

## Prevention of N-Methylnitrosourea-induced Colon Carcinogenesis in F344 Rats by Lycopene and Tomato Juice Rich in Lycopene

Tomio Narisawa,<sup>1,4</sup> Yoko Fukaura,<sup>1</sup> Makiko Hasebe,<sup>1</sup> Seiko Nomura,<sup>1</sup> Shunji Oshima,<sup>2</sup> Hideki Sakamoto,<sup>2</sup> Takahiro Inakuma,<sup>2</sup> Yukio Ishiguro,<sup>2</sup> Junko Takayasu<sup>3</sup> and Hoyoku Nishino<sup>3</sup>

<sup>1</sup>Akita University College of Allied Medical Sciences, Hondo 1-1-1, Akita 010-8543, <sup>2</sup>Research Institute, Kagome Co., Nishitomiya 17, Nishinasuno, Tochigi 329-2762 and <sup>3</sup>Department of Biochemistry, Kyoto Prefectural University of Medicine, Kajii-cho 465, Kamigyo-ku, Kyoto 602-0841

Epidemiological studies have suggested a protective effect of lycopene and lycopene-rich tomatoes against various cancers. Here, the inhibition of colon carcinogenesis by lycopene and tomato juice was investigated. Seven-week-old female F344/NSlc rats received an intrarectal dose of 2 mg (experiment I) or 4 mg (experiment II) of N-methylnitrosourea 3 times a week for 3 weeks, and had free access to one of 4 drinking fluids: plain water (control group), 17 ppm lycopene water solution (Ly group), and diluted tomato juice containing 17 ppm (Tj group) or 3.4 ppm (tj group) lycopene, throughout the experiments. The colon cancer incidence at week 35 was significantly lower in the Tj group, but not in the Ly group, than in the control group: 21% and 33% vs. 54%, in experiment I (24 rats in each group). It was significantly lower in the Tj group than in the tj and control groups, 40% vs. 72% and 84%, in experiment II (25 rats in each group). An appreciable amount of lycopene (0.02  $\mu\text{g/g}$ ) was detected in the colon mucosa of rats in the Tj group, but not in the tj group. The results suggest that tomato juice rich in lycopene may have a protective effect against colon carcinogenesis.

Key words: Tomato — Lycopene — Colon cancer — Cancer prevention

Epidemiological studies have suggested a protective role of vegetables and fruits against certain types of cancer, including colon cancer.<sup>1)</sup> The studies focused on the role of micronutrients such as ascorbic acid,  $\alpha$ -tocopherol and  $\beta$ -carotene in the diet. Lycopene, as well as  $\beta$ -carotene, is a major carotenoid in the diet and in the blood in humans.<sup>2)</sup> It has a high singlet oxygen quenching capability, amounting to twice that of  $\beta$ -carotene *in vitro*.<sup>3)</sup> Lycopene occurs predominantly in tomatoes and tomato-based products in human diets.<sup>2)</sup> In epidemiological studies, an inverse correlation between dietary and blood levels of lycopene, and the risk of cancer in various organs including the colon has been observed,<sup>4-14)</sup> although in some cases the difference did not reach statistical significance.<sup>15-21)</sup> A similar negative association of the intake of tomatoes and tomato products and the risk of cancer was also observed. Thus, lycopene is thought to have an anticarcinogenic capability.

Our previous short-term experiment with rats showed that a small dose of lycopene (0.24 mg/day or 0.12 mg/day) administered by gavage inhibited the formation of aberrant crypt foci (putative preneoplastic lesions) induced by an intrarectal dose of a direct-acting carcinogen, N-methylnitrosourea (MNU), in the rat colon.<sup>22)</sup> These observations prompted us to examine lycopene and lycopene-rich tomato juice for a protective effect against colon tumorigenesis in rats. We found that a low concen-

tration of lycopene or diluted tomato juice given as drinking water suppressed the colon cancer development induced by MNU in F344 rats. This inhibitory effect appeared to be accompanied by lycopene accumulation in the colonic mucosa.

### MATERIALS AND METHODS

**Animals** Female F344/NSlc rats (Shizuoka Laboratory Animal Center, Hamamatsu), 7 weeks of age at the start of the experiment, were used. Five rats each were housed in plastic cages with sterilized wood-chip bedding in a specific-pathogen-free animal room under constant environmental conditions with a 12 h light and dark cycle, a temperature of 22°C and a relative humidity of 50%. They had free access to a standard laboratory pelleted chow CE-2 (CLEA Co., Tokyo) 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.

**Lycopene, tomato juice and drinking water** In experiment I, lycopene (Lycored Co., Beer-Sheva, Israel) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 0.85% (w/v), and this solution was added to tap water at a concentration of 0.2% (v/v) to prepare lycopene solution as drinking water for the Ly group of rats. This concentration of lycopene was designed to provide the same

<sup>4</sup> To whom correspondence should be addressed.

amount of lycopene as the effective dose provided by gavage in the preliminary short-term assay<sup>22)</sup> on inhibition of colonic aberrant crypt foci formation. Commercially available tomato juice (Kagome Co., Tochigi) was diluted 1:2 with tap water to prepare diluted tomato juice as drinking water for the Tj group. DMSO was added to the diluted tomato juice and to plain tap water used as drinking water for the control group at a concentration of 0.2% (v/v). Both lycopene solution and diluted tomato juice contained 17 ppm lycopene. In experiment II, the tomato juice was diluted 1:2 and 1:14 with tap water to provide diluted tomato juice as drinking water for the Tj and tj groups, respectively. These two fluids were the same dilution as that effective in experiment I and a greater dilution for comparison. The control group received plain tap water. The drinking fluids were freshly prepared and changed daily, and were shaded from light to prevent the decomposition of lycopene. The volume consumed was recorded. It was confirmed by a high performance liquid chromatography (HPLC) procedure that the contents of lycopene in the drinking fluids were unchanged after 24 h in the animal room. The nutritional ingredients of tomato juice and CE-2 chow used are listed in Table I.

**Animal treatments** In experiment I, 3 groups of 24 rats each, the control, Ly and Tj groups, had free access to the respective drinking fluids throughout the experiment for 35 weeks. All the rats received an intrarectal instillation of 0.5 ml of 0.4% aqueous solution of N-methyl-N-nitrosourea (MNU) (Nacalai Tesque, Kyoto) 3 times a week for weeks 1 to 3 as described previously.<sup>23)</sup> 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 infused. The solution filled the distal half of the colon, where the tumors developed. The feces from 5 rats of each group were collected for 4 days at week 25 and stored at  $-80^{\circ}\text{C}$  for lycopene analysis.

In experiment II, 3 groups of 25 rats each, the control, Tj and tj groups, had free access to the respective drinking fluids throughout the experiment for 35 weeks. All the rats received an intrarectal instillation of 0.5 ml of 0.8% aqueous solution of MNU 3 times a week for weeks 1 to 3 by the same procedure as in experiment I. The feces from 5 rats of each group were collected for 3 days at week 20 and stored at  $-80^{\circ}\text{C}$  for lycopene analysis.

**Tumor examination** In both experiments I and II, all the rats were killed at week 35 by withdrawal of blood from the abdominal aorta after laparotomy under intraperitoneal Nembutal anesthesia (40 mg/kg body weight). The blood was centrifuged, and the serum was collected. The colon was excised, opened lengthwise and inspected grossly. In experiment II, the mucosa of the colon without tumors was scraped with a blunt steel plate and collected after having been rinsed to remove debris with cold 0.9% NaCl

solution. The liver was also collected. The serum, colon mucosa and liver were stored at  $-80^{\circ}\text{C}$  for lycopene analysis. The segment of the colon with tumors was fixed in 10% formalin solution. All the tumors and grossly abnormal tissues or organs were examined histologically after standard processing, sectioning and staining with hematoxylin and eosin.

**Lycopene analysis** The assays of lycopene in the serum, liver, colon mucosa and feces were performed by HPLC as described.<sup>24)</sup> Briefly, the serum (200  $\mu\text{l}$ ) was mixed with ethanol (1.0 ml), then extracted twice with hexane and dichloromethane (4:1, v/v, 5.0 ml). The supernatant was evaporated to dryness, and the residue was reconstituted in a sufficient quantity of mobile phase to bring the sample to its original volume. The tissues and feces were homogenized and saponified by addition of 60% KOH and 3% butylated hydroxytoluene in ethanol (4:1, v/v), followed by heating at  $50^{\circ}\text{C}$  for 30 min. After repeated extraction with hexane and dichloromethane, the samples were dried and then reconstituted as described. The samples were analyzed by HPLC, using a Shimadzu SPD-M 10AVP diode array detector (Shimadzu, Kyoto) and a LiChrospher RP18-5 column (E. Merk, Darmstadt, Germany) (4.6 $\times$ 250 mm). The mobile phase was acetonitrile and methanol (1:1, v/v), and the flow rate was 2.0 ml/min.

**Statistical analysis** The significance of differences was tested statistically by use of the  $\chi^2$  test and Student's *t* test. The criterion of significance was taken as  $P < 0.05$ .

## RESULTS

**Body weight, diet and drinking fluid** The mean body weight gain, and the mean intakes of CE-2 chow and drinking fluids are summarized in Table II. They were approximately the same among the 3 groups in each of experiments I and II. The mean dosage of lycopene ingested from lycopene solution and diluted tomato juice

Table I. Contents of Macro- and Micro-nutrients in Tomato Juice and CE-2 Diet

	Tomato juice (100 g)	CE-2 diet (100 g)
Protein (g)	0.7	25.4
Fat (g)	—	4.4
Carbohydrate (g)	4.0	50.3
Fiber (g)	0.1	4.1
Ash (g)	0.5	6.9
Energy (kcal)	16	342
Ascorbic acid (mg)	9.0	19.0
$\alpha$ -Tocopherol (mg)	0.3	7.0
$\beta$ -Carotene (mg)	0.2	0.15
Lycopene (mg)	5.0	0.06
Lutein (mg)	0.04	0.40

Table II. Mean Values of Body Weight, Diet Intake, Drinking Fluid Consumption and Lycopene Dosage

Treatment groups <sup>a)</sup>	Initial/final body weight (g)	Diet (g/day/rat)	Drinking fluid (ml/day/rat)	Lycopene dosage	
				(mg/day/rat)	(mg/day/kg body weight)
Experiment I					
Control	111/219	9.3 <sup>b)</sup>	19 <sup>b)</sup>	—	—
Ly	111/223	9.6	19	0.32 <sup>b)</sup>	1.8 <sup>b)</sup>
Tj	110/219	9.4	18	0.30	1.7
Experiment II					
Control	115/217	9.8	20	—	—
Tj	116/217	9.5	20	0.33	1.8
tj	115/215	9.9	21	0.07	0.4

a) All rats received an intrarectal dose of MNU: 2 mg in experiment I and 4 mg in experiment II, 3 times a week for weeks 1–3, and had plain water (control group), 17 ppm lycopene water solution (Ly group), tomato juice diluted 1:2 with water (Tj group) and tomato juice diluted 1:14 with water (tj group) as drinking fluid throughout the experiment. Each group consisted of 24 (experiment I) or 25 (experiment II) rats. The experiment was terminated at week 35.

b) Mean amount throughout the experiment for 35 weeks.

Table III. NMU-induced Colon Tumors in F344 Rats Treated with Lycopene or Lycopene-rich Tomato Juice

Treatment <sup>a)</sup> groups	No. of rats examined	No. of rats with tumors	No. of tumors per rat	No. of tumors per tumor-bearing rat
Experiment I				
Control	24	13 (54%)	0.6±0.1 <sup>b)</sup>	1.2±0.1 <sup>b)</sup>
Ly	24	8 (33%)	0.4±0.1	1.3±0.2
Tj	24	5 (21%) <sup>c)</sup>	0.3±0.1 <sup>c)</sup>	1.2±0.2
Experiment II				
Control	25	21 (84%)	1.5±0.2	1.8±0.3
Tj	25	10 (40%) <sup>d)</sup>	0.8±0.2 <sup>e)</sup>	1.9±0.2
tj	25	18 (72%)	1.0±0.2	1.4±0.2

a) See Table II or text.

b) Mean±SE.

c) Significantly different from control group:  $P < 0.02$  and  $0.05$ .

d) Significantly different from control and tj groups:  $P < 0.01$  and  $0.05$ .

e) Significantly different from control group:  $P < 0.05$ .

Table IV. Lycopene Levels in Serum, Liver, Colon Mucosa and Feces

Treatment <sup>a)</sup> groups	Serum, $\mu\text{g/ml}$ (n=8)	Liver, $\mu\text{g/g}$ (n=8)	Colon mucosa, $\mu\text{g/g}$ (n=8)	Feces, $\mu\text{g/g}$ dry feces (n=5)
Experiment I				
Ly	<0.01	— <sup>b)</sup>	—	17.0±3.9 <sup>c)</sup>
Tj	<0.01	—	—	20.7±1.5
Experiment II				
Tj	<0.01	0.70±0.07 <sup>c)</sup>	0.02±0.007 <sup>c)</sup>	27.2±1.4
tj	<0.01	0.09±0.006	<0.01	10.2±0.4

a) See Table II or text. Samples were collected at week 35 (serum, liver and colon mucosa), and at weeks 20 and 25 (feces). No lycopene was detected in any samples from control groups.

b) Not examined.

c) Mean±SE.

was essentially the same in the Ly and Tj groups in experiment I. In experiment II, the mean dosage of lycopene in the Tj and tj groups was close to that intended in the experimental design.

**Colon tumors** The results of colon tumor development examined at week 35 are summarized in Table III. In experiment I, the tumor incidence and the number of tumors per rat were significantly lower and smaller, respectively, in the Tj group than in the control group. Those of the Ly group were decreased compared with the control group, but the difference did not reach statistical significance ( $0.1 < P < 0.2$ ). The number of tumors per tumor-bearing rat was similar among the 3 groups. In experiment II, the tumor incidence of the Tj group was significantly lower than those of the control and tj groups. The number of tumors per rat in the Tj group was significantly smaller than that in the control group. The number of tumors per tumor-bearing rat was not significantly different among the 3 groups.

All the tumors were located diffusely in the distal half of the colon, and were plaque-shaped or polypoid. Histologically, all the tumors except one from the Ly group in experiment I were well-differentiated adenocarcinomas. They were small, less than 10 mm in diameter, with minimum extension within the mucosa or the submucosa, and no metastases to lymph nodes or other organs, because the experiments were terminated early at week 35. No distinct differences among the groups throughout experiments I and II were observed in the location, shape, size, depth of invasion and histological type of tumors. One tumor, 15 mm in diameter, was mucinous adenocarcinoma with serosal invasion, peritoneal dissemination and lung metastases. There were no other abnormal findings in the gastrointestinal tract or other organs, except for mammary carcinomas in one rat of the control group in experiment I, and 2 rats of the control group and one rat of the tj group in experiment II.

Thus, the data clearly demonstrated that diluted tomato juice at the higher concentration (17 ppm lycopene) effectively, but 17 ppm lycopene fluid less effectively, inhibited MNU-induced colon carcinogenesis.

**Lycopene** Lycopene levels in the serum and feces were assayed in experiment I, and those in the serum, colonic mucosa, liver and feces in experiment II. No lycopene was detected in any samples from the control groups of experiments I and II. The mean values in the samples from the Ly, Tj and tj groups are summarized in Table IV. The levels in the serum were low or undetectable. An appreciable amount was detected in the colonic mucosa of the Tj group, but not in the tj group, in experiment II. A large amount or a small amount of lycopene was found to accumulate in the livers of the Tj and tj groups, respectively, in experiment II. A large amount of lycopene was recovered in the feces from all the groups. The fecal lev-

els of the Ly and Tj groups in experiment I were mostly similar.

## DISCUSSION

The present experiments provide evidence that tomato juice rich in lycopene, given as drinking water, can inhibit colon carcinogenesis in animal models. The difference of the incidence and number of colon tumors between the 2 experiments reflects the dosage of the carcinogen MNU, that is, 2 mg per dose in experiment I and 4 mg per dose in experiment II. The treatment with a high dose of tomato juice resulted in the inhibition of MNU-induced colon tumor development in the first experiment. This finding was confirmed by the second experiment, though a low dose of tomato juice did not affect the tumor development. The results are consistent with an epidemiological study showing that a high intake of tomatoes and tomato products reduced the risk of cancer, compared to a low intake.<sup>13)</sup> However, it was observed in the first experiment that lycopene solution was less effective, even though the amount of lycopene taken in and the fecal excretion were similar in the groups of rats given lycopene solution (Ly group) or diluted tomato juice (Tj group). In the rats given the effective dose of tomato juice, the increased intake of macro- and micro-nutrients and energy derived from tomato juice was not large, less than 5%, compared to the control rats, whereas there were marked increases in lycopene (55 times),  $\beta$ -carotene (90%) and ascorbic acid (30%). Lycopene solution contained only purified lycopene. Thus, it is likely that the difference in the inhibition of colon tumor development between the Ly and Tj groups reflects the combined effect of lycopene and other micro-constituents in tomato juice, such as  $\beta$ -carotene.

The treatment with the high dose of tomato juice caused an accumulation of a considerable amount of lycopene in the colonic mucosa and a large amount in the liver. These lycopene levels of the colon and liver are consistent with those found in a study using a single dose of <sup>14</sup>C-lycopene.<sup>25)</sup> Carotenoids are absorbed from the intestine via concentration-dependent passive transport.<sup>2)</sup> We found in rats that lycopene accumulated in the liver at 24 h after a single intrarectal administration of a large dose of lycopene (unpublished data). This observation indicates that the colon absorbs lycopene, presumably in a concentration-dependent fashion. Lycopene in the colon mucosa probably accounted in large part for the absorption from the colon *in situ*, because a large amount of lycopene was found in the feces, and affected an early phase of carcinogenesis as noted in our previous study on the inhibition of colonic aberrant crypt foci.<sup>22)</sup> On the other hand, the serum level of lycopene was very low even in the rats given the high (effective) dose of tomato

juice. This is consistent with the findings that in rats, lycopene was found in the blood within 4–8 h after a single dose and was eliminated rapidly, while in humans its maximum concentration was reached after 24–48 h with a half-life of 2–3 days.<sup>25, 26)</sup>

There have been a few animal studies on anticarcinogenic activity of lycopene. Spontaneous mammary tumors in SHN virgin mice were reduced by feeding of a diet rich in lycopene.<sup>27)</sup> Intraperitoneal injection of lycopene-enriched tomato extract reduced 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors in rats,<sup>28)</sup> and the ingestion of lycopene-containing drinking water decreased carcinogen-induced lung tumors in male B6C3F1 mice.<sup>29)</sup> Recent reports showed that 25 ppm lycopene solution and diluted tomato juice (25 ppm lycopene) given as drinking water reduced N-butyl-N-(4-hydroxybutyl)nitrosamine-induced urinary bladder cancer in rats, though tomato juice was much more effective than lycopene.<sup>30, 31)</sup> It is noteworthy that the results are consistent with our results in the present study. Furthermore, a mixture of vegetables and fruits (including tomatoes) in the diet reduced N-methyl-N'-nitro-N-nitrosoguanidine-induced colon cancer in rats fed with high fat diet.<sup>32)</sup> In *in vitro* studies, a potential anticancer activity of lycopene

was demonstrated against human endometrial, lung and breast cancer cells, whereas human fibroblasts were less sensitive to growth inhibition by lycopene.<sup>33)</sup> Furthermore, lycopene inhibited malignant transformation of C3H/10T1/2 cells initiated by 3-methylcholanthrene.<sup>34)</sup> Lycopene is known to have an antioxidative action, which may be related to its beneficial effect in preventing colon carcinogenesis. However, further studies are necessary to elucidate fully the mechanism(s) of the anticarcinogenic activity of lycopene, tomatoes and tomato products.

In conclusion, the present experimental results, along with epidemiological studies showing an inverse association of lycopene-rich diet and cancer risk, suggest that tomatoes and tomato-based products rich in lycopene may provide protection against colon carcinogenesis.

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