (0.50)
Small bowel resection	2.96 (0.11)	37.0 (3.7)	3.82 (1.11)
Small bowel resection+calcium	2.84 (0.15)	35.0 (4.4)	2.28 (0.41)

All comparisons NS.

TABLE VIII Results of mean (SD) total faecal fat excretion at 32 weeks

Group	Total faecal fat (μmol/g)
Control	50 (10)*
Control+calcium	103 (20)†
Small bowel resection	65 (20)‡
Small bowel resection+calcium	113 (20)§
1,2 dimethylhydrazine+small bowel resection	80 (10)
1,2 dimethylhydrazine+small bowel resection+calcium	100 (20)

* *v* † p<0.01; ‡ *v* § p<0.01; all other comparisons NS.

transection and with or without calcium supplementation; no carcinogen was given.²⁷ In the second study, tumour yield (but not CCPR) was determined in 53 similar animals (mean (SD) starting weight 150 (14) g) given azoxymethane for six weeks before using an identical protocol, but delaying sacrifice until 25–27 weeks later.²⁶ In contrast, we have used a single experiment. At 16 weeks, there was an increase in CCPR by 1,2 dimethylhydrazine or 70% small bowel resection and a decrease in CCPR by calcium; there were no interactions between these factors (Tables III & IV). The results at 32 weeks (Tables V and VI) confirmed the former findings but also showed significant interactions by calcium or small bowel resection with the carcinogen. Unlike Appleton *et al*,^{26,27} we did not find complete linkage between CCPR and tumour yield. This discrepancy might be explained by differences in the species or sex of the animals used, the extent of small bowel resection (70% *v* 80%), or the type of carcinogen used, although it should be noted that 1,2 dimethylhydrazine is the natural precursor of azoxymethane. Alternatively, the discrepancy may be explained by the experimental protocols since the studies of Appleton *et al*^{26,27} could not take into account the interactions between carcinogen and calcium or enterectomy.

The effects of calcium on tumour promotion may be the result of direct or indirect action. *In vitro*, high extracellular calcium concentrations inhibit the proliferation of human colonic epithelial cells⁷ and several colonic cancer cell lines.³⁷ A systemic mode of action has been suggested for calcium⁴ but this seems unlikely from the present study since the serum values of calcium and phosphate were similar in the different groups. Supplementary dietary calcium has been shown in some studies to reduce colonic cell proliferation in man.^{6,7,9,10} The indirect mechanism of action proposed to explain such an effect implicates the sequestration of bile acids³⁸ or other acidic lipids, such as fatty acids.³⁹ It seems likely that the proposed mechanisms of action of calcium, involving either the precipitation of calcium bound bile salts or fatty acids as soaps, and the negation of such actions by phosphate is an over simplification. For example, bile acids are probably precipitated from the water fraction of faeces as calcium-phosphate-bile salt complexes.⁴⁰ While increased dietary calcium may increase faecal calcium and the concentration of the bile acid deoxycholate in man, colonic mucosal proliferation may remain unaffected.⁸ In the present study, we have shown an increase in faecal fat excretion (albeit not significant) in the 1,2 dimethylhydrazine treated small bowel resection group given calcium while the CCPR was significantly reduced, yet the tumour incidence was similar.

A similar paradox pertains in the relationship between dietary fibre, colonic cell proliferation, and experimental carcinogenesis. Although most studies² show a protective effect by 20–40% dietary wheat bran, this also causes appreciable epithelial cell proliferation and mucosal hyperplasia.⁴¹ Oat bran has been shown to increase tumourigenesis yet it does not effect colonic cell proliferation.⁴² Indeed Galloway *et al*⁴³ reported the highest proliferation activity in a dietary

experimental group with the lowest incidence of tumours and the converse was also found.

A number of interactions were shown between crypt cell production, 1,2 dimethylhydrazine administration, small bowel resection, and different sites of the intestine. Small bowel resection results in significant changes in the delivery of a variety of luminal factors to the colon including fibre, bile salts, other fats, ions, and water. The precise mechanisms of action and interaction of these factors on carcinogenesis require elucidation in order to understand fully their relationship to colonic cell proliferation. It seems that while an increase in colonic cell proliferation usually precedes and accompanies colorectal carcinogenesis, a reduction of cellular proliferation is not of itself always sufficient to reverse the process. We and others have previously shown increased mucosal cell proliferation in patients with adenomatous polyps or cancer^{10,25,35} and a reduction of mucosal cell proliferation after calcium supplementation.^{6,7,9,10} Moreover, sulindac has been shown to cause regression of rectal polyps in familial adenomatous polyposis without there being an alteration in the Ki 67 index, an alternative marker of cellular proliferation.⁴⁴ Until long term studies are available, caution should be exercised in the use of colonic cell proliferation indices as markers of neoplastic potential in human dietary intervention studies.

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