Calcium phosphate

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Calcium phosphate: an alternative calcium compound for dietary prevention of colon cancer? A study on intestinal and faecal parameters in healthy volunteers

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In an effort to reduce the risk of colorectal cancer development, oral calcium carbonate supplementation has been used in previous studies for the precipitation of cytotoxic bile acids and fatty acids. In human intervention trials its effect on mucosal hyperproliferation in the colorectum has not always been satisfactory. Because the complexation of calcium and bile acids requires the formation of calcium phosphate, we performed an intervention study in 14 healthy volunteers, giving them 1,500 mg calcium as $Ca_3(PO_4)_2$ for 1 week. The effects of tricalcium phosphate on luminal and faecal parameters of cytolytic activity were evaluated before, during, and after calcium phosphate supplementation. The cytolytic activity of faecal water and intestinal alkaline phosphatase activity in faecal water were not affected by supplemental calcium phosphate. In duodenal bile, the proportion of cholic acid tended to increase, whereas that of chenodeoxycholic acid tended to decrease during calcium phosphate supplementation. Neither concentrations of total and individual faecal bile acids, nor that of faecal fat were affected during calcium phosphate supplementation. It is suggested that, although phosphate is involved in bile acid precipitation, phosphate competes for calcium in the binding of fatty acids. This might possibly explain the unchanged cytolytic potency of faecal water, and therefore does not make tricalcium phosphate a suitable calcium compound for dietary intervention.

Key words: Bile acids, calcium phosphate, colon cancer prevention, cytotoxicity tests, dietary intervention, fatty acids.

Introduction

The potential role of calcium in the prevention of colorectal cancer has become a research area of major interest during the last decade (Welberg et al, 1991). Newmark et al (1984) speculated that supplemental calcium could convert free bile acids and fatty acids in the colon to insoluble calcium soaps, thereby decreasing the damaging (and hence poten-tially tumour-promoting) effect of these acids on the colonic epithelium.

In animal experiments a number of different calcium salts have been used to study the role of calcium in colon cancer prevention. Calcium lactate (Wargovich et al, 1983, 1990; Appleton et al, 1987; Reshef et al, 1990), calcium carbonate (Reshef et al,

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1990; Wargovich *et al*, 1984), calcium gluconate (Pence and Buddingh, 1988), calcium monophosphate (Bird *et al*, 1986), and calcium biphosphate in combination with calcium carbonate (McSherry *et al*, 1989) have all been reported to reduce mucosal hyperproliferation or the development of adenocarcinomas in the colon, induced by the administration of colonic carcinogenic or tumour-promoting agents. Furthermore, calcium chloride has been shown to have a beneficial effect on mucosal damage produced by deoxycholate in a perfused rat colon model (Rafter *et al*, 1986).

Dietary intervention studies with calcium in man have mainly been performed with calcium carbonate (Lipkin and Newmark, 1985; Lipkin *et al*, 1989; Gregoire *et al*, 1989; Rozen *et al*, 1989; Stern *et al*, 1990; Wargovich *et al*, 1992; Kleibeuker *et al*, 1993), and have merely studied its effect on epithelial cell proliferation, an intermediate biomarker for the risk of colorectal cancer development. In some of these studies, performed on patients at high risk of developing colorectal cancer, a beneficial effect of calcium was found on the proliferative activity of rectal epithelial cells (Lipkin and Newmark, 1985; Lipkin *et al*, 1989; Rozen *et al*, 1989; Wargovich *et al*, 1992). However, two other studies (Gregoire *et al*, 1989; Stern *et al*, 1990), performed in high risk patients after surgical colonic resections, and one study evaluating epithelial cell proliferation in the sigmoid (Kleibeuker *et al*, 1993), did not show a beneficial effect of calcium carbonate. This apparent discrepancy in effects could indicate that calcium carbonate is not the optimal formula in which to administer the calcium ion. There are several reasons to prefer calcium phosphate, instead of calcium carbonate, for the purpose of intervention. Van der Meer *et al* (1990) have shown that the complexation of bile acids in the human colon depends on the formation of insoluble calcium phosphate, probably with the stoichiometry of tricalcium phosphate. Moreover, supplemental calcium phosphate, in contrast to calcium carbonate, is expected not to increase the pH of the luminal content. The potential of bile acids and fatty acids to become highly surfactant in the colon is governed by colonic pH (Thornton, 1981). A raised colonic pH increases bacterial 7α-dehydroxylase activity and also the amount of ionization of bile and fatty acids, two mechanisms which enhance the damaging effect of these acids on epithelial cells (Thornton, 1981; McJunkin *et al*, 1981; Rafter *et al*, 1986). Therefore, we decided to study the feasibility of nutritional intervention with calcium phosphate in healthy volunteers. The effects of calcium phosphate on

luminal and faecal surfactants, and epithelial cell lysis caused by the faecal content, were evaluated.

Subjects and methods

Study design

Fourteen healthy volunteers (10 males, four females) with a mean age of 30 years (range 25–37 years) participated in the study, which was approved by the Medical Ethical Committee of the University Hospital of Groningen. All subjects gave informed consent. A dietician obtained a dietary history from all subjects and calculated the habitual dietary intake of energy and nutrients according to the Dutch food composition table. Subjects were instructed to maintain a calcium constant diet based on their habitual diet for 3 weeks. The first and third week served as control periods, hereafter referred to as first and second control period respectively. During the second week the diet was supplemented with 39 mmol calcium and 26 mmol phosphate as tricalcium phosphate ($Ca_3(PO_4)_2$; Merck, Darmstadt, Germany) tablets daily. Compliance with the usage of calcium phosphate was measured by pill counts. At the end of each week (days 7, 14 and 21), faeces of one bowel movement were collected for the determination of faecal pH, and concentrations of bile acids and fat. In faecal water intestinal alkaline phosphatase (ALP) activity was measured as a marker for epithelial cell lysis (Lapré *et al*, 1993a,b), and cytolytic activity of faecal water was also measured. At the end of the first 2 weeks (days 7 and 14) duodenal bile was collected for duodenal bile acid analysis.

Analysis of faecal parameters

After determination of faecal wet weight, faeces were diluted with distilled water (1:1, w/v) and homogenized in a Waring blender. Subsequently, faecal pH was recorded and the faeces were dried under vacuum and stored at room temperature until further processing.

Faecal bile acids were determined enzymatically using a 3α-hydroxysteroid dehydrogenase, as well as by gas-liquid chromatography (GLC). Only the four major faecal bile acids (lithocholic, deoxycholic, chenodeoxycholic and cholic acid) were quantified by GLC, as described previously (Cats *et al*, 1992). No efforts were made to identify and quantify the minor faecal bile acids. Individual bile acids were expressed as percentages of the sum of the major bile acids determined by GLC.

Faecal fat content was determined titrimetrically according to Van de Kamer *et al* (1949).

For the determination of cytolytic and ALP activity, faecal water fractions were prepared by reconstituting freeze-dried faeces with double-distilled water to the original amount of water. After incubation for 1 hour at 37°C, the samples were centrifuged at 15,000x*g*. The supernatants were carefully aspirated and stored at −20°C until further use. Previous experiments had demonstrated that the cytolytic activity of faecal water prepared with freeze-dried faeces using this procedure was not different from that of water obtained from fresh faeces (Lapré *et al*, 1991).

The cytolytic activity of faecal water was tested with the use of isolated human erythrocytes as described previously (Lapré *et al*, 1993a,b). In brief, Fe release from erythrocytes incubated for 6 hours at 37°C with different dilutions of faecal water, was measured using atomic absorption spectrophotometry. The lytic activity of each faecal sample was quantified as the area under the lytic curve, and expressed as a percentage of the maximal area, which implies 100% lysis at each dilution of faecal water.

In faecal water, the activity of intestinal ALP, an apical membrane enzyme, was determined spectrophotometrically with *p*-nitrophenyl phosphate as the substrate and *p*-nitrophenol as the reaction product, as described previously (Lapré *et al*, 1993a,b). The difference between total (non-inhibited) ALP activity and that after inhibition of the intestinal isozyme with L-phenylalanine accounts for the activity of intestinal ALP. The activity of ALP was expressed as μmol *p*-nitrophenol/min/ml faecal water (U/ml).

Analysis of duodenal bile acids

Duodenal bile was collected using an encapsulated, woolly string, the Entero-Test (HDC Corp, Mountain View, CA) as described previously (Vonk *et al*, 1986; Cats *et al*, 1992). Four hours after swallowing the Entero-Test, the string was withdrawn and stored at −20°C until further processing. The adsorbed duodenal bile acids were analysed by capillary GLC as described earlier (Vonk *et al*, 1986).

Statistical analysis

Statistical analyses of faecal parameters were based on Friedman's two way analysis of variance. Results on duodenal bile acid composition were tested using the Wilcoxon matched pairs signed rank test. The level of significance was set at $P < 0.05$.

Results

The calculated habitual dietary intake of energy and nutrients of all 14 subjects is summarized in Table 1. The pattern of a typical mixed Western diet was observed.

Compliance with the required usage of calcium phosphate was excellent. None of the subjects complained of side effects during calcium phosphate supplementation. No gastrointestinal discomfort and constipation were reported specifically.

All subjects but one succeeded in collecting a faecal bolus at the end of each week. Table 2 shows the results of the faecal parameters for the three periods studied. The mean faecal wet and dry weight was not significantly altered during calcium phosphate supplementation, although a slight increase was noted. No difference in the average faecal pH between the two control and calcium phosphate supplementation periods was found. The concentration of bile acids in the faeces was not affected by supplemental calcium phosphate. Table 3 shows faecal bile acid composition in the three periods studied. Neither the percentages of individual bile acids in the first control period nor those in the second control period differed from the percentages of individual bile acids during calcium phosphate supplementation. However, the percentage of lithocholic acid in the second control period was significantly reduced compared to that in the first control period.

In nine of the 14 subjects studied, the Entero-Test was applied successfully and duodenal bile acid composition could be determined. In duodenal bile, no significant changes in the percentages of the major bile acids were noted during calcium phosphate supplementation. However, a slight increase

Table 1. Estimated dietary intake of 14 healthy volunteers

Type of intake	Mean ± SEM
Energy (MJ/day)	8.2 ± 0.5
Protein (energy %)	16.9 ± 0.7
Fat (energy %)	35.9 ± 1.6
Carbohydrate (energy %)	47.2 ± 1.5
Calcium (mmol/day)	36.6 ± 1.8
Phosphate (mmol/day)	55.8 ± 3.4

Table 2. Faecal parameters during two control (control₁ and control₂) periods and one calcium phosphate supplementation period (n = 13)

Faecal parameter	Control₁	Calcium phosphate	Control₂
Wet weight (g/bolus)	127 ± 19	150 ± 23	115 ± 23
Dry weight (g/bolus)	31 ± 4	35 ± 4	28 ± 4
pH	6.7 ± 0.1	6.6 ± 0.1	6.5 ± 0.1
Bile acids (μmol/g wet weight)	4.9 ± 0.6	4.4 ± 0.3	4.7 ± 0.5
Total fat (μmol/g wet weight)	0.8 ± 0.1	1.0 ± 0.2	0.9 ± 0.2
Lytic activity (%)	43 ± 7	35 ± 8	39 ± 7
Alkaline phosphatase (U/ml)	0.94 ± 0.28	0.99 ± 0.22	0.94 ± 0.21

Mean ± SEM.

Table 3. Effect of supplemental calcium phosphate on the molar composition of faecal bile acids (n = 13)

Bile acid	Bile acid composition (%)		
	Control₁	Calcium	Control₂
Cholic	4 ± 1	3 ± 1	4 ± 1
Chenodeoxycholic	10 ± 2	9 ± 1	13 ± 2
Deoxycholic	54 ± 2	53 ± 2	53 ± 2
Lithocholic	32 ± 2	35 ± 2	30 ± 2[a]

Mean ± SEM. [a] $P < 0.05$ compared with the first control period.

of the percentage of cholic acid, concurrent with a slight decrease of the percentage of chenodeoxycholic acid, was observed during calcium phosphate supplementation compared to the first control period (Table 4).

The concentration of faecal fat was the same before, during and after supplementation with calcium phosphate (Table 2).

The mean cytolytic activity of faecal water did not show a statistically significant alteration during calcium phosphate supplementation compared to the first and second control periods (Table 2). A minimal reduction of the mean cytolytic activity might be observed, but no uniform reaction of cytolytic activity on calcium phosphate could be

Table 4. Effect of supplemental calcium phosphate on the molar composition of duodenal bile acids (n = 9)

Bile acid	Bile acid composition (%)	
	Control₁	Calcium
Cholic	40 ± 5	47 ± 5
Chenodeoxycholic	41 ± 4	36 ± 3
Deoxycholic	16 ± 3	15 ± 2
Lithocholic	2 ± 0	1 ± 0

Mean ± SEM.

observed (see Figure 1). The intestinal ALP activity of faecal water was not affected during calcium phosphate supplementation compared to both the control periods (Table 2).

Discussion

This study was performed to evaluate the effect of supplemental calcium phosphate on intestinal and faecal parameters in healthy volunteers. An intervention period of 1 week was chosen, because we had previously demonstrated that metabolic alterations observed during such a short period are comparable to those after intervention of longer
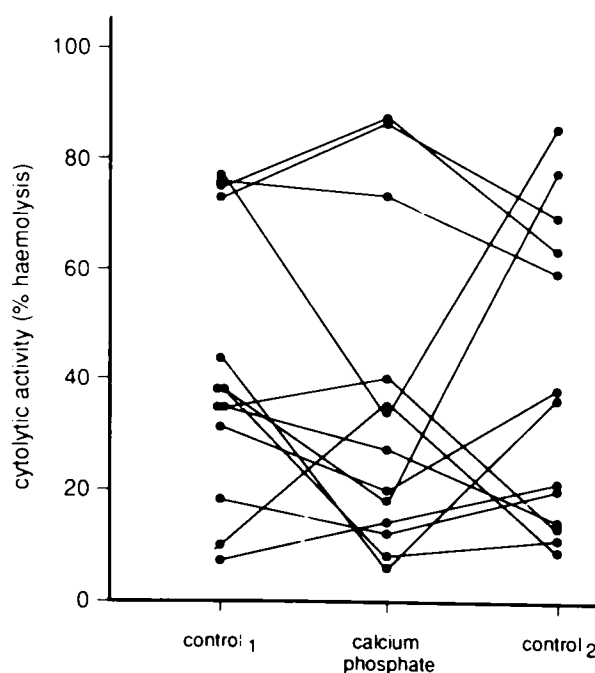

Figure 1. The effect of calcium phosphate on the cytolytic activity of faecal water (n = 13) during two control periods (control₁ and control₂) and one calcium phosphate supplementation period.

duration (Van der Meer *et al*, 1990; Welberg *et al*, 1993). The composition of the habitual diet, specifically the daily intake of calcium, phosphate and the macronutrients, was the same in subjects evaluated in the present study and in subjects evaluated in a former calcium carbonate supplementation study (Van der Meer *et al*, 1990; Lapré *et al*, 1993a).

As theoretically expected, calcium phosphate supplementation, in contrast to calcium carbonate, did not increase faecal pH (Gregoire *et al*, 1989; Van der Meer *et al*, 1990). An alkaline pH in the colon has been reported to have a stimulatory effect on bacterial degradation of primary bile acids into the more hydrophobic, and thus more cytotoxic, secondary bile acids (Thornton, 1981). Higher pH levels also increase the solubility of intestinal bile acids compared with lower pH values (McJunkin *et al*, 1981; Rafter *et al*, 1986). In view of this relationship between colonic pH and the damaging potential of intestinal bile acids, supplemental calcium phosphate, compared to calcium carbonate, might result in a less cytotoxic luminal content in the large bowel. However, despite the observed unaltered faecal pH in the present study, no effect of calcium phosphate on the measured cytotoxic parameters could be demonstrated. The cytolytic activity of faecal water during calcium phosphate supplementation was virtually unchanged, whereas calcium carbonate has previously been found to markedly decrease the cytolytic activity of faecal water (Lapré *et al*, 1993a). In addition, ALP activity in faecal water (representing the amount of epitheliolysis in the colon) was not affected by calcium phosphate, whereas it has been shown to decrease during calcium carbonate supplementation (Lapré *et al*, 1993a).

We do not think that these different effects of calcium carbonate and calcium phosphate on colonic epitheliolysis and cytolytic potency of the intestinal content are due to a different interaction between calcium and bile acids. Earlier *in vitro* studies have indicated that the binding of calcium to bile acids is due to adsorption of bile acid micelles to insoluble calcium phosphate (Van der Meer and De Vries, 1985; Van der Meer *et al*, 1991), while a close association between these compounds has been demonstrated in the human colonic content (Van der Meer *et al*, 1990). Furthermore, comparing the supplemental calcium carbonate study reported earlier (Van der Meer *et al*, 1990) with the present calcium phosphate supplementation study, we did not observe any definite differences in effects on intestinal and faecal bile acids which could possibly explain the unchanged cytolytic parameters in faecal water during calcium phosphate supplementation.

Although the duodenal bile acid composition did not show statistically significant changes during calcium phosphate supplementation, the proportion of the dihydroxy bile acid, chenodeoxycholic acid, tended to decrease, whereas simultaneously the proportion of the less cytotoxic trihydroxy bile acid, cholic acid, tended to increase. This trend is similar to the shift in the ratio of the di/trihydroxy bile acids observed in the calcium carbonate supplementation study (Van der Meer *et al*, 1990). Calcium phosphate did not affect faecal bile acid concentration and composition in this study. This is in accordance with our previous findings in adenoma patients (Welberg *et al*, 1993). In the latter study total bile acid excretion increased during calcium carbonate supplementation excretion, but this was parallelled by an increase in dry weight, and the concentration was not changed. Since faeces of only one bowel movement were collected in the present study, total excretion was not evaluated. It has been reported (Lapré *et al*, 1993a) that during calcium carbonate supplementation, the ratio of hydrophobic to hydrophilic bile acids in faecal water is decreased, thus reducing cytotoxicity, but not the total soluble bile acid concentration. The similarity of the effects of calcium phosphate, although less pronounced, to those of calcium carbonate on duodenal and faecal bile acids suggests a similar favourable change in soluble faecal bile acid composition during calcium phosphate supplementation.

In addition to its affects on bile acids, calcium is also a powerful chelator of fatty acids. Precipitation of fatty acids, which are involved in the induction of damage of cell membranes, may result in protection of the colon mucosa. Several studies have shown that supplemental calcium causes an increase of the total fatty acids in faeces, probably due to precipitation of calcium and fatty acids (Saunders *et al*, 1988; Wargovich *et al*, 1990; Appleton *et al*, 1991; Govers and Van der Meer, 1993). In our present study, however, the concentration of total fat in the faeces was not affected by calcium phosphate. Similar findings have recently been reported in a calcium intervention study in rats, in which high concentrations of supplemental dietary phosphate slightly counteracted the stimulation of faecal fatty acid excretion (Govers and Van der Meer, 1993). These findings might indicate that fatty acids, in contrast to bile acids, preferentially bind to ionized calcium rather than to calcium phosphate. It has been suggested that fatty acids are precipitated by calcium before absorption in the proximal small bowel can take place (Appleton *et al*, 1991; Govers and Van der Meer, 1993). As calcium phosphate starts

precipitating at a pH of about 5.5 (Van der Meer and De Vries, 1985), the intestinal pH causes precipitation of calcium and phosphate only shortly after entering the small intestine. Although phosphate is present in the human diet in at least equimolar amounts of calcium, it is possible that an excess of phosphate in the small intestine, due to orally added phosphate, inhibits the precipitation of ionized calcium and fatty acids by binding competitively to calcium. This phenomenon may thus explain the absence of a favourable effect of calcium phosphate supplementation on cytolytic and ALP activities of faecal water.

In conclusion, calcium phosphate supplementation does not affect the cytolytic properties of the colonic luminal content. Hence, no protective effect with regard to colonic cancer is to be expected from calcium phosphate. While bile acids are bound to calcium phosphate in a micellar mode, calcium phosphate might not be able to precipitate free fatty acids. Future studies should investigate whether calcium compounds other than calcium phosphate can overcome the unpredictable effect of calcium carbonate on colorectal epithelial cell proliferation. In addition, it may well be possible that differences between the effects of different calcium compounds on intestinal parameters can serve to clarify the various mechanisms involved in the influence of calcium on epithelial cell proliferation.

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