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Original Paper

Calcium Inhibits Colon Carcinogenesis in an Experimental Model in the Rat

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Different dietary factors can affect colorectal cancer incidence. However, the effect of increased levels of dietary calcium on neoplasms is unclear. The present study was designed to examine the effect of a low calcium supplement on experimental colon carcinogenesis induced by parenteral administration of dimethylhydrazine (DMH). One hundred and twenty 10-week-old Sprague–Dawley rats were divided into five groups of equal sex distribution. The 10 rats in group A (control group) received no treatment; the 30 rats in group B (DMH group) were injected subcutaneously with 18 weekly doses of 21 mg/kg DMH; the 20 rats in group C (EDTA control group) received EDTA solution only; the 30 rats in group D (calcium group) received calcium at 3.2 g/l by adding calcium lactate to the drinking water from the start until the conclusion of the experiment; and the 30 rats in group E (DMH + calcium group) received oral calcium supplements at the same dose as the rats in group D (calcium group) and the same DMH injections as the rats in group B (DMH group). The rats were sacrificed at 25–34 weeks. In group E, we observed a significant diminution in the number of tumours ($P = 0.01$); an increase in the number of tumour-free animals ($P = 0.006$); a change in tumour location towards the distal colon ($P < 0.025$); more adenomas ($P = 0.02$); and a diminution of adenocarcinomas and mucinous carcinomas, although this was not significant. We conclude that a low dietary calcium supplement in rats inhibits colon cancer carcinogenesis induced by DMH, and changes tumour location towards the distal colon.

Key words: calcium, carcinogenesis, colon, cancer, tumours, neoplasm
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INTRODUCTION

COLORECTAL CANCER has a high mortality rate in the Western world [1, 2]. The incidence of colon cancer has increased, but the colon cancer-derived mortality rate has not changed substantially over the past 25 years [2]. The causes of colon cancer have not been fully identified. Epidemiology [3–6], experimental [7–10] and genetic studies [11] suggest that colon cancer genesis may involve genetic susceptibility, carcinogens, promoters, and inhibitors. Environmental factors, particularly dietary factors such as fat intake, promote the development of colon cancer [2, 3, 6, 12]. Based on these observations, it would appear that prevention may offer the best opportunity of controlling the disease [3–5, 9, 10, 12].

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Different authors suggest that calcium reduces the risk of colon cancer, a theory supported by clinical and experimental studies [13–18]. It has been hypothesised that calcium reduces the risk of colon cancer by forming insoluble soaps with ionised fatty acids and secondary bile acids in the colon lumen, potentially diminishing the proliferative stimulus of these substances on the colon mucosa [17, 19]. A direct effect of calcium on the proliferative activity of the mucosa has also been suggested [20]. However, case–control and cohort studies of colorectal cancer are not conclusive; some studies reported an inverse association with calcium [21, 22], while others suggested no relationship [23]. Nevertheless, it is difficult to perform a prospective study on the influence of different environmental factors in human colon cancer [3, 4]. For these reasons, an experimental carcinogenesis model using rats with tumours induced by 1,2 dimethylhydrazine

(DMH) can be useful for obtaining results relevant to human disease [7, 8, 24]. Some authors have found a relationship between calcium supplements and diminution of invasive colonic tumour incidence [7, 14, 25] in experimental models, although their results were not conclusive and their use of high calcium doses makes it difficult to compare the results with the human situation. The aim of this study was to examine the effect of low dose calcium dietary supplement on experimental colon carcinogenesis.

MATERIALS AND METHODS

One hundred and twenty 10-week-old Sprague–Dawley rats, 60 males and 60 females of identical strain, were allocated to one of five groups with equal sex distribution. The 10 rats in group A (control group) received no treatment. The 30 rats in group B (DMH group) were injected subcutaneously with 18 weekly doses of 21 mg/kg DMH (Sigma, St Louis, Missouri, U.S.A.). The DMH was prepared using a dilution of 400 mg of DMH in distilled water containing 37 mg of EDTA (Farmitalia) as a stabilising agent, buffered to pH 6.5 with NaOH [26]. The solution was prepared weekly before injection into the lumbar zone of the rat. The 20 rats in group C (EDTA control group) received the same volume of EDTA solution only. The 30 rats in group D (calcium group) received calcium at 3.2 g/l by adding calcium lactate to the drinking water from the beginning until the conclusion of the experiment. The 30 rats in group E (DMH + calcium group) were treated with 18 weekly subcutaneous injections of DMH and 3.2 g/l of calcium by adding calcium lactate to the drinking water until the conclusion of the experiment. The daily calcium intake was quantified by controlling daily drinking water consumption. The rats' daily calcium intake was increased by approximately 40%.

All rats were fed on standard rodent diet (ITM-R20, Letica) with 3% fat and 0.8% calcium. The daily food consumption of the treated groups was controlled throughout the study. Fifty per cent of the animals were weighed every week until sacrificed.

The rats were sacrificed between week 25 and week 34 by lethal intraperitoneal injection of chloral hydrate at 4.5%. In order to avoid time effect variability, a fixed number of rats from each group was sacrificed during the same week. The weight of all the animals was recorded.

At autopsy, thoracic and abdominal cavities were examined. The colon and rectum were removed, opened along the antimesenteric border and gently cleaned of residue with water. The entire gastrointestinal tract was palpated for tumours, adhesions or other abnormalities. The number of tumours, their location and size were recorded. Tumours and normal colonic mucosa specimens taken from the caecum and ascendant (right colon) and from the transverse colon and from the descending rectum (left colon) were removed and fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin for histological observation. The tumours obtained were classified according to the degree of invasion, differentiation grade of adenocarcinomas, tumour size and macroscopic aspect, and the presence of associated lymphoid tissue. Metastasis, small intestine tumours and extra-intestinal tumours were also noted.

The chi-square test was performed on the DMH and DMH + calcium groups in order to compare tumour incidence, anatomopathological findings, total colorectal tumours, left versus right colon tumour totals, average tumour size, the presence of metastasis and tumour association with lymphoid tissue extension. In the event that the conditions for application were not fulfilled, Fisher's exact test was employed.

Multiple regression was used to evaluate time as a covariant in body weight and tumour incidence. The Mann–Whitney non-parametric U test was used to compare body weights at death. Throughout the study, the Kruskall–Wallis test was used to evaluate the food consumption and body weight of the animals in the treated groups.

RESULTS

Four male rats (3%) died before completion of the study, one from the DMH group, one from the calcium group and two from the DMH + calcium group. These rats were excluded from the analysis. The body weight at death of all animals from the DMH + calcium group was significantly lower than that of the animals from the other groups for both genders ($P < 0.01$). These data are presented in Table 1, where control groups A and C are combined to form a single control group, given that the differences in the body weight at death in both groups were negligible. No differences in body weight were observed for animals in group B (DMH) relative to animals in group D (calcium). However, a diminution of body weight was recorded for animals in group E (DMH + calcium) relative to animals in group B (DMH; $P = 0.0002$ for males and $P = 0.0001$ for females) or group D (calcium; $P = 0.005$ for males and $P = 0.0002$ for females). (Figure 1). With regard to food consumption, a diminution was observed in animals from group E (DMH + calcium) relative to group B (DMH; $P = 0.001$ for males and $P = 0.032$

Table 1. *Body weight at death*

Group	Sex	Number	Weight (g) mean ± S.D.
Control	Males	15	659.0 ± 52.82
(groups A + C)	Females	15	332.0 ± 26.78
Calcium	Males	14	596.4 ± 29.66
(group D)	Females	15	336.6 ± 39.65
DMH	Males	14	556.6 ± 91.54
(group B)	Females	15	355.3 ± 34.61
DMH + calcium	Males	13	457.7 ± 78.82*
(group E)	Females	15	296.6 ± 22.11**

Mann–Whitney U test: *$P < 0.005$ versus others groups; **$P < 0.002$ versus others groups. S.D., standard deviation; DMH, dimethylhydrazine.

Table 2. *Tumour incidence and distribution*

	Mean no. tumours/rat (± S.D.)	Total no. tumours	Tumour distribution in colon	
			Right	Left
DMH ($n = 29$)	2.24 ± 1.26	65	31 (48%)	34 (52%)
DMH + calcium ($n = 28$)	1.35 ± 1.20	38*	11 (29%)	27 (71%)**

*$P = 0.01$ versus DMH group; **$P < 0.025$ versus DMH group. S.D., standard deviation; DMH, dimethylhydrazine.

Table 3. Tumour classification

Group	Adenocarcinoma	Mucinous carcinoma	Carcinoma *in situ*	Adenoma	Dysplasia
DMH ($n=65$)	28 (43%)	20 (31%)	5 (8%)	1 (2%)	11 (17%)
DMH + calcium ($n=38$)	14 (37%)	7 (18%)	2 (5%)	7 (18%)*	8 (21%)

*$P=0.02$ versus DMH group. DMH, dimethylhydrazine.

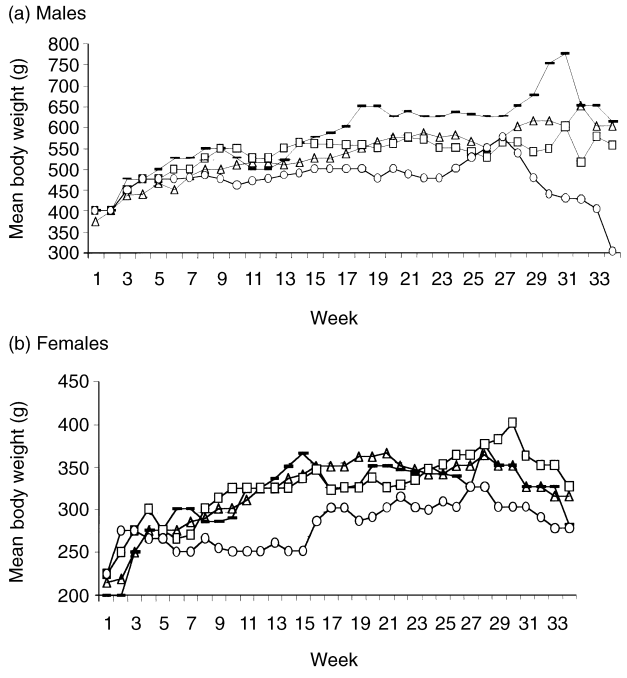

Figure 1. Changes in mean body weight. - ▬ - control, -△- calcium, -□- DMH, -○- CAL-DMH.

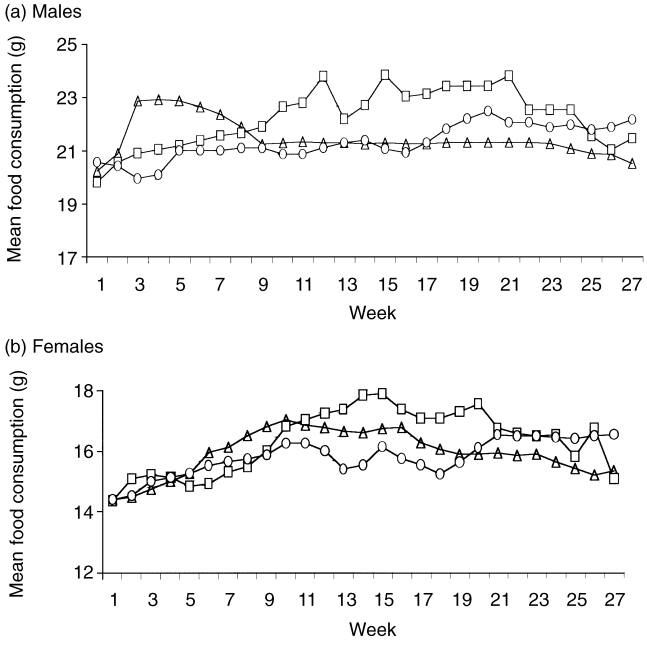

Figure 2. Mean food consumption of each group. -△- calcium, -□- DMH, -○- CAL-DMH.

in females) and in animals from group D (calcium) relative to group B (DMH; $P=0.004$ in males and $P=0.01$ in females). There was no significant difference between groups D and E (Figure 2).

Tumours appeared only in DMH treated groups, 65 in group B (DMH) and 38 in group E (DMH + calcium) ($P=0.01$; Table 2). In the DMH + calcium treated group, the majority of tumours (71%) were located in the left colon ($P<0.025$), whereas in the DMH treated group, tumours were distributed equally between the right and left colons. DMH + calcium treated animals had significantly more adenomas ($P=0.02$) and less adenocarcinomas and mucinous carcinomas than animals in the DMH treated group, although this variable only approached significance ($P=0.067$; Table 3).

There were a higher number of tumour-free rats in group E, (DMH + calcium) compared with group B (DMH treated; $P=0.006$; Table 4). Furthermore, the number of rats which developed two or more colon tumours was lower in group E than in group B, 11 in the DMH + calcium treated group compared with 20 in the DMH treated group ($P<0.01$; Table 4). There was no difference in tumour size (68% tumours < 0.8 mm in the DMH + calcium group compared with 71% in the DMH group), in the appearance of metastasis (55% in the DMH + calcium group compared with 49% in the DMH group) or in associated lymphoid tissue (18% in the DMH + calcium group compared with 9% in the DMH group). However, a reduction in the number of small bowel tumours in animals from the DMH + calcium group was detected: an increment of tumours in the ileum ($P=0.003$) was accompanied by a reduction of tumours in the duodenum and jejunum, but the result only approached significance ($P=0.06$; Table 5).

Table 4. Effect of calcium on dimethylhydrazine (DMH)-induced carcinogenesis

	Number of tumours		
Group	None	One	Two or more
DMH ($n=29$)	1 (3%)	8 (28%)	20 (69%)
DMH + calcium ($n=28$)	10 (36%)$^+$	7 (25%)	11 (39%)*

$^+P=0.006$ versus DMH group; *$P<0.01$ versus DMH group.

Table 5. Small bowel tumour incidence and distribution

Group	Duodenum	Jejunum	Ileum
DMH ($n=15$)	7 (47%)	7 (47%)	1 (7%)
DMH + calcium ($n=11$)	2 (18%)	2 (18%)	7 (64%)*

*$P=0.003$ versus DMH group (Fisher's exact test). DMH, dimethylhydrazine.

DISCUSSION

This study has shown that a low diet calcium supplement significantly reduced the development of colonic tumours induced by DMH in rats with a dietary intake of only 3% fat. Recent findings seem to demonstrate that calcium acts as a potential chemoprotective colon cancer factor [27–29]. Our results are similar to those obtained by Wargovich and colleagues [15], but differ from those published by Beaty and associates [30] and Sitrin and coworkers [31], whose studies did not reveal significant tumour reduction in groups treated with calcium.

In our study, a low-dose calcium supplement resulted in a decrease in the number of rats which developed multiple neoplasms and increased the number of tumour-free rats. Moreover, DMH + calcium treated rats also had significantly more adenomas and less, (non-significant) adenocarcinomas and mucinous carcinomas. Similar results have been obtained by others [15, 25, 31] who also found a diminution of tumours per rat in calcium treated animals. The reduction in the number of animals with multiple neoplasms could be due to individual predispositions at a genetic level [7]. Wargovich and colleagues [15] described a reduction in the number of carcinomas, but not in the average number of adenomas per animal whilst Sitrin and coworkers [31] found a decrease in tumour size as well as a tendency towards an increase in carcinomas *in situ* not seen in our study.

Our results suggest that calcium acts late in the pathogenic mechanism of colon cancer, diminishing the progression of adenomas to invasive adenocarcinomas [14]. Some authors have suggested that calcium acts late because it produces a decrease in initial malignant tumours without altering the appearance of adenomas and adenocarcinomas [14, 25].

In our study, calcium caused a relative increase in the number of tumours in the left colon. These data conflict with the results of others who found a reduction in the number of tumours in the distal colon, especially carcinomas *in situ* [30], with no significant differences. Such findings may be due to their use of a higher calcium dose which have a greater effect. It has been shown that under hypercalcaemia conditions increased calcium secretion occurs in the proximal colon mucosa [32], a fact which might facilitate the interrelation between the ion and harmful intestinal products. However, this has no relation to the interference of calcium in DMH metabolism; it has been proven that calcium alters luminal media when taken at high doses, with little systemic effect [27]. Therefore, it is unlikely that calcium could modify DMH metabolism, since its administration was parenteral.

A tumour location shift towards the distal colon could be explained by three mechanisms. First, cell turnover is dependent on colon site, being higher in the distal colon, and a low dose calcium supplement may be better able to inhibit less dynamic cell proliferation [17]. Cell proliferation increases with a hypocalcaemic diet, but decreases with oral calcium supplements [18]. Second, the fact that calcium significantly inhibited tumour development in the proximal colon may indicate that tumour production in this site is different from that in the distal colon, as others have suggested [33, 34]. Finally, deficient calcium secretion occurs in the distal colon when calcium is added to the diet [32].

We observed a reduction in body weight in animals treated with DMH + calcium in comparison with the other groups. Beaty and associates [30] described similar findings, although in their experiment they administered vitamin D simultaneously with calcium, observing a decrease in body weight in animals treated with low doses of vitamin D (0.1 mg/kg diet) and high doses of calcium (15 g/kg diet). Calcium intake has the effect of provoking a reduction in food consumption; animals in the calcium treated and DMH + calcium treated groups consumed less food. This may be explained in terms of calcium having an anorexigenic effect or causing defective fat absorption as the intestinal calcium concentration increases [25]. DMH had no effect over food intake, but it did bring about a decrease in animal body weight, probably due to its aggressive effect on the mucosa. Others did not report a reduction in body weight [15, 25, 31].

In our study, rats in the DMH + calcium group developed a lower number of extracolonic tumours than rats in the DMH group. These findings concur with those of others who found a non-significant reduction of duodenal tumours in rats that received calcium [25]. In our study, there was an increase in ileum tumours in the group that received calcium. The reason for this may lie in the ileal mucosa secretion which prevents calcium concentration by generating an ion deficiency to counteract the deleterious effect of certain metabolites [32], since the ileum has the largest concentration of biliary salts and toxic substances which are associated with the pathogenesis of colon cancer [35].

We conclude that a low-dose dietary calcium supplement has an inhibitory effect on colon cancer carcinogenesis induced by DMH, shifting the location of the tumours towards the distal colon and increasing the number of tumour-free rats. Calcium intake also seems to have an influence on the production and location of small bowel tumours.

1. National Cancer Institute. Division of Cancer Prevention and Control Surveillance Program. Cancer statistics review 1973–88. US Department of Health and Human Services, National Institutes of Health. *NIH Publication* 1991, **91**, 2789.
2. Greenwald P. Colon cancer overview. *Cancer* 1990, **70**, 1206–1215.
3. Weisburger JH. Causes, relevant mechanisms and prevention of large bowel cancer. *Semin Oncol* 1991, **18**, 316–336.
4. Trock B, Lanza E, Greenwald P. Dietary fiber, vegetables and colon cancer: critical review and metaanalyses of the epidemiologic evidence. *J Natl Cancer Inst* 1990, **82**, 650–661.
5. Birt DF. Effects of the intake of selected vitamins and minerals on cancer prevention. *Magnesium* 1989, **8**, 17–30.
6. Wilett WC. Relation of meat fat and fiber intake to the risk of colon cancer in a prospective study among women. *N Engl J Med* 1990, **323**, 1664–1672.
7. Pories SE, Ramchurren N, Summerhayes Y, Steele G. Animal models for colon carcinogenesis. *Arch Surgery* 1993, **128**, 647–653.
8. Amberger H. Different authoctonous models of colorectal cancer in the rat. *J Cancer Res Clin Oncol* 1986, **111**, 157–159.
9. Nelson RL. Dietary minerals and colon carcinogenesis. *Anticancer Res* 1987, **7**, 259–269.
10. Martin MS. Experimental intestinal carcinogenesis. *Cancer J* 1992, **5**, 5–10.
11. Bosman FT. Prognostic value of pathological characteristics of colorectal cancer. *Eur J Cancer* 1995, **31A**, 1216–1221.
12. McIntosh GH. Dietary modifications required for a balanced protective diet. *Prev Med* 1993, **22**, 767–774.
13. Pence BC. Role of calcium in colon cancer prevention: experimental and clinical studies. *Mut Res* 1993, **290**, 87–95.
14. Wargovich M, Lynch PM, Levin B. Modulating effects of calcium in animal models of colon carcinogenesis and short-term studies in subjects at increased risk for colon cancer. *Am J Clin Nutr* 1991, **54**, 202S–205S.
15. Wargovich MJ, Allnutt D, Palmer C, Anaya P, Stephens LC. Inhibition of the promotional phase of azoxymethane-induced

colon carcinogenesis in the F344 rat by calcium lactate: effect of simulating two human nutrient density levels. *Cancer Lett* 1990, **53**, 17–25.
16. Govers MJAP, Termont DSM, Van der Meer R. Mechanism of the antiproliferative effect of milk mineral and other calcium supplements on colonic epithelium. *Cancer Res* 1994, **54**, 95–100.
17. Newmark HL, Lipkin M. Calcium, vitamin D, and colon cancer. *Cancer Res* 1992, **52**(Suppl.), 2067s–2070s.
18. Nobre-Leitao C, Chaves P, Fidalgo P, *et al.* Calcium regulation of colonic crypt cell kinetics: evidence for a direct effect in mice. *Gastroenterology* 1995, **109**, 498–504.
19. Lipkin M, Friedman E, Winawer SJ, Newmark H. Colonic epithelial cell proliferation in responders and nonresponders to supplemental dietary calcium. *Cancer Res* 1989, **49**, 248–254.
20. Lipkin M, Newmark H. Effect of added dietary calcium on colonic epithelial cell proliferation in subjects at high risk for familial colonic cancer. *N Engl J Med* 1985, **313**, 1381–1384.
21. Garland C, Barkett-Connor E, Rossof AH, Shekelle RB, Criqui MH, Paul O. Dietary vitamin D and calcium and risk of colorectal cancer: a 19-year prospective study in men. *Lancet* 1985, **36**, 307–309.
22. Barsoum GH, Hendrickse C, Winslet MC, *et al.* Reduction of mucosal crypt cell proliferation in patients with colorectal adenomatous polyps by dietary calcium supplementation. *Br J Surg* 1992, **79**, 581–583.
23. Negri E, La Vecchia C, D'Avanzo B, Franceschi S. Calcium, dairy products, and colorectal cancer. *Nutr Cancer* 1990, **13**, 255–262.
24. Viñas J, Fortuny JC, Panades MJ, *et al.* Appearance of ear tumours in Sprague–Dawley rats treated with 1,2 dimethylhydrazine when used as a model for colonic carcinogenesis. *Carcinogenesis* 1992, **13**, 493–495.
25. Appleton GVN, Davies PW, Bristol JB, Williamson RNC. Inhibition of intestinal carcinogenesis by dietary supplementation with calcium. *Br J Surg* 1987, **74**, 523–525.
26. Barkla DH, Tutton PJM. Ultrastructure of 1,2 dimethylhydrazine-induced adenocarcinomas in rat colon. *J Natl Cancer Inst* 1978, **61**, 1291–1298.
27. Appleton GVN, Owen RW, Wheeler EE, Challacombe DN, Williamson RCN. Effect of dietary calcium on the colonic luminal environment. *Gut* 1991, **32**, 1374–1377.
28. Appleton GVN, Bristol JB, Williamson RCN. Increased dietary calcium and small bowel resection have opposite effects on colonic cell turnover. *Br J Surg* 1986, **73**, 1018–1021.
29. Reddy BS. Micronutrients as chemopreventive agents. In Stewart BW, McGregor D, Kleihues P, eds. *Principles of Chemoprevention*. Lyon, IARC Scientific Publications no. 139, 1996, 221–235.
30. Beaty MM, Lee EY, Glauert HP. Influence of dietary calcium and vitamin D on colon epithelial cell proliferation and 1,2-dimethylhydrazine-induced colon carcinogenesis in rats fed high fat diets. *J Nutr* 1993, **123**, 144–152.
31. Sitrin MD, Halline AG, Abrahams C, Brasitus TA. Dietary calcium and vitamin D modulate 1,2-dimethylhydrazine-induced colonic carcinogenesis in the rat. *Cancer Res* 1991, **51**, 5608–5613.
32. Krishnamra N, Angkanaporn K, Deenoi T. Comparison of calcium absorptive and secretory capacities of segments of intact or functionally resected intestine during normo-, hypo-, and hypercalcemia. *Can J Physiol Pharmacol* 1994, **72**, 764–770.
33. McGarrity U, Peiffer LP, Colony PC. Cellular proliferation in proximal and distal rat colon during 1,2-dimethylhydrazine-induced carcinogenesis. *Gastroenterology* 1988, **95**, 343–348.
34. Heitman DW, Grubbs BG, Heitman TO, Cameron IL. Effects of 1,2-dimethylhydrazine treatment and feeding regimen on rat colonic epithelial cell proliferation. *Cancer Res* 1983, **43**, 1153–1162.
35. Van Der Meer R, Welberg JWM, Huipers F, *et al.* Effects of supplemental dietary calcium on the intestinal association of calcium, phosphate, and bile acids. *Gastroenterology* 1990, **99**, 1653–1659.

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