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Decrease of intestinal tumors induced by 1,2-dimethylhydrazine in rats fed with cow milk and buffalo milk

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ABSTRACT: Epidemiological studies in human beings and experimental studies in laboratory animals suggest that milk and dairy products can inhibit effects on the development of some kinds of tumors. Cow milk contains sphingomyelin, butyric acid, conjugated linoleic acid, calcium, vitamin A, carotene and vitamin D. All of these components are known to inhibit the process of carcinogenesis. Our objective was to determine the effect of cow milk and water buffalo milk on the development of colon neoplasias in an experimental model of carcinogenesis in rats induced with 1,2-dimethylhydrazine (DMH). Three-month-old Wistar male rats with an average body weight of 180 g were given a nutritionally adequate diet and drinking water *ad-livitum*, cow milk or water buffalo milk. The milk diets were provided two weeks before the first DMH treatment and their administration was continued during the 10 weeks of DMH treatment. Milk administration finished two weeks after the last DMH doses treatment. Four months after the last carcinogen injection, all surviving animals were sacrificed and examined for intestinal tumors. The number, size, and location of the tumors were recorded and gross pathology was described. Small tumors (< 2.5 mm) were examined by Scanning Electron Microscopy (SEM). Significantly fewer tumors were observed in both groups treated with DMH and supplemented with milk, than in the group treated with DMH without milk administration.

Introduction

In the human being, the tumors of the large bowel are an important cause of morbidity and mortality in great part of the World. The highest incidence of colon cancer is observed in the United States, Canada, Australia, Sweden, and other affluent countries. Its incidence is substantially lower – up to 60-fold – in Japan, Africa, and South America with the exception of Uruguay and Argentina, where the intake of red meat is high (Castro, 1986).

Environmental and dietary factors are responsible for 85-90% of all cases (Vargas and Alberts, 1992).

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Among the dietary components suggested as colon cancer promoters are excessive fat and calories and low intake of various dietary fibers, vegetables, and micronutrients such as antioxidant vitamins (e.g., vitamins C and E, selenium, and β-carotene) and calcium (Byers and Perry, 1992). In the human being, low fiber and high animal-fat intake has been implicated in the cause of colon cancer (Reddy and Wynder, 1973; Suzuki and Mitsuoka, 1992; Weisburger and Wynder, 1991; Wynder and Shigematsu, 1967; Wynder and Reddy, 1973).

The relationship between colorectal cancer and dietary components, such as fat, vegetal fibers, vitamins and other elements, has been evaluated in epidemiological and experimental animal studies (Burkitt, 1971; Cotran *et al*., 2000; Greenwald, 1992; Sloan *et al*., 1993; Thorup *et al*., 1992).

Epidemiological studies in human beings and experimental studies in laboratory animals suggest that

milk and dairy products can inhibit the development of some kinds of tumors. Cow milk contains sphingomyelin, butyric acid, conjugated linoleic acid, calcium, vitamin A, carotene, and vitamin D. These components potentially inhibit the process of carcinogenesis (Imaizumi *et al*., 1992; Ustarroz, 2003).

The effects of a milk diet on colon experimental carcinogenesis in rats were studied by our laboratory. In that experience, we observed less tumors in rats treated with skimmed milk powder than in those treated with whole milk powder (Sánchez Negrette *et al*., 2005a-b).

Our objective in the present study was to determine the effect of cow milk and water buffalo milk on the development of colon neoplasias in an experimental model of carcinogenesis in rats induced with 1,2-dimethylhydrazine (DMH).

Materials and Methods

Three-month-old Wistar male rats with average body weight of 180 g, were given a nutritionally adequate diet and drinking water *ad-livitum*, cow milk or water buffalo milk (Holando argentina breed and Murrah respectively). We randomly divided 90 rats into three experimental groups of 20 rats each for carcinogen administration (DMH + water: DMH-alone group; DMH + cow milk: CM group; DMH + buffalo milk: BM group), and three similar control groups of 10 rats each without carcinogen injection (with water, cow milk or buffalo milk). Body weight was assessed twice a month until the end of the estudy.

Intestinal tumors were induced by 1,2-dimethylhydrazine (DMH) given subcutaneously in 10 weekly doses of 20 mg/kg of body weight. The carcinogen (DMH) solution was prepared just before its administration. The DMH solution used for injection was prepared with 400 mg of DMH dissolved in 100 ml of demineralized water containing 37 mg of ethylene-diamine tetraacetic acid (EDTA). The pH was raised to 6.5 with sodium hydroxide. Control animals were given the same volume of EDTA at pH 6.5. The rats were housed in individual cages, with a temperature of 22ºC plus or minus 1ºC.

The milk diet (cow milk or buffalo milk) was provided two weeks before the first DMH treatment and its administration was continued up to two weeks after the last DMH doses treatment. Four months after the last carcinogen injection, all surviving animals were sacrificed by cervical dislocation after etherization and examined for intestinal tumors.

We performed a full autopsy on each animal and paid particular attention to the large bowel, which was removed, opened along its length, and pinned to a corkboard with its mucosal surface up. The entire large bowel was then fixed in 10% neutral buffered formalin for 24 h.

Small tumors (< 2.5 mm) were examined by Scanning Electron Microscopy (SEM). Preparation for SEM was deliberately kept as simple as possible to reduce the development of artifacts. The specimens were first deshydrated by passing them through a graded acetone immersion. They were critical point dried, mounted on 0.5-inch aluminum stub, and coated with gold palladium. Finally, they were examined by using a JEOL 5800 LV SEM.

TABLE 1.

Number and distribution of intestinal tumors in rats subjected to weekly subcutaneous injection (n= 10) of 1,2-dimethylhydrazine.

Group	**DMH-alone**	**CM +DMH**	**BM +DMH**
Nº of rats	n= 20	n= 20	n= 20
Nº of tumors	137	61	67
Maximum Nº tumors/rat	15	9	10
Average tumors/rat	6,85	3,05	3,35
Localization			
Rectum	32 (23.35)	7 (11.47)	13 (19.40)
Distal colon	68 (49.63)	28 (45.90)	27 (40.29)
Proximal colon	15 (10.95)	22 (36.06)	23 (34.32)
Cecum	22 (16.05)	4 (6.55)	4 (5.97)

Numbers in parenthesis are percentages of tumors in each group. CM: cow milk. BM: buffalo milk.

For each animal we recorded the total length of the fixed colon and determined the number, the size, the gross characteristic appearances, and the location of the tumors in the different segments of colon.

The tumors were grossly classified into 1) polypoid-type growth (pedunculated lesions, sessile lesions, or broad-based lesions), 2) or non polypoid-type growth without intramucosal protuberant growth (flat tumors with plaque-shaped or ulcerative-infiltrative carcinomas).

We used the Kruskall-Wallis test to analyze tumor data for each rat and for each treatment group. We used a program for the analysis of variance (ANOVA) to analyze the weight-gain data for each treatment group.

Results

The number of intestinal tumors in both groups (> 50%) given the milk supplements (CM group and BM group) was significant lower than in DMH alone group. We observed fewer tumors in the experimental group supplemented with cow milk (61) than with that supplemented with buffalo milk (67) (Table 1). However, the difference in the number of tumors observed between the CM group and BM group was not statistically significant (Table 1).

Both the CM group and BM group gained more weight than the DMH alone group. Also, the average body weights of the experimental and control groups (with and without carcinogen administration) of the CM group and BM group were similar.

In the CM group and BM group, intestinal tumors were frequently located in the distal and proximal colon (Fig. 1), whereas in the DMH alone group, they were frequently located in the distal colon and rectum. Both the CM group and BM group had a lower percentage of tumors in the cecum than those in DMH alone group.

The ultrastructural study allowed us to identify the smaller tumors (< 2.5 mm) and differentiate them from gut-associated lymphoid tissue (Figs. 2 and 3).

The frequency of polypoid and nonpolypoid growth is shown in Table 2. The CM group had more polypoid sessile tumors, whereas the BM group and the DMH alone group had more flat tumors.

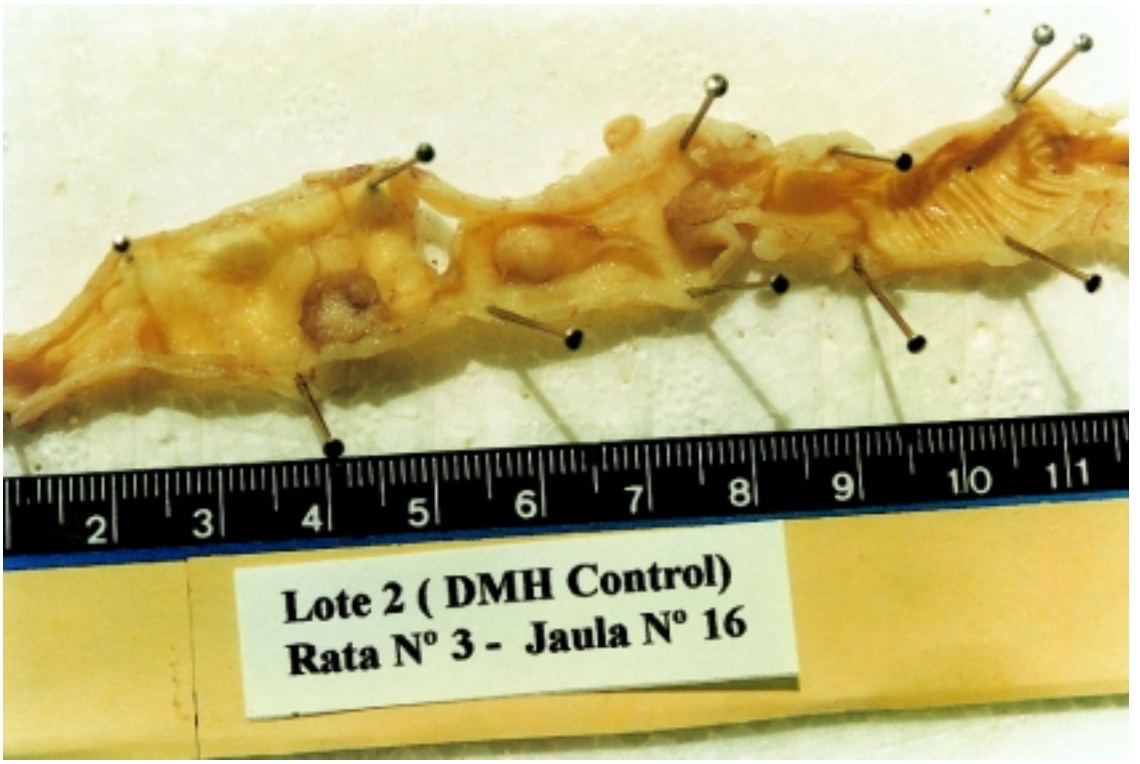

FIGURE 1. Multiple tumors in the distal colon and the proximal colon of an animal treated with DMH and observed for 26 weeks after the first application of carcinogen.

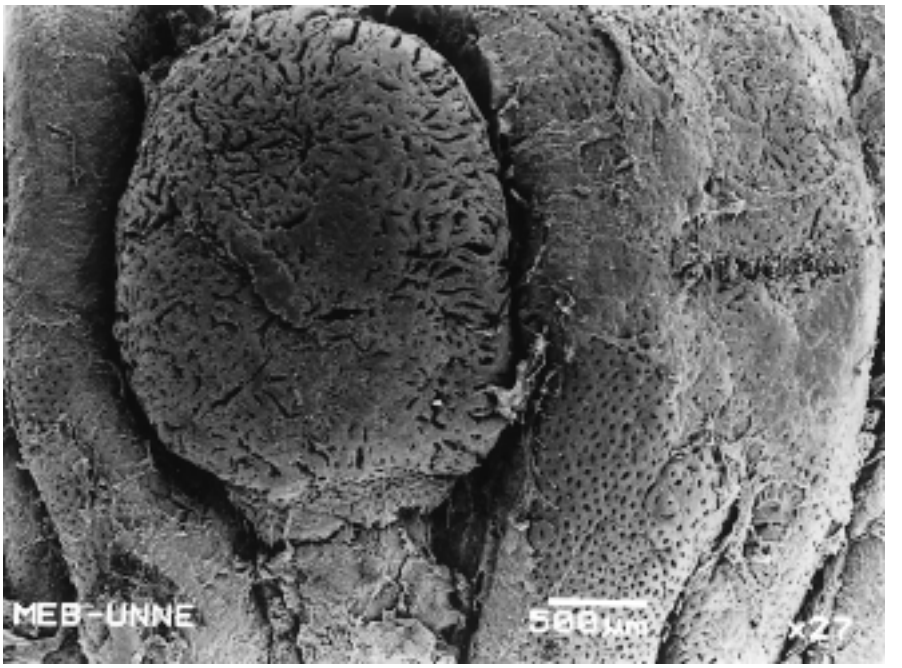

FIGURE 2. Small tumor (< 2.5 mm), located in the distal colon between two longitudinal mucosal folds (SEM X 27,000).

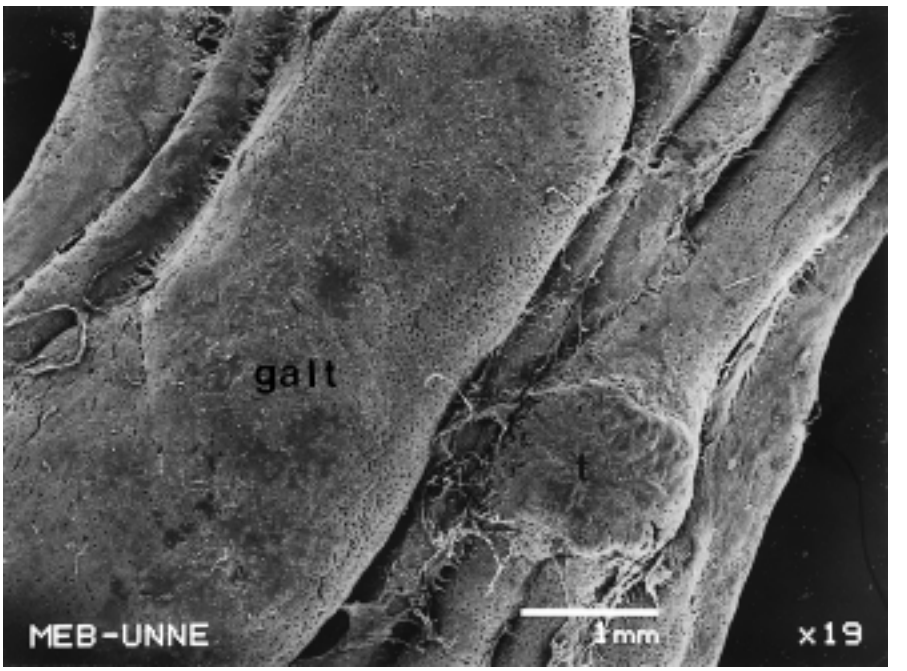

FIGURE 3. Small tumor located in the distal colon in longitudinal mucosal folds (t), nearly to gut-associated lymphoid tissue (galt) (SEM X 19,000).

The majority of tumors were between 1 and 5 mm in diameter. The size of the tumors was similar among the three experimental groups (Table 3). The BM group had the largest percentage of tumors between 1 and 5 mm in diameter.

The DMH alone group had two rats with pulmonary metastases. No rats in the CM group and BM group had pulmonary metastases.

Discussion

Clearly colorectal cancer is a disease of affluence, and it is generally accepted that about 80% of cases of colorectal cancer is attributable in some way to diet (Bingham, 2000). Among the dietary components suggested as colon cancer promoters are excessive fat and calories and a low intake of various dietary fibers, veg-

etables, and micronutrients, such as the antioxidant vitamins (Byers and Perry, 1992). Milk contains components, such as calcium, casein, butyric acid, ether lipids, sphingomyelin, conjugated linoleic acid, vitamin A, β-carotene, and vitamin D, that have the potential to inhibit the process of carcinogenesis (Parodi, 1996; Papenburg *et al*., 1990).

Sphingomyelin and other phospholipids account for 0.2 to 1.0% of the total lipids in milk (Christie *et al*., 1987), where they are associated with the milk-fat globule membrane. The use of sphingomyelin as a dietary chemopreventive supplement was reported by Dillehay *et al*. (1994). Using the 1,2-DMH model of colonic adenocarcinoma, these investigators showed that sphingomyelin, which was from commercially available nonfat dry milk, inhibits colon cancer in CF1 mice.

Milk fat is the richest natural dietary source of conjugated linoleic acid (CLA). Depending upon pasture conditions, milk fat may contain up to 30 mg CLA/g fat. CLA has been shown to inhibit initiation of mouse skin carcinogenesis induced by 7,12-dimethylbenz(a) anthracene (DMBA) (Cope and Reeve, 1994), mouse forestomach neoplasia induced by benz(a)pyrene and rat mammary tumorigenesis induced by DMBA (Carroll, 1991).

Bovine milk fat contains from 7.5 to 13.0 mole % butyric acid (Parodi, 1996). Butyric acid is an inhibitor of cell proliferation and a potent inducer of differentiation *in vitro* for a wide variety of neoplastic cells, including colon, rectum, liver, leukemia, lymphoma, cervix, and ovarian cells (Chen and Breitman, 1994). Butyric acid induces apoptosis and modulates the expression of oncogenes and suppressor genes in several cell types (Heerdt *et al*., 1994).

One of the most daunting aspects of the relationship between nutrition and cancer is the sheer complex-

TABLE 2.

Frequency of tumor types in the three groups treated with DMH.

Tumor Types	Polypoid Pedunculated	Polypoid Sessile	Flat	Ulcerative Infiltrative	Total
Group					
DMH-alone	6 (4.32)	58 (42.33)	70 (51.10)	3 (2.18)	137
CM+DMH	15 (24.59)	26 (42.62)	17 (27.86)	3 (4.91)	61
BM+DMH	13 (19.40)	23 (34.32)	28 (41.79)	3 (4.47)	67
Total	34 (12.83)*	107 (40.37)*	115 (43.39)*	9 (3.39)*	265

* Percentage over total of 265 tumors.
Numbers in parenthesis are percentages of tumors in each group. CM: cow milk. BM: buffalo milk.

TABLE 3.

Frequency and size of tumors in the three groups treated with DMH.

Group	DMH-alone	CM +DMH	BM +DMH
Size			
1-5mm	58 (42.33)	32 (52.45)	57 (85.07)
6-10mm	58 (42.33)	22 (36.06)	8 (11.94)
11-15mm	17 (12.4)	7 (11.47)	2 (2.98)
16-20mm	1 (0.72)	—	—
+ 20mm	3 (2.18)	—	—
Total	137	61	67

Numbers in parenthesis are percentages of tumors in each group. CM: cow milk. BM: buffalo milk.

ity of human diets (Johnson, 2004). Many studies about milk components with possible anticarcinogenic properties have employed a reductionist approach, in which cultured tumor cells or animal models have been exposed to single compounds, often at concentrations much higher than those that are likely to be encountered through normal dietary exposure. Experiments of this type are often essential to demonstrate proof-of-principle or to explore mechanistic events in a controlled manner.

In this study, rats treated with 1,2-DMH that were supplemented with cow milk and buffalo milk had fewer tumors than those without milk administration. These results are consistent with another study where rats treated with skimmed milk powder had fewer tumors than those not supplemented with milk (Sánchez Negrette *et al*., 2005b). These authors assumed that these findings were probably a result of the synergistic mechanism of the diverse components of the milk, such as calcium, vitamin A, β-carotene, vitamin D, casein, sphingomyelin, butyric acid, and conjugated linoleic acid, which all potentially inhibit carcinogenesis. On the other hand, our results suggest that the diverse components of cow milk and buffalo milk may play an important role in inhibiting colon tumor growth and may themselves provide an effective means of chemoprevention. Future studies should be carried out to determine the antitumorigenic potential of individual components, perhaps by addition to or subtraction from a "basic" milk fat. Hence, the cooperative synergistic and combined effect of these components could be determined.

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