

Effects of Combination of Calcium and Aspirin on Azoxymethane-Induced Aberrant Crypt Foci Formation in the Colons of Mice and Rats

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Human intervention studies have suggested an exciting synergistic action between calcium supplementation and aspirin intake in reducing the risk of colorectal cancer. The aim of this study was to determine whether such a synergy can be demonstrated on azoxymethane (AOM)-induced colon aberrant crypt foci (ACF) formation in mice and rats. Female CF-1 mice and male F344 rats were injected subcutaneously with AOM and then received diet treatments for 8 wk. The basal control diet contained high fat (20% mixed lipids by weight) and low calcium (1.4 mg/g diet) to mimic the average Western diet. The treatment diets contained enriched calcium (5.2 mg calcium/g diet), aspirin (0.2 mg aspirin/g diet), or calcium plus aspirin (5.2 mg calcium plus 0.2 mg aspirin/g diet). Treatment with calcium, aspirin, or their combination significantly decreased the number of total ACF and aberrant crypt per mouse (by 43–59%) or rat (by 23–38%), but statistically significant differences among the 3 groups were not observed. A hint of additivity between calcium and aspirin was observed in mice but not in rats. These results indicate that the combination of calcium and aspirin did not produce a synergistic effect on the ACF formation in AOM-treated mice and rats.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer death in the Western world, and the occurrence of this cancer is increasing rapidly in many Asian countries (1,2). The risk of this cancer is strongly related to the dietary pattern. High fat, low fiber, and low calcium contents of a Western diet are associated with a higher risk for colorectal cancer (1–4). These etiological factors suggest that colorectal cancer is preventable, and the prevention of this disease is an important public health issue. Previously, epidemiological studies have consistently shown a modest inverse association between calcium intake and colorectal can-

cer (4–8). Epidemiological studies have also shown a consistent 40–50% decrease in relative risk of colorectal cancer in individuals taking nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, compared to those not taking these agents (9–13). In the Calcium Polyp Prevention Trial on subjects with a recent history of colorectal adenomas, calcium supplementation (1,200 mg/day) has been found to decrease the risk of all types of neoplastic and hyperplastic polyps, especially on more advanced colorectal lesions (14,15). Two randomized, placebo-controlled trials also showed that aspirin reduced the risk of colorectal adenomas in populations with an increased risk of developing adenomas (16,17). Furthermore, data from 2 different randomized clinical trials as analyzed by Grau et al. (18) suggested a synergistic interaction between calcium supplementation and the use of NSAIDs. Grau et al. (18) found that calcium reduced the risk of advanced adenomas by 65% among frequent users of NSAIDs, and there was a strong interaction between calcium and frequent NSAIDs use. Low dose aspirin (81 mg daily) resulted in a risk reduction of 80% for advanced adenomas among calcium users vs. a nonsignificant 32% reduction among nonusers.

The possible synergistic action between calcium and aspirin in human colon polyp prevention trials is exciting. It would be very interesting if such synergy could also be demonstrated in animal models to verify the biological effect; however, such data are not available. In an azoxymethane (AOM)-induced cholic acid-promoted colon tumorigenesis model in rats, Pence et al. (19) reported inhibitory action by calcium, but not by aspirin; and combination of aspirin with calcium did not produce additional protective effects beyond calcium. Molck et al. (20) reported that high calcium appeared to decrease AOM-induced aberrant crypt foci (ACF) formation, but enhanced colon tumor formation in rats; and the effect of aspirin was unclear. These studies (19,20), however, might not have used experimental diets that closely mimic the human low-calcium, high-fat diet and levels of aspirin intake. Individually, both calcium and aspirin have been shown to inhibit colon tumorigenesis in rodent models. In the AOM-induced or 1,2-dimethylhydrazine-induced colon ACF and tumorigenesis rat model, the inhibitory effect of dietary

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TABLE 1
Composition of Diet^a

Diet Ingredients	Basal High-Fat, Low-Calcium Diet (1.4 mg Calcium/g Diet)	Calcium-Enriched Diet (5.2 mg Calcium/g Diet)	Aspirin-Enriched Diet (1.4 mg Calcium and 0.2 mg Aspirin/g Diet)	Combination Diet (5.2 mg Calcium and 0.2 mg Aspirin/g Diet)
Casein	23.80	23.54	23.80	23.53
DL-methionine	0.36	0.35	0.36	0.35
Corn starch	24.16	23.89	24.16	23.88
Maltodextrin	11.90	11.77	11.90	11.77
Dextrose	9.16	9.06	9.16	9.06
Cellulose	5.95	5.88	5.95	5.88
Mixed lipid ^b	20.24	20.02	20.24	20.01
Vitamin mix	1.19	1.18	1.19	1.18
Choline bitartrate	0.24	0.24	0.24	0.24
Mineral mix ^c	0.60	0.59	0.59	0.59
Potassium phosphate, monobasic	1.62	1.60	1.62	1.60
Sodium chloride	0.31	0.30	0.31	0.30
Calcium phosphate, dibasic	0.47	0.46	0.47	0.46
Calcium carbonate	—	1.12	—	1.12
Aspirin	—	—	0.02	0.02

^aComposition is expressed as a percentage by weight.

^bMixed lipid contains 16% beef tallow, 10% lard, 12% butter fat, 30% hydrogenated soybean oil, 27% corn oil, and 5% peanut oil.

^cMineral mix contains 8.4% magnesium oxide, 51.5% magnesium sulfate heptahydrate O, 0.4% chromium potassium sulfate, 0.2% cupric carbonate, 0.7% potassium iodate, 4.2% ferric citrate, 2.5% manganous carbonate, 0.7% sodium selenite, 1.1% zinc carbonate, and 31.7% sucrose.

calcium was demonstrated (21). Calcium supplementation was also shown to inhibit hemin-induced colon epithelial hyperproliferation and ACF formation (22). Aspirin was shown to decrease ACF incidence and crypt multiplicity in an AOM-induced rat model (23). The inhibition of tumorigenesis by aspirin was also shown in *Apc*^{min/+} mice (24).

Calcium and aspirin are expected to have their own set of mechanisms for colon cancer chemopreventive activities. When the mice are on a high-fat and low-calcium diet (comparable to the human situation with Western diet), there may be an insufficient amount of extracellular levels of calcium to stabilize the E-cadherin, a tumor suppressor, at the cell adherence junction. Dietary calcium supplementation may increase the expression of E-cadherin directly or through the action of calcium-sensing receptor (25–27). This would reduce the cellular and nuclear level of β -catenin, which attenuates the Wnt signaling and activation of many growth promoting genes such as cyclooxygenase (COX). The best recognized mechanism of aspirin action is inhibition of the COX-1 and COX-2, which decreases the formation of proinflammatory and procarcinogenic eicosanoids such as prostaglandin E2 (9). It may also enhance the expression of E-cadherin and lower the level of β -catenin or directly inhibit the activation of nuclear factor- κ B and activator protein-1 (28–30). With the two agents used in combination, an additive effect or even a synergistic effect may occur, leading to enhanced inhibition of colon carcinogenesis.

The present study was designed to examine the inhibitory effect of a combination of calcium and aspirin in AOM-induced colon tumorigenesis models in mice and rats maintained on a Western-style diet containing a low-calcium and high-fat diet.

MATERIALS AND METHODS

Animal Treatment

Female CF-1 mice (Charles River Laboratories, Wilmington, MA) were acclimated for 1 wk with the basal, high-fat and low-calcium diet modified from the AIN-76A diet (20% mixed lipids and 1.4 mg calcium/g diet). At the age of 5 wk, the mice received two consecutive weekly subcutaneous injections of AOM (from the National Cancer Institute Repository, Bethesda, MD) at 10 mg/kg body weight. AOM was dissolved in sterilized saline and kept on ice throughout the procedure. Equal volumes of saline were given to the negative control mice. Male F344 rats (from Taconic Farms, German Town, NY), at the age of 7 wk, were given two weekly injections of AOM (15 mg/kg). The AOM-treated animals were randomized into 4 groups (10 mice or 5 rats in each group) and received the following dietary treatments: 1) control diet (the basal high-fat and low-calcium diet), 2) calcium enriched diet (5.2 mg calcium/g diet), 3) aspirin diet (0.2 mg aspirin/g diet), and 4) calcium and aspirin combination diet (5.2 mg calcium plus 0.2 mg aspirin/g diet). Compositions of all the 4 diets are shown in Table 1. Diet

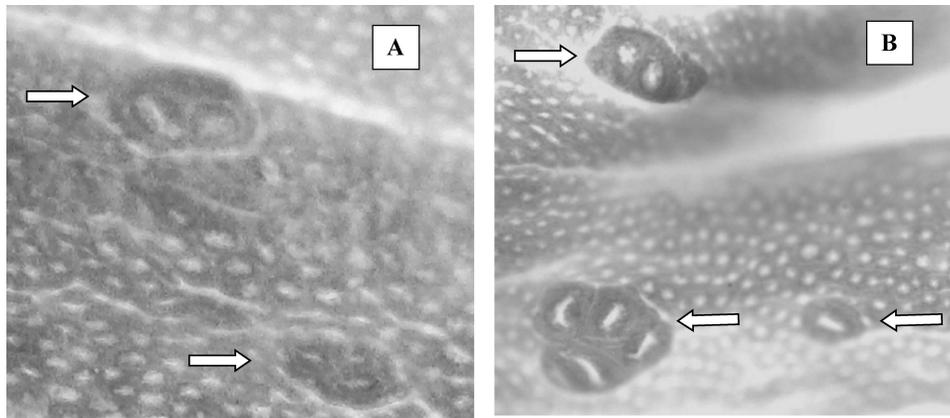


FIG. 1. Typical ACF induced by AOM treatment in mice (A) and rats (B). Colons were collected after sacrifice and flattened on filter paper. After fixation in 10% buffered formalin, colon segments were stained by methylene blue, and then examined under microscope. ACF were characterized in by criteria described in Materials and methods. A. ACF containing 2 AC (upper arrow) or 3 AC (lower arrow); X40. B. ACF containing 2 AC (upper arrow), 4 AC (lower left arrow), or 1 AC (lower right arrow); X40.

treatments were initiated 3 days after the final AOM injection and continued for 8 wk until the study was terminated. Body weight as well as food and fluid intake were measured weekly. Animals were sacrificed by CO₂ asphyxiation.

Analysis of ACF

ACF were analyzed in colon tissues following a previously established procedure (31). In brief, the colons were immediately removed and rinsed with ice-cold saline solution, slit open longitudinally, fixed flat between wet (saline) filter paper for 24 h in 10% buffered formalin, and then placed in 70% ethanol solution at room temperature until they were stained with 0.2% methylene blue for 3 to 5 min. The number of ACF per colon as well as the number of aberrant crypt (AC) per focus were determined under a microscope at a magnification of 40-fold to 200-fold. ACF were distinguished from surrounding normal crypts by increased size, elongated luminal opening, increased distance from luminal to basal surface of cells, thickened epithelial cell lining, and enlarged pericryptal area relative to surrounding normal crypts (Fig. 1).

Statistical Analyses

Differences in body weight, fluid and food intake, and the number of AC and ACF among groups were compared by 1-way analysis of variance (ANOVA) combined with Tukey's honestly significantly different post hoc test. Difference in incidence of ACF containing larger than ≥ 3 AC among groups was compared by Fisher exact probability test. For simple comparisons between two groups, 2-tailed Student's *t*-test was used.

RESULTS

Body weights and fluid intake were not significantly different among groups throughout the experiment with both mice and rats. Food intake of the mice was found to be slightly higher

(by 9%) in the combination group as compared to the control group ($P < 0.05$), but a difference in food intake was not found in rats. Treatment of mice and rats with AOM resulted in 100% incidence of ACF, whereas no ACF were found in the saline-treated control group. In both mice and rats, most ACF were distributed in the distal and middle colon, and only a few ACF were found in the proximal colon. The result is consistent with other studies in AOM-induced ACF murine models (32).

The effects of calcium and aspirin treatment on the development of AOM-induced ACF in mice are summarized in Table 2. Treatment of the AOM-treated mice with calcium, aspirin, or their combination significantly decreased the total number of ACF per colon (by 43%, 40%, or 50%, respectively) and the total number of AC per colon (by 53%, 50%, or 59%, respectively). The decrease by the combination of calcium and aspirin treatment appeared greater than that by either agent individually; however, no statistically significant differences among the 3 treatment groups were observed. Because studies have shown that the number of large ACF correlates more closely with subsequent development of colon cancer, the numbers of 1 crypt/focus, 2 crypts/focus, and more than 3 crypts/focus were evaluated. The number of large foci (containing more than 3 crypts/foci) was significantly decreased by treatment with calcium, aspirin, and their combination (by 58%, 66%, and 79%, respectively), but there was no statistical significant differences among the 3 treatment groups. However, the combination treatment group was the only group that significantly decreased the incidence of the large ACF. The treatment did not significantly affect the number of smaller ACF.

The effects of the treatments on ACF formation in AOM-treated rats are shown in Table 2. Similar to the results from studies in mice, all 3 treatment diets significantly decreased the total numbers of AC per rat and ACF per rat, but there were no statistically significant differences among the 3 groups. The number of large ACF (≥ 4 AC/focus) was significantly de-

TABLE 2
Effects of Calcium and Aspirin Treatments on ACF Formation in AOM-Treated Mice and Rats

Group (N)	Total No. of AC/Mouse ^c	Total No. of ACF/Mouse ^c	No. of Foci Containing				Incidence of ACF Containing ≥ 3 AC ^d
			1AC	2AC	≥ 3 AC ^c		
1. AOM-treated mice ^a							
Control (7)	25.57 \pm 7.07 ⁺	13.57 \pm 2.88 ⁺	6.86 \pm 3.63	3.14 \pm 1.95	3.57 \pm 1.90 ⁺	100% ⁺	
Calcium (10)	12.40 \pm 7.29 [≠]	7.70 \pm 4.40 [≠]	4.80 \pm 2.82	1.40 \pm 1.71	1.50 \pm 0.97 [≠]	80% ^{+≠}	
Aspirin (9)	12.78 \pm 7.71 [≠]	8.33 \pm 4.77 ^{+≠}	5.44 \pm 3.24	1.56 \pm 1.33	1.22 \pm 1.56 [≠]	78% ^{+≠}	
Calcium + Aspirin (8)	10.13 \pm 6.70 [≠]	6.75 \pm 3.58 [≠]	4.00 \pm 2.33	1.75 \pm 1.67	0.75 \pm 1.17 [≠]	38% [≠]	
Group (N)	Total No. of AC/Rat ^c	Total No. of ACF/Rat ^c	No. of Foci Containing				
			1AC	2AC	3AC	≥ 4 AC ^c	
2. AOM-treated rats ^b							
Control (4)	567 \pm 55 ⁺	199 \pm 15 ⁺	24 \pm 12	88 \pm 7	40 \pm 5	47 \pm 12 ⁺	
Calcium (5)	353 \pm 33 [≠]	141 \pm 17 [≠]	22 \pm 9	68 \pm 7	26 \pm 8	24 \pm 5 [≠]	
Aspirin (4)	425 \pm 48 [≠]	149 \pm 12 [≠]	19 \pm 8	66 \pm 17	27 \pm 11	38 \pm 10 ^{+≠}	
Calcium + Aspirin (5)	402 \pm 77 [≠]	154 \pm 24 [≠]	21 \pm 6	68 \pm 3	38 \pm 8	28 \pm 9 [≠]	

^aAbbreviations are as follows: ACF, aberrant crypt foci; AC, aberrant crypt; AOM, azoxymethane. Female CF-1 mice were treated with 2 weekly subcutaneously (sc) injections of AOM and then maintained on the experimental diet for 8 weeks. Values are means \pm SD of the number mice (N) analyzed.

^bMale F344 rats were treated with 2 weekly sc injections of AOM and then maintained on the experimental diet for 8 weeks. Values are mean \pm SD of the number of rats (N) analyzed.

^cOne-way ANOVA; values with different superscripts (⁺, [≠]) differ significantly ($P < 0.05$).

^dFisher exact probability test; $P < 0.05$. ⁺, [≠]Values with different superscripts differ significantly.

creased by treatment with calcium, but not further decreased by its combination with aspirin. The results agree with the report by Pence et al. (19) in an AOM-induced, cholic acid-promoted, colon carcinogenesis rat model showing that the inhibitory effect of calcium was not enhanced by its combination with aspirin.

DISCUSSION

Calcium has practically no toxicity or side effects at the levels that have been demonstrated to have a colorectal cancer preventive effect. Aspirin is another relatively safe agent when used at low doses. Therefore, a possible synergistic action between calcium and a nontoxic dose of aspirin may provide a very promising, convenient, and inexpensive means for colon cancer prevention. We decided to reexamine the inhibitory effect of a combination of calcium and aspirin in AOM-induced colon tumorigenesis models in mice and rats because we thought the previous studies on this topic (19,20) might not have used experimental diets that closely mimic the human low-calcium and high-fat diet and levels of aspirin intake. The U.S. diet typically contains high fat, which contributes to 35–40% of the total caloric intake, as well as low calcium levels. The estimated human intake of calcium in the United States is 600 mg/day (33) or 600 mg/2,000 kcal (for a person consuming 2,000 kcal per day);

that is equivalent to 0.3 mg/kcal. This calcium level corresponds to 1.4 mg/g diet (1.4 mg/4.6 kcal = 0.3 mg/kcal) in a high-fat rodent feed, based on the calorie density concept (34). The AIN-76A diet contained 5.2 mg calcium/g diet (1.1 mg/kcal), which can be considered a calcium-enriched diet, corresponding to humans taking about 2,200 mg calcium/day, below the upper limit of the current Dietary Reference Intake of 2,500 mg calcium/day (35). The aspirin dose we used was 0.2 mg/g (0.2 mg/4.6 kcal = 87 mg/2,000 kcal), which approximates the dose of 81 mg/day used in human study (14,15). The present dietary design is based on this consideration.

In the study with mice, the combination treatment of calcium and aspirin appeared to cause a more pronounced decrease in the total number of AC and ACF per colon than either agent alone, especially with regard to the incidence and number of large ACF (≥ 3 AC/focus). Significant differences among the 3 treatment groups, however, were not found. The results from the rat experiment also did not demonstrate an enhanced inhibition of colon ACF formation by the combination of calcium and aspirin, and there was not even a hint of additivity. The results are similar to those reported by Pence et al. (19). The results that the number of large ACF was lower in the treatment groups in both mice and rats suggest that the fast-growing aberrant foci might be the target of calcium and aspirin treatment.

In human studies, a synergistic effect between calcium and aspirin has been suggested in reducing the risk of advanced adenomas (18). In both the calcium and the aspirin studies, the overall effect of the agents was stronger for advanced adenomas (15,17), suggesting that the agents inhibit the progression of early adenomas to more severe lesions. The possible interpretations for not detecting a synergistic or clear additive effect in our study are 1) the treatment period, 8 wk, may not be long enough to produce the effect; 2) ACF are lesions not advanced enough to receive the benefit of the combination treatment; and 3) there is no synergistic effect in the combination of calcium and aspirin in our experimental systems.

In summary, the present study shows that both calcium and aspirin can inhibit ACF formation in AOM-treated mice and rats, but a synergistic or a clear additive effect was not observed. Further studies are needed to better characterize the possible interactions between calcium and aspirin.

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