Jpn. J. Cancer Res. **89**, 22–26, January 1998

Inhibitory Effect of Tomato Juice on Rat Urinary Bladder Carcinogenesis after *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine Initiation

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The effects of tomato juice on urinary bladder carcinogenesis were studied in male Fischer 344 rats initiated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) in rats. The animals (6 weeks old) were given 0.05% BBN in their drinking water for 8 weeks, followed by diluted tomato juice for 12 weeks, and killed at 20 weeks after the beginning of the experiment. Lycopene concentrations in the livers of rats given tomato juice were elevated. Histopathological analysis of urinary bladder lesions revealed the numbers, but not incidences, of urinary bladder transitional cell carcinomas (TCCs) to be decreased in the group given tomato juice. No influence on the incidence of simple and nodullopapillary hyperplasias, invasion or differentiation of TCC was noted. These results indicate that tomato juice, presumably the contained lycopene and other anti-oxidants in combination, exerts an inhibitory effect on the development of TCCs in the rat urinary bladder.

Key words: Tomato juice — Rat urinary bladder carcinogenesis — *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine

Despite recent progress in therapeutic approaches for urinary bladder carcinomas, the recurrence rate of papillary, non-invasive TCCs in man is high, sometimes with an increase in the level of malignancy of the tumor.[1)] In order to decrease this risk, it would be helpful to identify environmental factors which may prevent tumorigenesis. For this purpose the BBN rat urinary bladder carcinogenesis model is well established.[2)]

Tomatoes and tomato products including tomato juice, a popular soft drink world-wide, contain a large amount of lycopene and are the major source of lycopene in the human diet.[3)] Lycopene is a carotenoid without provitamin A activity, but with efficient singlet oxygen quenching and radical scavenging activities.[3)] Recent cohort studies have shown a low risk of urinary bladder[4)] and prostate[5)] cancer in people with a high serum level of lycopene or with a regular high intake of lycopene or tomatoes. Possible inhibitory mechanisms are enhancing effects of lycopene on gap junctional communication,[6)] its suppression of tumor cell prolife-ration[7)] and a positive influence on immunological surveillance.[8)] Astaxanthin, another carotenoid without provitamin A activity,[9)] but not β-carotene,[10)] exerts a chemopreventive effects against the development of invasive bladder cancers in mice. Recently we studied the effects of β-carotene and lycopene on rat superficial urinary bladder carcinogenesis by BBN and found that β-carotene is ineffective and lycopene is only an equivocal chemopreventor.[11)]

In the present study, we investigated the influence of tomato juice, which contains lycopene and other anti-oxidants in their natural state, on the post-initiation development of superficial urinary bladder cancers in rats.

MATERIALS AND METHODS

Chemicals and tomato juice BBN was purchased from Tokyo Kasei Kogyo (Tokyo). *Trans*-8′-apocarotenal was obtained from Sigma (St. Louis, MO), and the other reagents used for the carotenoid estimation were all of special reagent grade. Tomato juice for animal experiments, which was prepared by diluting commercially available tomato juice with an equal amount of distilled water, was manufactured by Kagome Co., Ltd. Its contents are listed in Table I. Details of their estimation have already been reported.[12)] Tomato juice used in the present study, including the commercially manufactured product, contained no extra additives except distilled water. For experiments, the prepared tomato juice was diluted again with the same amount of distilled water twice a week just before the use, to a quarter (the concentration of lycopene being 25 ppm) of the commercially available concentration, and given in light-opaque bottles.

Animals and treatment A total of 48 male Fischer 344 rats (Japan SLC Inc., Hamamatsu), 6 weeks old at the commencement were used in the experiments. The animals

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Abbreviations: BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; TCC, transitional cell carcinoma; SH, simple hyperplasia; NPH, nodulopapillary hyperplasia; DTPA, diethylenetriaminepentaacetic acid; HPLC, high-performance liquid chromatography.

were housed 4 to a plastic cage, with hardwood chips for bedding, and were given free access to water and diet under conditions of controlled temperature (23±1°C), humidity (60±10%) and lighting (12 h-12 h light-dark cycle). Throughout the experimental period, the animals were observed daily to assess thier general condition. Body weights and consumption of food and drinking water were measured weekly. The basal diet, CE-2, was purchased from Japan Clea Co., Ltd. (Tokyo) and was available together with the prepared tomato juice during the animal experiment.

Experimental protocol The experimental protocol is shown in Fig. 1. In experiment 1, 24 rats were randomly divided into 2 groups of 12, and both were given 0.05% BBN in the drinking water for the first 8 weeks. This was followed by tap water or tomato juice for 12 weeks, respectively, in groups 1 and 2. All animals were killed under ether anesthesia 20 weeks after the beginning of the experiment. Their urinary bladders were ligated at the neck, inflated by intraluminal injection of 10% phosphate-buffered formalin, quickly resected and immersed in fixative. In addition to the urinary bladders, the livers were also removed, weighed and subjected to histological examination. Parts of the livers from 8 rats in each group were frozen in liquid nitrogen for the measurement of carotenoid contents. Experiment 2, using the same protocol as for experiment 1, was conducted to confirm the reproducibility of the results.

Histopathological examination After overnight fixation, bladders were longitudinally disected and macroscopically observed, and the lesions were recorded as a guide to histological examination. Sections through the bladders and the liver were routinely prepared and stained with hematoxylin and eosin for histological examination. Histopathological classification of bladder lesions was performed according to the criteria of Oyasu *et al.* as described previously.[13] Carcinomas were divided into 3 grades as follows: grade 1 tumor, either papillary or nodular, showing minimal cytological atypia and infrequent mitotic figures. The nuclei of grade 2 carcinomas were larger and more pleomorphic than were those of grade 1, and nucleoli were prominent. Mitotic figures were readily detectable. Grade 3 carcinomas were characterized by marked cytological and architectural abnormalities. Depending upon the depth of wall invasion, tumors were divided into Ta, confined to the epithelium or to the tumor stroma only; T1, extension to lamina propria; T2, tunica muscularis; or T3, tunica adventitia.

Measurement of lycopene Levels of lycopene in the liver tissues were estimated individually according to the methods of Oshima *et al.*[14] Briefly, after adding 1 ml of ethanol solution containing *trans*-β-8′-apocarotenal as an internal standard, wet liver tissues were immersed in 5.0 ml of ethanol containing 0.02% butylated hydroxytoluene, and minced with scissors. Minced liver tissues were immersed in 1 ml of 60% KOH followed by addition of 1 ml of 0.9% NaCl solution (saline) containing 0.5 m*M* DTPA, and incu-

Table I. Contents of 100 g of Tomato Juice[a)]

Water	94.7 g
Protein	0.7 g
Fiber	0.5 g
Saccharide	4.0 g
β-Carotene	0.2 mg
Lycopene	5.0 mg
Vitamin C	9.0 mg
Vitamin E	0.3 mg
Minerals	0.5 g
Ca	4.7 mg
K	270 mg
Cu	47 μg
Zn	113 μg
Mn	67 μg

a) Values are contents in the original tomato juice manufactured for the present study. It was diluted half for the experiment (see text).

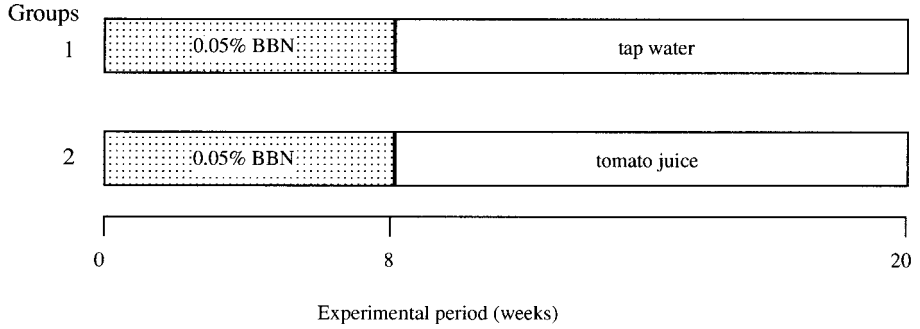

Fig. 1. Experimental protocol.

bated at 50°C for 30 min. The mixture was adjusted to a total volume of 10 ml with saline, and 1 ml aliquots were added to 5 ml of *n*-hexane and dichloromethane (4:1, v/v), then centrifuged at 3000 rpm for 5 min. A 4 ml aliquot of the supernatant was dried by evaporation under nitrogen gas, and the residue was dissolved in a mixture of methanol, acetonitrile, dichloromethane and water (7:7:2:0.16, v/v/v/v) for HPLC analysis. The levels of carotenoids were measured by HPLC on the basis of their retention time, using a Lichrospher RP 18-5 column (E. Merck, Darmstadt, Germany) with an eluting solvent of methanol, acetonitrile, dichloromethane and water (7:7:2:0.16, v/v/v/v) containing 50 m*M* $NaClO_4$ and 2.0 m*M* DTPA at the constant flow rate of 1.0 ml/min. The effluent was monitored at 450 nm using a Shimadzu SPD-10 AV spectrophotometric detector (Shimadzu, Kyoto).

Statistical analysis Quantitative differences between group values were analyzed for statistical significance using the unpaired Student's *t* test and Fischer's exact test.

RESULTS

Details of experiments Initial and effective numbers of rats in experiments 1 and 2 are shown in Table II. Four rats of group 1 in experiment 1 died with pneumonia. Final body weights were not significantly different between the

Table II. Body and Relative Liver Weights for Rats Given BBN Followed by Tap Water or Tomato Juice and Liver Lycopene Concentrations

Group	Treatment	No. of rats		Body weight (g)		Relative liver weight to body weight (g/kg)	Lycopene concentration (ng/g wet liver tissue)
		Initial	Effective[a)]	at 8 wk	at 20 wk		
Experiment 1							
1	None	12	8	289± 7	371±15	31.5±1.5	ND
2	Tomato juice	12	12	291±10	370±14	29.5±2.2	199.8±96.6
Experiment 2							
1	None	12	12	284±10	374±18	33.3±2.8	ND
2	Tomato juice	12	12	277±10	360±14	30.6±1.3	247.6±30.8
Sum of both experiments							
1	None	24	20	286±10	373±17	32.6±2.5	ND
2	Tomato juice	24	24	284±15	365±15	30.1±1.8	228.5±67.6

a) Based on histological examination.
ND, not detected.

Table III. Incidences of Urinary Bladder Lesions Including TCCs and Their Invasiveness and Differentiation in Rats Given BBN Followed by Tap Water or Tomato Juice

Group	Treatment	Effective No. of rats[a)]	Incidence(%)						
			SH[b)]	NPH[c)]	TCC[d)]	TCC			
						Invasion[e)]		Differentiation[f)]	
						Ta	Tl	Grade 1	Grade 2
Experiment 1									
1	None	8	8(100)	8(100)	7(95)	1(14)	6(86)	3(43)	4(57)
2	Tomato juice	12	12(100)	11(92)	10(83)	3(30)	7(70)	3(32)	7(68)
Experiment 2									
1	None	12	12(100)	12(100)	12(100)	2(17)	10(83)	3(25)	9(75)
2	Tomato juice	12	12(100)	11(92)	9(75)	0(0)	9(100)	3(32)	6(68)
Sum of both experiments									
1	None	20	20(100)	20(100)	19(95)	3(16)	16(84)	6(32)	13(68)
2	Tomato juice	24	24(100)	22(92)	19(79)	3(16)	16(84)	6(32)	13(68)

a) Based on histological examination.
b) Simple hyperplasia.
c) Nodulopapillary hyperplasia.
d) Transitional cell carcinoma.
e) Ta, No invasion; T1, Invasion to the lamina propria.
f) Grade 1, Either papillary or nodular, showing minimal cytological atypia and infrequent mitoses; Grade 2, Larger and more pleomorphic than grade 1 carcinomas, with prominent nucleoli and readily detectable mitotic figures.

Table IV. Numbers of TCCs and Their Invasiveness and Differentiation of Rats Given BBN Followed by Tap Water or Tomato Juice

Group	Treatment	No. of TCCs		Invasion (%)[a)]		Differentiation (%)[a)]	
		Per group	Per rat[b)]	Ta	T1	Grade 1	Grade 2
Experiment 1							
1	None	18	2.25±1.3	7(39)	11(61)	11(61)	7(39)
2	Tomato juice	13	1.08±0.8[c)]	5(38)	8(62)	9(69)	4(31)
Experiment 2							
1	None	26	2.17±1.5	8(31)	18(69)	11(42)	15(58)
2	Tomato juice	15	1.25±1.1[c)]	1(7)	14(93)	3(20)	12(80)
Sum of both experiments							
1	None	44	2.20±1.4	15(34)	29(66)	22(50)	22(50)
2	Tomato juice	28	1.17±0.9[c)]	6(21)	22(79)	12(43)	16(57)

a) Numbers and percentages of TCCs demonstrating Ta and T1 invasion or grade 1 and grade 2 differentiation.
b) Values are mean±SD.
c) Significantly different from group 1 ($P<0.05$ by Student's *t* test).

groups in experiment 1 or 2. Average of intakes of tap water and tomato juice were 70.2±13.5 and 70.8±12.8 ml/day/kg body weight in group 1 and 71.0±17.5 and 70.9±17.6 ml/day/kg body weight in group 2, respectively.

Lycopene was not detectable in the liver of rats given BBN followed by tap water (detection limit, <10 ng/g wet tissue), but large amounts were found in all animals given the tomato juice, with values of 199.8±96.6 ng/g wet tissue in experiment 1, and 247.6±30.8 ng/g in experiment 2.

Incidences and numbers of urinary bladder lesions The incidences of rats bearing urinary bladder lesions are summarized in Table III. Lesions including SH, NPH and TCC were encountered. The incidences of these lesions were not affected by the administration of tomato juice in either of the experiments. Invasive or poorly differentiated carcinomas classified as T2, T3 or grade 3 were not found and the incidences of Ta and T1 and grade 1 and 2 lesions did not differ between the groups.

The numbers of TCCs per rat and their classification with regard to invasion and differentiation are given in Table IV. A significant decrease in multiplicity was apparent in rats treated with tomato juice. However, the invasiveness and differentiation of TCC cells were not affected.

DISCUSSION

The present investigation of the effects of tomato juice on urinary bladder carcinogenesis demonstrated a reproducible inhibition of TCC development in terms of the numbers of lesions, but no effect on the incidence or differentiation. The average daily intake of 4-times-diluted tomato juice per kg body weight in this study, 71 ml, corresponds to 17.8 ml of commercially available tomato juice. Thus, the average daily intakes of lycopene, β-carotene, vitamin C and vitamin E per kg body weight were 1.78 mg, 0.07 mg, 3.2 mg and 0.11 mg, respectively. Extrapolating the value of 17.8 ml to an average human being, it represents 1068 ml, approximately 5 cans of commercially available tomato juice per individual per day.

The concentration of lycopene used in the present study was the same as that used in our previous experiment, in which pure lycopene was given.[11)] The amount of lycopene consumed per rat was similar,[11)] but the inhibitory effects were far clearer in the persent case. Interestingly, the lycopene concentration was higher, at 228.5 ng/g wet liver tissue as opposed to 99.5 ng/g wet liver tissue, suggesting that there may be a difference in the stability of purified lycopene and its form in tomato juice. In this context, the metabolism of lycopene needs further study.

The situation is complicated by the fact that many other components are contained in tomato juice in addition to lycopene. They include anti-oxidants, vitamin C (ascorbic acid) and vitamin E (alpha tocopherol). Reportedly, neither vitamin C[15)] nor vitamin E[16)] exert any inhibitory effect on rat urinary bladder carcinogenesis. Vitamin C, in combination with either sodium or potassium salt, rather exerts an enhancing effect on rat urinary bladder carcinogenesis.[15, 17)] The present results might have been due to the combined effects of lycopene and anti-oxidants such as vitamins C and E, which might explain the lack of clear inhibitory potential of pure lycopene for rat urinary bladder carcinogenesis observed earlier.[11)] An involvement of other ingredients in tomato juice can not be excluded.

While tomato juice is a popular drink all over the world, five cans per day is well beyond the consumption of ordinary people. However, the inhibitory effects on urinary bladder carcinogenesis observed here support the epidemiological finding of a low risk of TCC in people with a high serum level of lycopene or with a high regular intake of tomatoes and tomato products. The possibility of supplementation for high-risk individuals clearly warrants further attention

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid from the Ministry of Health and Welfare for the Second Term Comprehensive 10-Year Strategy for Cancer Control, Japan and a Grant-in-Aid for Scientific Research (A) from the Ministry of Education, Science, Sports and Culture, Japan.

(Received September 11, 1997 / Revised October 22, 1997 / Accepted October 27, 1997)

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