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Chemoprevention of 4-Nitroquinoline 1-Oxide-induced Oral Carcinogenesis by Dietary Curcumin and Hesperidin: Comparison with the Protective Effect of β-Carotene[1]

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ABSTRACT

The modifying effects of two natural products, curcumin and hesperidin, given during the initiation and postinitiation phases of oral carcinogenesis initiated with 4-nitroquinoline 1-oxide (4-NQO) were investigated in male F344 rats and compared with that of β-carotene. At 6 weeks of age, rats were divided into experimental and control groups and fed the diet containing β-carotene, hesperidin, or curcumin at a dose of 0.5 g/kg diet (500 ppm). At 7 weeks of age, all animals except those treated with each test chemical alone and control groups were given 4-NQO (20 ppm) in the drinking water for 8 weeks to induce oral cancer. Seven days after the 4-NQO exposure, groups of animals fed the diets containing test chemicals were switched to the basal diet and continued on this diet until the end of the study. Starting 1 week after the stop of 4-NQO exposure, the groups given 4-NQO and a basal diet were switched to the diets containing β-carotene, hesperidin, and curcumin and maintained on these diets for 22 weeks. The other groups consisted of rats given 500 ppm β-carotene, hesperidin, or curcumin alone or untreated rats. All animals were necropsied at the termination of the experiment (week 32). The incidences of tongue neoplasms and preneoplastic lesions, polyamine levels in the tongue tissue, and cell proliferation activity estimated by bromodeoxyuridine-labeling index and by morphometric analysis of silver-stained nucleolar organizer region proteins were compared among the groups. Feeding of curcumin and β-carotene during the initiation and postinitiation phases and hesperidin at the initiation stage caused a significant reduction in the frequency of tongue carcinoma (41–91% reduction, $P < 0.05$) and the order of chemopreventive efficacy was curcumin > β-carotene > hesperidin. The incidences of oral preneoplasia in rats fed the diets mixed with these compounds were also decreased ($P < 0.05$). There were no such lesions in rats treated with test compounds alone or those in an untreated control group. Dietary administration of these compounds significantly decreased the labeling index of bromodeoxyuridine and the number and area of silver-stained nucleolar organizer region proteins per cell nucleus that are proliferation biomarkers, of the tongue squamous epithelium ($P < 0.05$). In addition, polyamine levels in the oral mucosa were lowered in rats treated with 4-NQO and three test compounds when compared to those give 4-NQO alone ($P < 0.05$). These results indicated that natural compounds curcumin and hesperidin inhibited rat oral carcinogenesis initiated with 4-NQO (relatively weak in hesperidin) as did β-carotene, and such inhibition might be related to suppression of cell proliferation.

INTRODUCTION

Oral cancer is a common neoplasm in Asia and the Pacific Islands, particularly in India, Sri Lanka, South Vietnam, Papua New Guinea, the Philippines, Hong Kong or Taiwan, China, and parts of Brazil (1–4). Although Japan has one of the lowest incidence of oral and pharyngeal cancer in the world, the patients with these malignancies have recently been increasing (4). The variation in the incidence of oral cancer in the world is related to exposure to known etiologic agents (5). It is generally believed that oral mucosal carcinomas are caused predominantly by chemical carcinogens, although there is evidence implicating viral, fungal, and physical stimuli in the genesis of some oral neoplasms (6). Epidemiological data provide strong support for exogenous factors such as tobacco and alcohol use as being major causative agents (6, 7). Recently, an increased incidence of oral cancer, especially tongue cancer, was indicated among young adults and this might be related to the use of smokeless tobacco (8–10). Neoplasms in the head and neck including oral cavity possess some biological characteristics that are multistage and multifocal carcinogenesis as revealed by histological, experimental, and molecular studies (11–13). In fact, it has been reported that patients with oral cancer have an increased incidence of second primary tumors of the oral cavity (14, 15).

The term "cancer chemoprevention" refers to the prevention of cancer by intervention using nontoxic synthetic chemicals or chemicals from natural substances before malignancy (16). Epidemiological observations suggest a statistically significant inverse association between oral cancer and the consumption of fruits and/or vegetables (17, 18). A number of micronutrients, macronutrients, and nonnutrients have been reported as inhibiting or chemopreventive agents in chemical carcinogenesis in rodents (19–21). Experimental studies on oral cancer chemoprevention in the oral cavity have mainly been conducted using hamster buccal pouch carcinogenesis model with a carcinogen 7,12-dimethylbenz(*a*)anthracene and chemopreventives have been limited to some vitamins including β-carotene (22, 23). Another experimental oral carcinogenesis model was developed by continuous application of a water-soluble carcinogen 4-NQO[3] to rats. As reviewed by Gerson (24), oral lesions in rats produced by 4-NQO are more comparable to human lesions since many ulcerated and endophytic tongue lesions develop when the 4-NQO is in the drinking water (25). Because of easy accessibility to examine and follow-up of the lesions in the oral cavity, oral cavity is one of the excellent target organs for experimental chemoprevention studies. Our previous works using 4-NQO-induced rat oral carcinogenesis model revealed inhibitory and chemopreventive effects of several natural and synthetic compounds (26–31). Among them, plant phenolics (caffeic, chlorogenic, ferulic, and protocatechuic acids) and synthetic compounds (butylated hydroxyanisole and butylated hydroxytoluene) are potent antioxidants. Protocatechuic acid (3,4-dihydroxybenzoic acid), a phenolic antioxidant (32), could also inhibit liver and colon tumorigenesis (33, 34).

Carotenoids including β-carotene are versatile antioxidants (35). β-Carotene, containing the highest potential vitamin A activity (one-sixth that of retinol), is well known to have a preventive effect on hamster buccal pouch carcinogenesis and to retard the progressive changes of premalignant lesions in the oral cavity of humans (22, 23).

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[3] The abbreviations used are: 4-NQO, 4-nitroquinoline 1-oxide; ODC, ornithine decarboxylase; BrdUrd, 5-bromodeoxyuridine; AgNORs, silver-stained nucleolar organizer region proteins; 4-HAQO, 4-hydroxyaminoquinoline 1-oxide.

Curcumin is the major pigment in turmeric (the ground rhizome of *Curcuma longa* L.) which is widely used as a spice and coloring agent in curry, mustard, and other foods and has been reported to possess antioxidative and antiinflammatory activity (36). Extensive works by Huang's group (37) demonstrated a protective effect of curcumin (diferuloylmethane) on tumor initiation by benzo(*a*)pyrene and tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in female CD-1 mice. Hesperidin, a major citrus flavonoid, is the 7-β-rutinoside (6-*O*-α-L-rhamnosyl-β-D-glucoside) of the flavone hesperitin. Hesperidin is reported to inhibit 2-aminoanthracene-induced mutagenesis in *Salmonella typhimurium* (T-98) (38) and to suppress the *in vitro* effects of the tumor promoter teleocidin, such as the incorporation of $^{32}P_i$ into phospholipids of HeLa cells, as did another antitumor promoter, quercetin (39). Hesperidin and hesperitin are weak antioxidants (40). However, there were no studies on the modifying effect of hesperidin on *in vivo* experimental carcinogenesis including oral carcinogenesis. These findings led us to investigate the modifying (possibly inhibiting) effects of curcumin and hesperidin on experimental oral carcinogenesis initiated with 4-NQO, since oral neoplasms induced by 4-NQO mimic those of humans.

In the present study, possible inhibitory effects of dietary exposure of curcumin and hesperidin during the initiation and postinitiation stages on 4-NQO-induced oral carcinogenesis were investigated in male F344 rats and compared that of β-carotene, a known chemopreventive agent against oral cancer (22, 23). Also, the effects of these compounds on the proliferation biomarkers of polyamine levels, BrdUrd-labeling index, and AgNORs number were assessed to clarify the underlying mechanism(s) of modification.

MATERIALS AND METHODS

Animals, Diets, and Carcinogen. Male F344 rats, 4 weeks old, were purchased from Japan SLC, Inc. (Hamamatsu City, Japan). After a 2-week quarantine, they were transferred to the holding room under controlled conditions at 23 ± 2°C (SD) temperature, 50 ± 10% humidity, and a 12-h light/dark cycle and randomized into experimental and control groups. They were housed three or four to a wire cage. Powdered CE-2 (CLEA Japan, Inc., Tokyo, Japan) was used as basal diet during the experiment. It contained 50.4% crude carbohydrate, 24.8% crude protein, 4.6% crude fat, 7.2% ash, 4.2% crude cellulose, and 8.8% water but no compounds present in the plant foods. 4-NQO (CAS: 56–57–5, 98% pure) was obtained from Wako Pure Chemical Ind. Co., Ltd., Osaka, Japan. Curcumin (C.I. 75300, >96% pure) and hesperidin (>80% pure) were purchased respectively from Fluka Chemie AG, CH-9470 Buchs, Switzerland, and Nacalai Tesque, Inc., Kyoto, Japan. Experimental diets mixed with test compounds at a dose of 500 ppm and 4-NQO solution (20 ppm) was prepared on a weekly basis and stored in a cold room (4°C) until used. The stability test of test chemicals in the diets at room temperature was not done since these chemicals are stable.

Experimental Procedure. The experiment was designed to examine the modifying effects of β-carotene, hesperidin, and curcumin during the initiation and postinitiation phases of 4-NQO-induced oral carcinogenesis in male F344 rats (Fig. 1).

A total of 173 rats were divided into 11 groups as shown in the tables. At 7 weeks of age, rats in groups 1 through 7 were given 4-NQO (20 ppm) in the drinking water for 8 weeks. Groups 2, 3, and 4 were respectively given the diets containing 500 ppm β-carotene, hesperidin, and curcumin, starting at 6 weeks of age until 1 week after the stop of the carcinogen exposure. They were then switched to the basal diet and maintained on this diet for 22 weeks. Groups 5, 6, and 7 were fed the diets mixed with curcumin, hesperidin, and β-carotene, respectively, at a concentration of 500 ppm, starting 1 week after the cessation of 4-NQO treatment and continued on these diets for 22 weeks. Groups 8–10 were fed the diets containing test compounds alone during the experiment. Group 11 was given the basal diet and tap water throughout the experiment and served as an untreated control.

All rats were carefully observed daily and consumption of the drinking water containing 4-NQO or the diets mixed with test chemicals was recorded to estimate intake of chemicals. The experiment was terminated at 32 weeks and all animals were sacrificed by decapitation between 9 a.m. and noon to evaluate the incidences of preneoplastic and neoplastic lesions in the oral cavity. At necropsy, all organs, especially the oral cavity, were grossly examined and all organs except for the tongue were fixed in 10% buffered formalin. Tongues were cut approximately into two halves; one portion was used for polyamine assays and the other was used for histopathology and cell proliferation counts. The tongues used for these measurements basically did not contain tumorous lesions. For histopathological confirmation, tissues and gross lesions were fixed in 10% buffered formalin, embedded in paraffin blocks, and processed by the conventional histological methods using hematoxylin and eosin stain. Epithelial lesions (hyperplasia, dysplasia, and neoplasm) in the oral cavity were diagnosed according to the criteria described by Bánóczy and Csiba (41) and WHO (42).

Polyamine Levels of Tongue Tissue. The polyamines in the oral cavity tissues were measured by the method of Koide *et al.* (43). The results obtained were confirmed to be correlated well with those by high performance liquid chromatography. At sacrifice, one-half of the tongues of all rats were collected and the amounts of diamine, spermine, and spermidine were determined by an enzymatic differential assay.

Determination of Proliferative Activity in the Tongue Epithelium by AgNORs Enumeration and BrdUrd-labeling Index. To assess the proliferative activity of squamous epithelium of the tongue, the number of AgNORs per nucleus and the BrdUrd-labeling index of all animals was quantified according to the methods described previously (13). For measurement of BrdUrd-incorporated nuclei, the animals were given an i.p. injection of 50 mg/kg body weight BrdUrd (Sigma Chemical Co., St. Louis, MO) 1 h prior to killing. The tongue was removed and cut in two. One half was used for polyamine assay and the other was fixed in 10% buffered formalin for histopathology, AgNORs counting, and BrdUrd-labeling index. Three serial sections (3 μm thick) were made after embedding in paraffin. On one section, a one-step silver colloid method for AgNORs staining (13) was carried out, and computer-assisted image analysis quantification using an image analysis system SPICCA II (Japan Abionics Co., Tokyo, Japan) with a Olympus BH-2

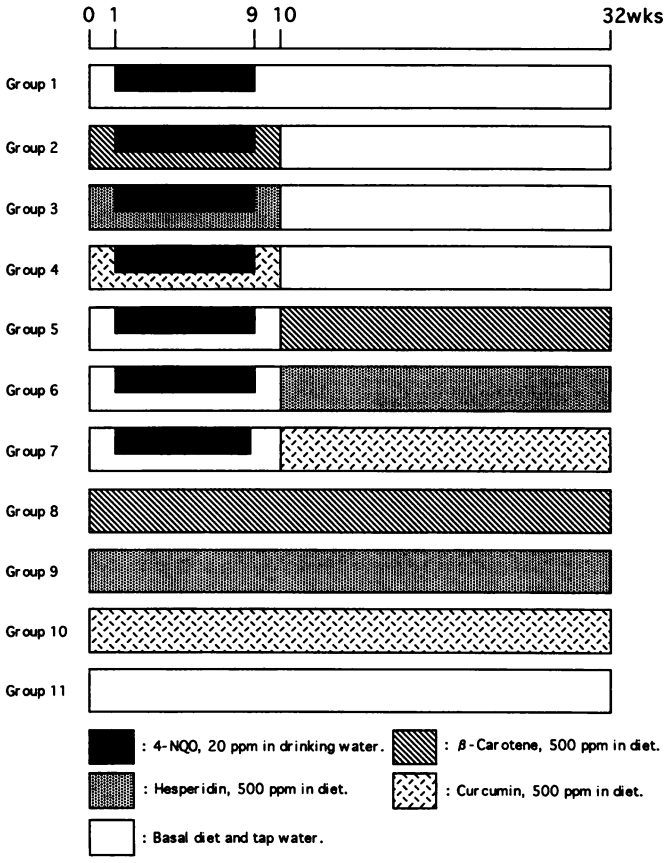

Fig. 1. Experimental protocol.

Table 1 *Body, liver, and relative liver weights in each group*

Group	Treatment	No. of rats examined	Body wt (g)	Liver wt (g)	Relative liver wt (g/100 g body weight)
1	4-NQO	24	320 ± 31[a,b]	12.3 ± 1.5	3.85 ± 0.31
2	4-NQO + β-carotene (initiation phase)	17	323 ± 25	11.4 ± 1.5	3.53 ± 0.37[c]
3	4-NQO + hesperidin (initiation phase)	18	336 ± 21[c]	12.3 ± 1.4	3.67 ± 0.26
4	4-NQO + curcumin (initiation phase)	19	333 ± 16	12.2 ± 0.9	3.66 ± 0.29[c]
5	4-NQO → β-carotene (postinitiation phase)	17	328 ± 24	11.7 ± 1.2	3.57 ± 0.36[c]
6	4-NQO → hesperidin (postinitiation phase)	19	325 ± 26	11.7 ± 1.5	3.59 ± 0.34[c]
7	4-NQO → curcumin (postinitiation phase)	20	317 ± 22	12.3 ± 1.5	3.87 ± 0.36
8	β-Carotene	11	323 ± 44	11.8 ± 1.5[b]	3.65 ± 0.16[b]
9	Hesperidin	10	335 ± 12	12.6 ± 0.8	3.76 ± 0.24
10	Curcumin	11	307 ± 27[b]	11.8 ± 1.3[b]	3.87 ± 0.41
11	No treatment	13	343 ± 18	13.2 ± 1.2	3.83 ± 0.24

[a] Mean ± SD.
[b] Significantly different from group 11 by Dunnett's *t* test ($P < 0.05$).
[c] Significantly different from group 1 by Dunnett's *t* test ($P < 0.05$).

microscope (Olympus Optical Ind. Co., Ltd., Tokyo, Japan) and a color-charged coupled device camera (Hamamatsu Photonics Co., Hamamatsu City, Japan) was performed on 100 nuclei of interphase cells from nonlesional areas (44). The other section was used for immunohistochemical detection of BrdUrd incorporation using an immunohistochemical analysis kit (Amersham, United Kingdom). The labeling indices of BrdUrd (%) were calculated by counting for labeled nuclei of 100 cells from each rat at ×400. The remaining section was used for histopathological diagnosis.

Statistical Analysis. Statistical analysis on the incidence of lesions was performed using Fisher's exact probability test or χ^2 test, and the data for body weight, liver weight, polyamine assay, AgNORs enumeration, and BrdUrd-labeling index were compared by Dunnett's *t* test. The results were considered statistically significant if the *P* was 0.05 or less.

RESULTS

General Observations. Animals in groups 1–10 tolerated well the oral administration of 4-NQO and/or test compounds. There were no significant differences on total intakes of 4-NQO or test compounds/rat among ten groups (groups 1–10; data not shown). The mean body and liver weights at the end of the study are indicated in Table 1. The mean body weights of rats in group 1 given 4-NQO alone was significantly lower than that of a control group (group 11) ($P < 0.01$). The mean body weight of rats in group 10 (curcumin alone) was significantly smaller than that of group 11 ($P < 0.001$). The mean liver weights of animals in groups 8 (β-carotene alone) and 10 were significantly smaller than that of group 11($P < 0.02$). The average relative liver weights (g/100 g body weight) of groups 2, 4, 5, and 6 were significantly smaller than that of group 1 ($P < 0.005$, $P < 0.05$, and $P < 0.02$) and the value of group 8 was significantly lower than that of group 11 ($P < 0.05$).

Incidences of Tumors and Preneoplastic Lesions. In this study, endophytic and exophytic tumors occurred only in the oral cavity, especially the dorsal site of the tongue of rats in groups 1–7. The former were microscopically well-differentiated squamous cell carcinoma and latter were squamous cell papilloma. The incidences of tongue tumors (squamous cell papilloma and carcinoma) in all groups are shown in Table 2. In group 1 (4-NQO alone), the incidences of tongue squamous cell carcinoma and squamous cell papilloma were 54 and 17%, respectively. The combined incidence of tumors in this group was 58%. On the other hand, only a few rats given test compounds during 4-NQO administration (groups 2–4) or those fed diets mixed with test chemicals after 4-NQO exposure (groups 5–7) possessed tongue neoplasms. No neoplasms developed in rats of groups 8–11. Statistical analysis revealed significant decrease in the incidences of tongue carcinoma in groups 2, 3, 4, 5, and 7 when compared to that of group 1 ($P < 0.05$). The incidence of tongue carcinoma in rats fed hesperidin diet after 4-NQO exposure (group

Table 2 *Incidence of tongue neoplasms in rats of each group*

Group	Treatment	No. of rats examined	No. of rats with tongue neoplasms (%)		
			Total	Papilloma	Carcinomas
1	4-NQO	24	14 (58)	4 (17)	13 (54)
2	4-NQO + β-carotene (initiation phase)	17	4 (24)[a]	2 (12)	3 (18)[a]
3	4-NQO + hesperidin (initiation phase)	18	6 (33)	2 (11)	4 (22)[a]
4	4-NQO + curcumin (initiation phase)	19	1 (5)[a]	0 (0)	1 (5)[a]
5	4-NQO → β-carotene (postinitiation phase)	17	5 (29)[a]	2 (12)	4 (24)[a]
6	4-NQO → hesperidin (postinitiation phase)	19	9 (47)	1 (5)	8 (42)
7	4-NQO → curcumin (postinitiation phase)	20	4 (20)[a]	2 (10)	3 (15)[a]
8	β-Carotene	11	0 (0)	0 (0)	0 (0)
9	Hesperidin	10	0 (0)	0 (0)	0 (0)
10	Curcumin	11	0 (0)	0 (0)	0 (0)
11	No treatment	13	0 (0)	0 (0)	0 (0)

[a] Significantly different from group 1 by Fisher's exact probability test ($P > 0.05$).

Table 3 *Incidence of preneoplastic lesions of tongue in rats of each group*

Group	Treatment	No. of rats examined	No. of rats with hyperplastic lesions (%)		
			Simple hyperplasia	Papillary hyperplasia	Dysplasia
1	4-NQO	24	23 (96)	9 (38)	23 (96)
2	4-NQO + β-carotene (initiation phase)	17	15 (88)	1 (6)[a]	12 (71)[a]
3	4-NQO + hesperidin (initiation phase)	18	10 (56)[a]	1 (6)[a]	11 (61)[a]
4	4-NQO + curcumin (initiation phase)	19	15 (79)	7 (37)	11 (58)[a]
5	4-NQO → β-carotene (postinitiation phase)	17	16 (94)	2 (12)	13 (76)
6	4-NQO → hesperidin (postinitiation phase)	19	16 (84)	3 (16)	13 (69)[a]
7	4-NQO → curcumin (postinitiation phase)	20	18 (90)	4 (20)	13 (65)[a]
8	β-Carotene	11	0 (0)	0 (0)	0 (0)
9	Hesperidin	10	0 (0)	0 (0)	0 (0)
10	Curcumin	11	0 (0)	0 (0)	0 (0)
11	No treatment	13	0 (0)	0 (0)	0 (0)

[a] Significantly different from group 1 by Fisher's exact probability test ($P < 0.05$).

6) was smaller than that of group 1, but the difference was not significant.

Besides these neoplasms, a number of hyperplasia and dysplasia that are considered to be preneoplastic lesions for oral cancer were present in the tongue of rats in groups 1–7, but not in rats of groups 8–11. The incidences of such lesions are listed in Table 3. As for squamous cell hyperplasia, the incidence in group 1 was 96% (96% with simple hyperplasia and 38% with papillary hyperplasia). In rats in groups 2–7, such incidences were smaller than those in group 1, especially in the rats fed hesperidin together with 4-NQO exposure (group 3). The frequencies of dysplasia in groups 2, 3, 4, 6, and 7 (58–71%) were significantly lower than that of group 1 (96%) ($P < 0.05$).

Polyamine Levels. The results of polyamine assay of tongue epithelium are shown in Table 4. Total polyamine (diamine plus spermidine plus spermine), spermidine, and spermine levels in rats of group 1 were significantly greater than those of group 11 ($P < 0.01$ and $P < 0.05$). Those values in groups 3–7 were significantly smaller than those of group 1. Values in group 2 were almost comparable to those in group 1.

Enumeration of AgNORs Number and BrdUrd-labeled Cells. The results of morphometric analysis of AgNORs and BrdUrd-labeling indices in the nonlesional squamous epithelium are summarized in Table 5. The mean number of AgNORs and BrdUrd-labeling index in the tongue epithelium exposed to 4-NQO alone (group 1) were significantly higher than those of untreated control (group 11) ($P < 0.05$ and $P < 0.001$). Dietary administration of three test chemicals (groups 2–7) significantly decreased those values ($P < 0.05$-$P < 0.001$). The mean total areas of AgNORs/nucleus in groups 2–7 were smaller than that in group 1, but the differences were not significant. The average numbers, total areas of AgNORs, and BrdUrd-labeling indices in group 8–10 (each test compound alone) were almost similar to those of group 11 (untreated control).

DISCUSSION

The results in the present study demonstrated that dietary β-carotene and curcumin during both initiation and postinitiation phases, and hesperidin feeding during initiation phase effectively suppressed oral carcinogenesis initiated with 4-NQO as revealed by reduced incidences of neoplasms and preneoplasia in the tongue. The order of inhibitory potencies was curcumin > β-carotene > hesperidin. Feeding diets mixed with these compounds also suppressed the polyamine levels and cell proliferation biomarker expression.

There are a substantial number of natural products that could suppress the development of oral cancer induced by 7,12-dimethylbenz(*a*)anthracene or 4-NQO (45). Among them, natural or synthetic retinoids and β-carotene were well studied to inhibit chemically induced oral carcinogenesis (22, 23). The reduction of oral tumors by feeding of β-carotene diet in the present study confirmed previous reports using hamster buccal pouch carcinogenesis model (23). Regardless of possessing provitamin A activity, β-carotene, and other carotenoids have common biological functions such as photoprotection, antioxidants property, immunomodulation, and anticancer activ-

Table 4 *Polyamine levels of tongue of rats in each group*

Group	Treatment	No. of rats examined	Polyamine levels (nmol/mg protein)			
			Diamine	Spermidine	Spermine	Total
1	4-NQO	24	0.25 ± 0.36[a]	1.77 ± 0.40[b]	2.01 ± 0.30[b]	4.03 ± 0.72[b]
2	4-NQO + β-carotene	17	0.22 ± 0.22	1.75 ± 0.45	1.89 ± 0.51	3.86 ± 0.86
3	4-NQO + hesperidin	18	0.23 ± 0.19	1.17 ± 0.30[c]	1.61 ± 0.32[c]	3.01 ± 0.47[c]
4	4-NQO + curcumin	19	0.17 ± 0.14	1.18 ± 0.38[c]	1.65 ± 0.30[c]	3.00 ± 0.59[c]
5	4-NQO → β-carotene	17	0.16 ± 0.14	1.46 ± 0.41[c]	1.84 ± 0.35[c]	3.46 ± 0.64[c]
6	4-NQO → hesperidin	19	0.23 ± 0.14	1.52 ± 0.36[c]	1.72 ± 0.27[c]	3.46 ± 0.47[c]
7	4-NQO → curcumin	20	0.15 ± 0.15	1.37 ± 0.53[c]	1.66 ± 0.41[c]	3.18 ± 0.88[c]
8	β-carotene	11	0.15 ± 0.12	1.20 ± 0.27	1.63 ± 0.34	2.98 ± 0.57
9	Hesperidin	10	0.16 ± 0.13	1.28 ± 0.26	1.82 ± 0.34[b]	3.26 ± 0.45[b]
10	Curcumin	11	0.21 ± 0.17[b]	1.20 ± 0.32	1.75 ± 0.33	3.16 ± 0.37[b]
11	No treatment	13	0.09 ± 0.13	1.21 ± 0.26	1.59 ± 0.23	2.88 ± 0.36

[a] Mean ± SD.
[b] Significantly different from group 11 by Dunnett's *t* test ($P < 0.05$).
[c] Significantly different from group 1 by Dunnett's *t* test ($P < 0.05$).

Table 5 *AgNORs counting and BrdUrd-labeling index of non-lesional areas of tongue squamous epithelium*

Group	Treatment	No. of rats examined	No. of AgNORS/ nucleus	Total area of AgNORs/nucleus (μm^2)	BrdUrd-labeling index (%)
1	4-NQO	24	3.00 ± 0.99[a]	3.16 ± 1.29	13.3 ± 2.1
2	4-NQO + β-carotene	17	2.21 ± 0.96[b]	2.90 ± 0.73[b]	9.4 ± 2.9[b]
3	4-NQO + hesperidin	18	2.30 ± 0.77[b]	2.95 ± 1.03[b]	9.6 ± 2.5[b]
4	4-NQO + curcumin	19	2.02 ± 0.89[b]	2.68 ± 1.12[b]	8.9 ± 1.5[b]
5	4-NQO → β-carotene	17	2.34 ± 1.21[b]	2.91 ± 0.52[b]	9.2 ± 2.1[b]
6	4-NQO → hesperidin	19	2.45 ± 1.02[b]	2.93 ± 0.78[b]	9.9 ± 3.2[b]
7	4-NQO → curcumin	20	2.13 ± 0.96[b]	2.71 ± 0.95[b]	8.8 ± 2.2[b]
8	β-Carotene	11	2.34 ± 0.34	2.63 ± 0.44	6.3 ± 0.7
9	Hesperidin	10	2.33 ± 0.45	2.64 ± 0.51	6.1 ± 0.9
10	Curcumin	11	2.35 ± 0.21	2.64 ± 0.24	6.2 ± 0.9
11	No treatment	13	2.34 ± 0.11	2.65 ± 0.32	6.1 ± 1.3

[a] Mean ± SD.
[b] Significantly different from group 1 by Dunnett's *t* test ($P < 0.05$).

ity (46). The anticancer property of carotenoids is currently considered to be independent of their provitamin A activity (47) and appears to be related to their effectiveness as an antioxidant and free radical scavenger (48). In fact, carotenoids without provitamin A activity canthaxanthin and astaxanthin could inhibit oral or bladder carcinogenesis in rodents (49, 50). Recently, new biological functions of certain carotenoids (β-carotene and canthaxanthin) have been found. These include the activation of the expression of genes which encode the message for production of a protein, connexin 43, which is an integral component of the gap junctions required for cell-cell communications (51), and the capacity to modulate the enzymatic activities of lipoxygenase (52). These properties may also contribute to the protective effect of β-carotene in 4-NQO-induced oral carcinogenesis.

A natural dye curcumin, composed of two ferulic acid moieties joined by a methylene bridge, is usually the main constituent *Curcuma* species and has various pharmacological effects including inhibition of arachidonic acid pathway (lipoxygenase and cyclooxygenase) and ODC activity (37, 53, 54). Our previous work revealed a protective effect of ferulic acid on 4-NQO-induced tongue carcinogenesis in rats (29). The products and intermediates (hydroxyeicosatetraenoic acids and prostaglandin E_2) of the lipoxygenase and cyclooxygenase pathways have been implicated in tumor promotion (55, 56). Moreover, a positive correlation between the levels of 8(*S*)-hydroxyeicosatetraenoic acid and degree of inflammation, hyperproliferation, clastogenicity, and tumor development in two stage mouse skin carcinogenesis (57). Previously, we reported that nonsteroidal antiinflammatory agents, indomethacin and piroxicam, that are potent inhibitors of arachidonic acid cascade and an irreversible ODC inhibitor, DL-α-difluoromethylornithine, inhibited 4-NQO-induced oral tumorigenesis in rats (27, 30). Thus, the results in the current study give additional evidence that some arachidonic cascade and/or ODC inhibitors cause the inhibition of 4-NQO-induced neoplasms. Reactive oxygen species produced also play a role in several stage of carcinogenesis (58). Recent work by Shin and Lin (59) demonstrated that curcumin inhibits 12-*O*-tetradecanoylphorbol-13-acetate-induced lipid peroxidation and 8-hydroxyguanosine formation in mouse fibroblasts. Curcumin could inhibit benzo(*a*)pyrene-mediated DNA adduct formation in mouse epidermis, the initiation and promotion in two stage skin carcinogenesis induced by benzo(*a*)pyrene and 12-*O*-tetradecanoylphorbol-13-acetate (60). Curcumin also inhibits preneoplastic lesions of colon cancer (61, 62).

Although it is not known whether hesperidin possess the modulating ability of arachidonic acid pathway, hesperidin could inhibit permeability of small vessels, suggesting its capability of inhibition of the certain step of arachidonic acid pathway. Hesperidin is a predominant flavonoid in lemons and sweet oranges (*Citrus sinensis*). In 1967, Van Duuren (63) reported that hesperidin did not act as tumor promoter in skin carcinogenesis initiated with 7,12-dimethylbenz(*a*)anthracene in ICR/Ha Swiss mice. Fujiki *et al.* (39) indicated antitumor promoter activity of hesperitin. Both hesperitin and hesperidin have been reported to have antioxidative activity (40). Previously we have reported that some natural antioxidants from plants exert protective effects against chemical carcinogenesis in several organs including oral cavity (26, 29, 31). Hesperidin might join with such types of chemopreventives.

In this study, all tested compounds inhibited polyamine levels and cell proliferation induced by 4-NQO. These results were comparable to our earlier experiments testing the chemopreventive efficacy of several natural phenolic antioxidants (28, 29, 31, 44) and a synthetic compound DL-α-difluoromethylornithine (30). In rodents and human oral carcinogenesis, increased polyamine levels and/or ODC activity that are essential for cellular proliferation were reported (64). ODC, a rate-limiting enzyme in polyamine biosynthesis, has been correlated with the rate of DNA synthesis and cell proliferation in several tissues (64) and an important role for ODC in tumor promotion has been indicated in various carcinogenesis models (64). It is also known that agents which inhibit ODC activity or polyamine levels are effective tumor inhibitors (30, 64–66). Cell proliferation is suggested to play an important role in multistage carcinogenesis including oral tumorigenesis (65–68). Galligan *et al.* (69) reported that the administration of α- or β-carotene to the NCI-H69 lung cancer cell line resulted in a decrease in growth in a dose-dependent manner and the decrease in growth was preceded by a decrease in N-*myc* and c-*jun* in RNA. Presently, dietary curcumin, hesperidin, and β-carotene administration in either the initiation or the postinitiation stage reduced cell proliferation in the tongue epithelium and polyamine levels in the tongue tissue with or without preneoplastic and neoplastic lesions. Indole-3-carbinol, sinigrin, and protocatechuic acid possessing antioxidative properties and antitumor activity in oral carcinogenesis also exert their antitumor effects through suppression of cell proliferation in the oral squamous epithelium (28, 31). Thus, the suppressive effects of curcumin, hesperidin, and β-carotene in the present study might be due to lowered cell proliferation caused by the feeding of these chemicals through the above described mechanism(s). As for 4-NQO-induced carcinogenesis, metabolic activation by DT-diaphorase [NAD(P)H:dehydrogenase] in several organs including liver, lung, and stomach to 4-HAQO, which is considered to be a proximate carcinogen (70); adduct formation of 4-HAQO with DNA (N^2-guanine, C^8-guanine, and N^6-adenine adducts) (71); and access of 4-NQO and/or 4-HAQO to the target tissues are necessary. The water-soluble carcinogen 4-NQO also produces intracellular oxidative stress (72). Therefore, the mechanism(s) by which curcumin, hesperidin, or β-carotene feeding during initiation stage suppressed tongue neoplasms might be its blocking capability of one or more of such processes.

Curcumin is suspected to modulate the enzyme activities in the target organ (61). Although the effect of curcumin on DT-diaphorase activity has not been reported, Azuine and Bhide (73) described recently that dietary exposure of turmeric whcih contains 1–5% curcumin decrease cytochrome b_5 and cytochrome P-450 levels and increased glutathione content and glutathione *S*-transferase activity in the liver of female Swiss mice. It may be likely that the drug-metabolizing system in the tongue will behave in a manner similar to that in the liver, which in turn may influence the tumorigenicity of 4-NQO. The results showing that administration of test chemicals with antioxidative effects in the diet during the initiation phase inhibited oral carcinogenesis may support the hypothesis that some plant antioxidative phenolics act as blocking agents (65, 66, 74).

The findings that curcumin and β-carotene exerted their chemopreventive effects in the initiation and postinitiation phase of 4-NQO-induced oral carcinogenesis and the effect of curcumin was greater than that of β-carotene were of interest, since curcumin is widely used as a spice and a coloring agent and is regularly consumed in relatively high concentrations by the general population. Since an important element in the evaluation of the possible role of chemopreventive compounds is the assessment of preclinical toxicity (65, 66), the safety and toxicity of the possible chemopreventives deserve more attention. In the current study, no histological findings indicating toxicity in the liver, kidney, and lung of rats fed curcumin, hesperidin, or β-carotene alone were found. Although additional studies on dose-dependent efficacy should be done, the results described here indicate that these compounds might be candidates for a chemopreventive agent against oral carcinogenesis.

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