Prevention of N-Methylnitrosourea-Induced Colon Carcinogenesis in Rats by Oxygenated Carotenoid Capsanthin and Capsanthin-Rich Paprika Juice (44523)

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Abstract. **Epidemiological and animal studies have provided evidence that dietary carotenoids may reduce the risk of certain types of cancer. An inhibitory activity of oxygenated carotenoid capsanthin, a potent antioxidant, and paprika juice rich in capsanthin (3.54 mg/100 ml) against colon carcinogenesis was investigated in F344 rats. In Experiment I (short-term assay), six rats each were given a gavage of 5 mg, 0.2 mg, or 0.008 mg capsanthin six times a week for Weeks 2–6 after receiving three intrarectal doses of 4 mg N-methylnitrosourea in Week 1. The number of colonic aberrant crypt foci, preneoplastic lesions, at Week 6 was significantly fewer (by 42%) in the 0.2 mg capsanthin group, but not in other groups, than the control group. In Experiment II (long-term assay), five groups of 30 or 25 rats each received an intrarectal dose of 2 mg N-methylnitrosourea three times a week for Weeks 1–3, and had either of 10 p.p.m. or 2 p.p.m. capsanthin solutions, 1:2.5 and 1:16.7 diluted solution of paprika juice (containing 10 p.p.m. or 2 p.p.m. capsanthin), and tap water (control fluid) as drinking fluid throughout the experiment. The experimental groups were fed 0.2 mg or 0.04 mg capsanthin/day/rat. The colon cancer incidence at Week 30 was significantly lower in the highly diluted paprika juice group (40%), but not in the moderately diluted paprika juice group (60%) and the capsanthin solution groups (68% and 68%) than the control group (83%). The results suggested that paprika juice may affect colon carcinogenesis. However, capsanthin alone failed to inhibit colon tumorigenesis, in spite of suppression of aberrant crypt foci formation in the short-term assay. Further studies are needed to explain this discrepancy.**

[P.S.E.B.M. 2000, Vol 224:116–122]

Epidemiological studies have suggested a protective role of vegetables and fruits against certain types of cancer including colon cancer (1, 2). The studies focused on the role of micronutrients such as retinoic acid, ascorbic acid, and α-tocopherol. Certain non-nutrient phytochemicals have been explored as possible components contributing to an anticarcinogenic activity of plant foods (3–5). An inverse association between dietary intake and blood level of carotenoids such as β-carotene and lycopene, and the risk of cancer in various organs has been observed in epidemiological studies (6–12), although some studies did not demonstrate such effects (13, 14). Thus, β-carotene, lycopene, lutein, and β-cryptoxanthin, which are major carotenoids in the diet and in the blood in humans, have been investigated extensively for their anticarcinogenic properties (15–17). The anticarcinogenic activity of these natural pigments appears to be attributable to their antioxidative potential. The antioxidant functions are associated with lowered DNA damage, diminished membrane lipid peroxidation, or inhibited malignant transformation *in vitro* (18, 19). It is possible to suggest that carotenoid-rich vegetables

This work was supported by the Research Fund of Akita University College of Allied Medical Sciences.

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Received June 30, 1999. [P.S.E.B.M. 2000, Vol 224]
Accepted February 7, 2000.

0037-9727/00/2242-0116$15.00/0
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and fruits may provide a mechanism for preventing oxidative damage *in vivo*, and thus carcinogenesis.

About 20 carotenoids, including the metabolites of a variety of ingested carotenoids, have been identified in human blood (20). Oxygenated carotenoids (xanthophylls) such as β-cryptoxanthin and lutein are possibly linked to a lower risk of cancer as well as hydrocarbon carotenoids such as β-carotene and lycopene (21). Cultured cell studies have shown that some carotenoids other than β-carotene possess anticarcinogenic properties equivalent or superior to β-carotene (22, 23). A xanthophyll capsanthin, which is a typical and major carotenoid in red paprika (sweet pepper), inhibited more effectively the photooxidation of linoleic acid, polyunsaturated fatty acid, than β-carotene, lycopene, and lutein (24), and acted as an antioxidant against free radical attack and singlet oxygen exposure in plasma lipoprotein (25). Capsanthin appears to rank highest among natural carotenoids in its capacity for quenching singlet oxygen and scavenging free radicals. Thus, it is supposed that capsanthin also has an anticarcinogenic potential as other antioxidant carotenoids have. In our previous study (26), capsanthin was not detected in the plasma in humans, because of low intake and fast clearance rather than poor absorption. In the same subjects, an appreciable plasma level of capsanthin (0.10–0.12 μ*M*) was identified within a few days after supplementing paprika juice (160 ml equivalent to 5.4 μmole capsanthin at every meal). These findings prompted us to investigate an anticarcinogenic activity of capsanthin and paprika juice rich in capsanthin in an animal model.

The present study was conducted to examine a chemopreventive activity of capsanthin and paprika juice against colon carcinogenesis in rats. The first set (short-term assay) of two experiments was carried out to select a dose of capsanthin for the following long-term assay, using colonic aberrant crypt foci (ACFs) known as preneoplastic lesions and an early intermediate biomarker of colon carcinogenesis (27). The next long-term assay experiment investigated the efficacy of capsanthin in the dosage, which was based on the results of the first experiment, and of paprika juice containing the same quantity of capsanthin, against colon cancer development. The results showed that paprika juice effectively prevented the colon cancer development, whereas capsanthin alone was not effective. This discrepancy in chemopreventive potential between capsanthin and capsanthin-rich paprika juice was discussed in association with other active phytochemicals in paprika juice.

Materials and Methods

Animals. Female F344/NSlc rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan), 7 weeks of age at the start of the experiment, were housed in plastic cages with sterilized wood-chip bedding in a specific-pathogen-free animal room under constant environmental conditions with a 12 :12-hr light:dark cycle, a temperature of 22°C, and a relative humidity of 50%. They had free access to a standard pelleted laboratory chow CE-2 (CLEA Co., Tokyo, Japan) and drinking water. The body weight and food intake were measured once a week. The rats were maintained according to the standards set forth in the *Guidelines for the Care and Use of Laboratory Animals of the Experimental Animal Facility,* Akita University School of Medicine.

Capsanthin and Paprika Juice. Capsanthin (purity 99.0%) was extracted from commercially available paprika juice (Kagome Co., Nishinasuno, Tochigi, Japan) by one of the authors, using hexane, acetonitrile, ethanol, and toluene (10:7:6:7, v/v/v/v) after saponification with KOH (10.7 *M*) (26). The contents of carotenoids in paprika juice used and CE-2 chow (Table I) were determined by high power liquid chromatography (HPLC) as described (26). Briefly, the samples were homogenized and saponified by addition of 60% KOH and 3% butylated hydroxytoluene in ethanol, followed by heating at 50°C for 30 min, then extracted twice with hexane, acetonitrile, ethanol, and toluene (10:7:6:7, v/v/v/v). The supernatant was dried, and then reconstituted in a solvent mixture of methanol, acetonitrile, dichloromethane, and water (7:7:2:3.2, v/v/v/v) as a mobile phase for HPLC. The analysis was performed with a Shimadzu SPD-10AV spectrophotometric detector (Shimadzu, Kyoto, Japan) and a Lichosper RP18–5 column (E. Merck, Darmstadt, Germany) at a flow rate of 1.5 ml/min. The nutritional ingredients of paprika juice and CE-2 chow are listed in Table I.

ACF Examination (Experiment I). Four groups of six rats each received three intrarectal instillations of 0.5 ml of freshly prepared 0.8% aqueous solution of N-methylnitrosourea (MNU) (Nacalai Tesque, Kyoto, Japan), a direct-acting carcinogen not requiring metabolic activation, in Week 1. Briefly, a metal feeding tube 8 cm long was inserted two-thirds of the way into the colon lumen through the anal orifice, and the solution was injected (28). The solution filled the distal half of the colon where the tumors develop. Then, the rats were given an intragastric gavage of

Table I. Contents of Macronutrients, Antioxidant Vitamins, and Carotenoids in Paprika Juice and CE-2 Chow

	Paprika juice (100g)	CE-2 chow (100g)
Protein (g)	0.9	25.4
Fat (g)	—[a]	4.4
Carbohydrate (g)	5.3	50.3
Fiber (g)	—	4.1
Ash (g)	0.5	6.9
Energy (kcal)	21.0	342.0
Ascorbic acid (mg)	74.3	19.0
α-Tocopherol (mg)	1.4	7.0
β-Carotene (mg)	0.46	0.19
Lutein (mg)	0.07	0.91
Lycopene (mg)	—	—
β-Cryptoxanthin (mg)	0.72	0.04
Capsanthin (mg)	3.54	—

[a] Not detected.

5 mg (Caps(H) group), 0.2 mg (Caps(M) group), or 0.008 mg (Caps(L) group) of capsanthin dissolved in 0.2 ml of corn oil (Nacalai Tesque, Kyoto, Japan) through a metal feeding tube six times a week during Weeks 2 and 6 (postinitiation phase). The rats of the control group were given 0.2 ml of corn oil. The experiment was terminated at Week 6, and all the rats were sacrificed. The colon was excised and cut open along its length, and the distal half of the colon, 10 cm long, was flattened on filter paper and placed in 10% neutral formalin solution for 1 day. The fixed colon was stained with 0.2% methylene blue dye solution for 5 min, and ACFs were scored under light microscopy at x40 magnification by two of the authors. The criteria for aberrant crypt (AC) consisting of ACFs were enlarged crypt opening, thicker surface epithelial cell lining, and extended pericryptal zone. An average of two observers' results was recorded as the number of ACFs in each colon.

Drinking Fluids for Experiment II. Capsanthin was dissolved in ethanol at concentrations of 1.0% and 0.2% (weight/vol), and these stock solutions were added to tap water at a concentration of 0.1% (v/v) to prepare 10 p.p.m. (Caps(h) group) and 2 p.p.m. (Caps(l) group) capsanthin solutions as drinking fluids. Paprika juice was diluted 1:2.5 (Pj(h) group) and 1:16.7 (Pj(l) group) with tap water to prepare diluted paprika juice as drinking fluids containing 10 p.p.m. and 2 p.p.m. capsanthin, respectively. The control group received tap water. These drinking fluids were freshly prepared, changed daily, and protected from light to prevent the decomposition of carotenoids by covering a drinking bottle with a steel can. The volume consumed was recorded. It was confirmed by HPLC that the content of capsanthin in the drinking fluids was unchanged after 24 hr in the animal room.

Tumor Examination (Experiment II). Five groups of 30 or 25 rats each had free access to one of the respective drinking fluids throughout the experiment for 30 weeks (starting at Day 1). All the rats received an intrarectal instillation of 0.5 ml of 0.4% aqueous solution of MNU three times a week for Weeks 1–3 (starting at Day 2), as described above. At Week 30, the experiment was terminated, and all the rats were sacrificed. At autopsy, the colon was excised, cut open along its length, and inspected grossly. The location, shape, and size of tumors were recorded. Then, the colon was fixed in 10% formalin solution. All the tumors and grossly abnormal tissues and organs were examined histologically after standard processing, sectioning, and staining with hematoxylin and eosin. One rat in the control group, which died early during the experiment, was excluded from the present study.

Statistical Analysis. Data were tested for statistical significance by use of the χ^2-test and Student's t test. The criterion of significance was a $P < 0.05$.

Results

Colonic ACFs. In the short-term assay for 6 weeks (Experiment I), the body weight gain was similar among all the groups. All the rats formed ACFs in the distal colon. The total number of ACFs/colon was significantly smaller in the Caps(M) group than in the control and Caps(H) groups (Table II). It was noted that the numbers of both the small-sized ACFs with one to three ACs and the large-sized ACFs with four or more ACs were reduced in the Caps(M) group by 42% and 45%, respectively, compared with the control group. The total numbers of ACFs/colon in the Caps(H) and Caps(L) groups were numerically altered in comparison with the control group, but these apparent differences did not reach statistical significance. The largest ACF consisted of 10 ACs over all the groups.

The dosage of 0.2 mg capsanthin/day/rat, which showed significant effect against ACF formation, was followed to test for antitumorigenic activity in Experiment II.

Colon Tumors. In Experiment II of the long-term assay for 30 weeks, the mean body weight gain (100–102 g/rat at Week 1 and 201–209 g/rat at Week 30), and the mean food intake (9.5–10.0 g/day/rat throughout the experiment) were similar among all the groups. The drinking fluid consumption was similar in the control, Caps(h), Caps(l), and Pj(l) groups (18–19 ml/day/rat), whereas it was larger in the Pj(h) group (23 ml/day/rat). However, the dosage of capsanthin fed from the capsanthin fluids and paprika juice

Table II. Number of N-Methylnitrosourea-Induced Colonic Aberrant Crypt Foci in F344 Rats in Experiment I

Treatment groups[a]	Number of aberrant crypt foci/colon			
	1–3 crypts	4–6 crypts	7–10 crypts	Total (range)
Control	40.5 ± 15.5[b]	6.5 ± 3.1	0.8 ± 0.9	47.8 ± 15.6 (20–66)
Caps (H)	47.0 ± 7.6	5.0 ± 2.8	1.2 ± 1.0	53.2 ± 9.8 (35–63)
Caps (M)	23.5 ± 4.1	3.5 ± 3.3	0.5 ± 0.5	27.5 ± 4.5[c] (20–35)
Caps (L)	34.2 ± 16.9	3.5 ± 2.5	1.0 ± 0.7	38.7 ± 17.3 (15–62)

[a] All rats received three intrarectal doses of 4 mg MNU in Week 1 and were given daily an intragastric gavage of 5 mg (Caps (H) group), 0.2 mg (Caps (M) group), 0.008 mg (Caps (L) group), or 0 mg (Control group) capsanthin dissolved in 0.2 ml corn oil during Weeks 2 and 6. The experiment was terminated at Week 6, and colonic ACFs were examined. Each group consisted of six rats.
[b] Mean ± SD.
[c] Significantly different from Control and Caps (H) groups by Student's t Test: $P < 0.05$.

was close to that intended in the experimental protocol (0.2 mg/day/rat and 0.04 mg/day/rat) in all the experimental groups (Table III).

The colon tumor yield at Week 30 is summarized in Table IV. The incidence and multiplicity of tumors in the Pj(l) group were significantly lower and smaller than those in the control, Caps(h), and Caps(l) groups, whereas those of the Pj(h) group were not significantly different from the control group ($0.05 < P < 0.1$). The incidence in the Caps(h) and Caps(l) groups was lower than that of the control group, but the difference did not reach statistical significance. The number of tumors per tumor-bearing rat was similar among the groups except the Caps(l) group. The tumors were located diffusely in the distal half of the colon between the anus and 10 cm proximal to the anus, and were plaque-shaped or polypoid. Histologically, all the tumors were well-differentiated adenocarcinomas, except for three signet-ring cell carcinomas, one of which was in the control group and two in the Caps(l) group. Most of them were small in diameter, less than 10 mm, with minimum extension in the mucosa or submucosa (Table V). No metastases to lymph nodes or other organs were observed. There were no other pathologic findings in the gastrointestinal tract and other organs except malignant thymomas in one rat of the control group, two rats each of the Caps(h), Caps(l), and Pj(l) groups, and three rats of the Pj(h) group.

The data clearly demonstrated that diluted paprika juice with the low concentration of capsanthin (2 p.p.m.) inhibited MNU-induced colon tumorigenesis, whereas that with the high concentration of capsanthin (10 p.p.m.) was not effective. Also, both fluids with capsanthin alone (2 p.p.m. and 10 p.p.m.) were not effective.

Discussion

The results of the present experiments clearly demonstrated that paprika juice given as drinking solution inhibited MNU-induced colon cancer development in F344 rats, whereas capsanthin fluid was not effective even though this carotenoid significantly suppressed the formation of MNU-induced colonic ACFs in the short-term assay. Both kinds of solutions were prepared to contain capsanthin at the same concentration, and capsanthin was extracted from paprika juice. The results are consistent with those of our previous study, in which tomato juice rich in lycopene protected rats from MNU-induced development of colon cancer, whereas lycopene extracted from tomato juice was not effective even at a dosage that effectively suppressed the formation of colonic ACFs (29). The paprika juice–fed rats (Pj(h) and Pj(l) groups) had additional nutrients from the fluids in comparison with other groups of rats. The increased intake of macronutrients (protein, carbohydrate, and fat) and energy were not large, less than 4.0% of the amount taken from CE-2 chow as calculated from the volume of paprika juice consumed, and the body weight gain was similar in all the groups. On the other hand, the Pj(l) group ingested β-cryptoxanthin in a larger amount relative to the capsanthin groups as well as the control group, and the Pj(h) group had ascorbic acid, β-carotene, and β-cryptoxanthin in much larger amounts (Table III). In the other study by us, the small doses of β-cryptoxanthin (0.053 or 0.014 mg/day/rat) supplemented in the diet, which were the same amounts as those ingested in the Pj(h) and Pj(l) groups of the present experiment, were slightly but not significantly active against MNU-induced colon tumorigenesis in rats (30).

Antioxidants, when combined, may show great antioxidative capacity, thereby yielding a better overall protection of colon carcinogenesis than what would be expected from their individual activity (31, 32). Also, overwhelming evidence from epidemiological studies indicates that complex interactions among multiple antioxidant compounds in vegetables and fruits may contribute to anticarcinogenic properties with far more significance than a single compound (6, 7, 10–12). Thus, the antitumorigenic properties of paprika juice may be speculated to be responsible for a synergistic action of capsanthin and other antioxidative compounds, particularly β-cryptoxanthin. The combined effect with other compounds such as antioxidative polyphenols abundant in vegetables and fruits (33) should be subject to further studies.

The suppression of colonic ACF formation has been established as a short-term assay to screen candidate compounds for chemopreventive activity in colon carcinogenesis study in rats (34). It was reported that the predictive value of the azoxymethane-induced ACF assay for colon cancer development was estimated to be 70% from the studies of 40 chemopreventive agents (35). Retinoids inhibited azoxymethane-induced ACFs in the rat colon, but 2-(carboxyphenol) retinamide failed to prevent colon cancer development (36). We had the same observation in the lycopene study as described above (29). Also in the present experimental results, capsanthin given in the postinitiation phase was able to prevent ACFs in the short-term assay,

Table III. Average Intake of Antioxidant Compounds from CE-2 Chow, Capsanthin Fluids, and Paprika Juice in Experiment II

	Treatment groups[a]				
	Control	Caps (h)	Caps (l)	Pj (h)	Pj (l)
Ascorbic acid	1.87[b]	1.80	1.88	6.74	2.73
α-Tocopherol	0.69	0.66	0.69	0.76	0.72
β-Carotene	0.019	0.018	0.019	0.048	0.024
Lutein	0.090	0.086	0.090	0.092	0.092
Lycopene	—	—	—	—	—
β-Cryptoxanthin	0.004	0.004	0.004	0.052	0.012
Capsanthin	—	0.181	0.039	0.235	0.039

[a] All rats received an intrarectal dose of 2 mg MNU 3 times a week during Weeks 1–3 and had either 10 ppm (Caps (h) group) or 2 ppm (Caps (l) group) capsanthin fluids, and 1/2.5 (Pj (h) group) or 1/16.7 (Pj (l) group) diluted paprika juice as drinking fluids throughout the experiment. Control group had tap water. The experiment was terminated at Week 30 when all rats were sacrificed and autopsied.
[b] mg/day/rat

Table IV. Incidence and Multiplicity of N-Methylnitrosourea-Induced Colon Tumors in F344 Rats in Experiment II

Treatment[a] groups	No. or rats examined	No. of rats with tumors	No. of tumors per rat	No. of tumors per tumor-bearing rat (range)
Control	29	24 (83%)	1.6 ± 1.2[b]	1.9 ± 1.1[b] (1–4)
Caps (h)	25	17 (68%)	1.3 ± 1.2	1.9 ± 1.0 (1–4)
Caps (l)	25	17 (68%)	1.7 ± 1.5	2.5 ± 1.2 (1–5)
Pj (h)	25	15 (60%)	1.0 ± 1.0	1.6 ± 0.9 (1–4)
Pj (l)	25	10 (40%)[c]	0.7 ± 1.1[d]	1.8 ± 1.0 (1–4)

[a] See Table III or text.
[b] Mean ± SD.
[c] Significantly different from Control, Caps (H) and Caps (l) groups by χ^2 test: $P < 0.01$ or 0.05.
[d] Significantly different from Control and Caps (l) groups by Student's t test: $P < 0.02$.

Table V. Size and Invasion of Colonic Tumors in Experiment II

Treatment[a] groups	Size (diameter)			Depth of invasion		
	~2.9 mm	3.0–5.9 mm	~6.0 mm	Mucosa	Submucosa	Muscle ~ serosa
Control	17	23	5	27	16	2
Caps (h)	11	13	9	20	12	1
Caps (l)	10	23	9	24	14	4
Pj (h)	5	14	5	6	13	5
Pj (l)	3	11	4	7	10	1

[a] See Table III or text.

whereas capsanthin fed throughout both initiation and postinitiation phases did not inhibit cancer development in the long-term assay. This unexpected result is likely to show that capsanthin alone may suppress the early phase of promotion of carcinogenesis, but could be too weak in activity to inhibit the events occurring in the late phase of promotion and/or progression in the present animal model. However, in contrast to our experimental results, dietary feeding of two xanthphylls, astaxanthin and canthaxanthin, inhibited azoxymethane-induced colon cancer development in rats (37). Further studies with a precise analysis of carcinogens and antioxidants used, and their dosage, bioavailability and metabolism are needed to understand this discrepancy.

An antitumor effect of lutein was investigated using transplantable mammary cancer cell line in mice (38). Low levels of dietary lutein (20 p.p.m. and 200 p.p.m.) lowered the incidence and growth rate of the tumor, whereas high levels (2000 p.p.m. and 4000 p.p.m.) were less effective. It was noted further that the low doses protected lipid peroxidation in the tumors, but the high doses did not. A low dose of green tea polyphenols in drinking water showed the same or greater potency for preventing MNU- and azoxymethane-induced colon cancer in rats as compared with a high dose (39, 40). Both lycopene and β-carotene at relatively low concentrations, which are comparable with those in the blood of individuals having a carotenoid-rich diet, afforded protection against oxidative damage of DNA and membrane of human colon cancer HT29 cells *in vitro*, whereas at higher concentrations their effectiveness against such oxidative damage was lost (41). It is well known that antioxidants in large doses act as prooxidants under such conditions as the presence of metal ions and the presence of nitric oxide (31, 42). This prooxidant hypothesis is of great interest because in the present study, the feeding of highly diluted paprika juice in the Pj(l) group was much more effective than the feeding of the less diluted one in the Pj(h) group (this group was fed not only a high dose of capsanthin, but also large amounts of ascorbic acid, β-carotene and β-cryptoxanthin from paprika juice). Also, the medium dose of capsanthin (Caps(M) group) suppressed ACF formation, but the high dose (Caps(H) group) did not. Furthermore, in our previous study (43), a medium dose of lycopene markedly suppressed ACF formation, but a high dose did not show such activity. These findings seem to suggest that there is a proper low dosage of antioxidative compounds that offers protection from carcinogenesis. Further studies are needed to determine whether these findings can be applied as cancer chemoprevention strategy.

In conclusion, the present study suggested that capsanthin-rich paprika juice affected MNU-induced colon carcinogenesis in F344 rats. Its protective effect could be involved in the synergistic antioxidative impact of capsanthin and other active constituents. It might be proposed that the foods containing multiple antioxidant phytochemicals,

which play a role in attenuating the cancer risk, are far more effective for cancer prevention than a single chemical constituent in the foods (e.g., paprika juice versus capsanthin in the present study). To our knowledge, there have been no reports to investigate an anticarcinogenic effect of capsanthin, and paprika or paprika products rich in capsanthin in animal models.

The authors thank Mr. T. Toita for his excellent technical assistance, and Dr. John H. Weisburger, American Health Foundation, Valhalla, NY, for his critical reading of the manuscript and valuable comments.

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