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Chemopreventive Effect of a Xanthine Oxidase Inhibitor, 1′-Acetoxychavicol Acetate, on Rat Oral Carcinogenesis

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The effect of a xanthine oxidase inhibitor, 1′-acetoxychavicol acetate (ACA), on 4-nitroquinoline 1-oxide (4-NQO)-induced oral carcinogenesis was investigated in male F344 rats. All rats except those in the ACA-alone and untreated groups were given 4-NQO (20 ppm) in the drinking water for 8 weeks to induce oral cancer. Starting 1 week before the 4-NQO exposure, animals were fed diet containing 100 ppm or 500 ppm ACA for 10 weeks, followed by the basal diet without ACA for 22 weeks. Other groups were fed the diet containing ACA at 100 ppm or 500 ppm for 22 weeks, starting 1 week after the cessation of 4-NQO exposure. The remaining groups consisted of rats given 500 ppm ACA alone or untreated rats. At the termination of the experiment (32 weeks), the incidences of tongue neoplasms and preneoplastic lesions, polyamine levels in the tongue tissue, and cell proliferation activity estimated in terms of 5-bromodeoxyuridine (BrdU)-labeling index and by morphometric analysis of silver-stained nucleolar organizer regions' protein (AgNORs) were compared among the groups. Feeding of ACA at the two doses during initiation or postinitiation significantly decreased the development of tongue carcinoma (93–100% reduction, $P<0.001$) and preneoplasia (43–50% reduction for hyperplasia and 34–48% reduction for dysplasia, $P<0.05$). There were no such lesions in rats fed ACA alone or those in the untreated control group. The number of AgNORs per cell nucleus was significantly decreased by feeding of ACA at a high dose (500 ppm) (29% inhibition, $P<0.05$). The BrdU-labeling index was also reduced by dietary administration of ACA (23–32% inhibition, $P<0.01$). In addition, ACA feeding reduced tongue polyamine levels (35–40% inhibition, $P<0.05$). These results indicate that ACA inhibited rat oral carcinogenesis, and such inhibition might be related to suppression of cell proliferation in the oral mucosa by the xanthine oxidase inhibitor.

Key words: 1′-Acetoxychavicol acetate — Xanthine oxidase inhibitor — 4-NQO — Oral carcinogenesis — Rat

Oral cancer is a common neoplasm in Asia and the Pacific Islands, particularly in Southern Asia, China, parts of Brazil, and parts of Europe.[1,2] Although the incidence of oral and pharyngeal cancer is low in the world, the numbers of patients with these malignancies have been increasing.[2] In the United States, oral cancer is responsible for more than 8,000 deaths each year, more than cervical cancer or malignant melanoma, and the incidence of this malignancy appears to be increasing in specific population groups such as older females and black males.[3] In Europe, oral cancer is the first-ranked cancer of males in Calvados, France, even above lung cancer, and substantial increases are occurring in most European countries.[4] The high-risk behaviors associated with oral cancer are considered to relate to the use of alcohol and tobacco,[5] including smokeless tobacco.[6] Several studies have revealed that head and neck cancer, including oral cancer, reflect multistage and multifocal carcinogenesis.[7,8] Field cancerization is defined as the increased risk of cancer development in the entire upper aerodigestive tract after prolonged exposure to carcinogens.[7] The use of the co-carcinogens tobacco and alcohol increases the risk of developing simultaneous or subsequent second primary epithelial cancers of the head and neck, including oral cavity.[9]

The term "cancer chemoprevention" refers to the prevention of cancer by intervention using nontoxic synthetic chemicals or natural substances before malignancy.[10] A number of micronutrients,[11] macronutrients,[12] and nonnutrients[13] have been reported as inhibitors or chemopreventors of chemical carcinogenesis in rodents. In our search for cancer chemopreventive agents using experi-

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mental animal models, some natural products from edible plants, herbs, or fruits have appeared to exert tumor-inhibiting effects in the digestive organs.[14] Using a 4-nitroquinoline 1-oxide (4-NQO)-induced rat oral carcinogenesis model, we have found several natural and synthetic compounds possessing chemopreventive action against oral cancer.[15] Among them, some plant phenolics (caffeic, ellagic, chlorogenic, ferulic, and protocatechuic acids) and synthetic compounds (butylated hydroxyanisole and butylated hydroxytoluene) are potent antioxidants.[15] Thus, various natural and synthetic antioxidants could inhibit chemically induced carcinogenesis of the oral cavity[15] and other organs,[16] although the mechanism by which these agents prevent cancer is unknown.

Free radicals, including oxy radicals, may contribute to cancer development.[17, 18] Xanthine oxidase is a highly versatile enzyme that is widely distributed among species (from bacteria to human) and within various tissues of mammals.[19] The enzyme catalyzes the hydroxylation of many purine substrates and converts hypoxanthine to xanthine and then uric acid in the presence of molecular oxygen to yield superoxide anion (O_2^-).[20] Several natural products present in plants act as xanthine oxidase inhibitors.[21] Among them, quercetin, hesperidin, genistein, and a catechin-containing green tea extract are reported to have cancer chemopreventive activity.[14, 22, 23] 1′-Acetoxychavicol acetate (ACA) is present in seeds or a rhizome of *Languas galanga* (Zingiberaceae), which is a plant used as a ginger substitute and a stomachic medicine in Thailand.[24] ACA is reported to be an inhibitor of xanthine oxidase,[25] which generates superoxide anion, known to be associated with tumor development.[17, 18] Elevation of xanthine oxidase activity during carcinogenesis including promotion has been reported.[26] ACA could inhibit Epstein-Barr virus activation induced by a tumor promoter *in vitro*.[27] Although little is known about xanthine oxidase activities in tumors, an increase of this enzyme has been found in human brain tumors[28] and mouse skin tumors.[29] Certain antioxidative flavonoids with xanthine oxidase inhibitory effects have been reported to inhibit chemical carcinogenesis.[14, 21–23, 30, 31] The water-soluble carcinogen 4-NQO could produce intracellular oxidative stress which may be involved in 4-NQO-induced carcinogenesis.[32] ACA has antioxidative activity similar in degree to that of α-tocopherol.[33]

In the present study, a modifying effect of ACA on 4-NQO-induced oral carcinogenesis was found in male F344 rats. To clarify the underlying mechanism(s), the dose-related efficacy of ACA and the effects of ACA on polyamine levels in the oral cavity were studied, and the alteration of proliferative potential of the oral mucosa was examined by measuring 5-bromodeoxyuridine (BrdU)-labeling index and silver-stained nucleolar organizer regions' proteins (AgNORs).

MATERIALS AND METHODS

Animals, diets, and chemicals Male F344 rats (4 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu). After quarantine for 14 days, 134 rats 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) was used as the basal diet during the experiment. It contained 45.5% carbohydrate, 24.8% protein, 4.6% fat, 4.2% cellulose, 11.1% minerals, 1% vitamin mixture, and 8.8% water. 4-NQO (CAS: 56-57-5, 98% pure) was obtained from Wako Pure Chemical Ind. Co., Ltd., Osaka. ACA (>95% purity, Fig. 1) was synthesized according to the method described previously.[27] Experimental diet mixed with ACA at two concentrations (100 ppm and 500 ppm) was prepared by using a V-blender on a weekly basis and stored in a cold room (4°C) until used. The solution of 4-NQO was freshly prepared every week: 4-NQO (2 g) was mixed with 50 ml of absolute ethanol and 4,950 ml of distilled water using a magnetic stirrer for 24 h, and then diluted in tap water at a concentration of 20 ppm. Drinking water bottles were shielded with aluminum foil to exclude light.

Experimental procedure A total of 134 rats were divided into 7 groups as shown in Fig. 2. At 7 weeks of age, rats in groups 1 through 5 were given 4-NQO (20 ppm) in the drinking water for 8 weeks. Groups 2 and 3 were given the diets containing 100 ppm ACA and 500 ppm ACA, respectively, starting at 6 weeks of age until 1 week after the cessation of 4-NQO exposure. They were then switched to the basal diet and maintained on this diet for 22 weeks. Groups 4 and 5 were fed the diets mixed with ACA at concentrations of 100 ppm and 500 ppm, starting 1 week after the cessation of 4-NQO treatment and continued on these diets for 22 weeks. Group 6 was fed the diet containing 500 ppm ACA during the experiment. Group 7 was given the basal diet alone throughout the study and served as an untreated control.

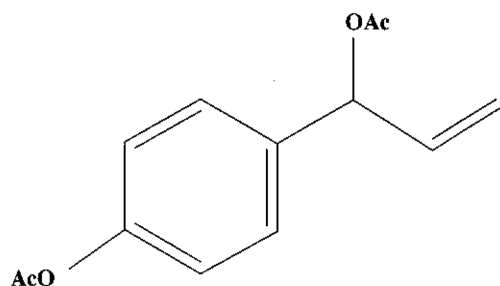

Fig. 1. Chemical structure of 1′-acetoxychavicol acetate.

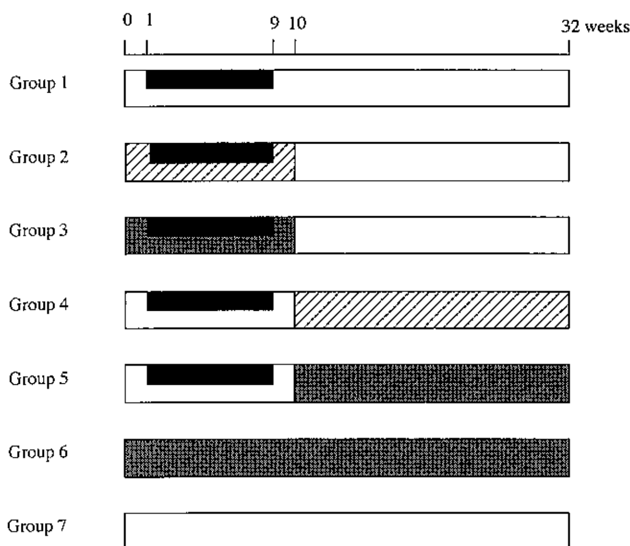

Fig. 2. Experimental protocol. ■ 4-NQO, 20 ppm in drinking water, ▨ ACA, 100 ppm in diet, □ Basal diet and tap water, ▩ ACA, 500 ppm in diet.

All rats were carefully observed daily, and consumption of the drinking water containing 4-NQO and the diets mixed with ACA was recorded to estimate intake of chemicals. The experiment was terminated at 32 weeks and all animals were killed by decapitation between 9:00 and noon. At necropsy, all organs including the oral cavity were examined grossly and all organs except for the tongue were fixed in 10% buffered formalin. Tongues were cut into two halves: one was used for polyamine assays and the other for histopathology and cell proliferation counts after fixation in 10% buffered formalin. For histopathological diagnosis, tissues and gross lesions were embedded in paraffin blocks, and processed by 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[34] and the WHO.[35]

Polyamine levels of tongue tissue Polyamines in the oral cavity tissue were measured by the method of Koide *et al.*[36] using one-half of the tongues. The amounts of diamine, spermine, spermidine, and the sum of these were determined.

Determination of proliferative activity in the tongue epithelium by AgNORs enumeration and BrdU-labeling index To assess the proliferative activity of tongue squamous epithelium, the number of AgNORs per nucleus and the BrdU-labeling index of five randomly selected animals from each experimental group were quantified according to the method described previously.[37] For measurement of BrdU-incorporated nuclei, the animals were given an i.p. injection of 50 mg/kg body weight BrdU (Sigma Chemical Co., Ltd., St. Louis, MO) 1 h prior to killing. The tongue was removed and cut into two. One half was used for polyamine assay and the other was fixed in 10% buffered formalin for histopathology, AgNORs counting, and determination of BrdU-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 was applied, and computer-assisted image analysis quantification using an image analysis system SPICCA II (Japan Avionics Co., Tokyo) with an Olympus BH-2 microscope (Olympus Optical Ind. Co., Ltd., Tokyo) and a color-charged coupled device camera (Hamamatsu Photonics Co., Hamamatsu) was performed on 100 nuclei of interphase cells from nonlesional areas. The other section was used for immunohistochemical detection of BrdU incorporation using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). The labeling indices of BrdU (percentage) were calculated by counting labeled nuclei of 100 cells at $\times$400. The remaining section was used for histological diagnosis.

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

RESULTS

General observations Animals in groups 1–6 readily tolerated the p.o. administration of 4-NQO and/or ACA. There were no significant differences in the mean intake of 4-NQO/rat/day among the five groups (groups 1–5; data not shown). The average intakes of the ACA-containing diet/rat/day in groups 2 through 5 were similar (data not shown). The mean body and liver weights at the end of the study are listed in Table I. The mean body weights of rats in all groups given 4-NQO and/or ACA, except group 6, were comparable with the control value (group 7). The mean body weight of rats in group 6 (ACA alone) was significantly smaller than that in group 7 ($P<0.01$). The mean liver weights of rats in all groups given 4-NQO and/or ACA were comparable with the control (group 7).

Incidences of tongue tumors and preneoplastic lesions In this study, exophytic tumors occurred only in the oral cavity. They were located at the dorsal site of the tongue and were histologically squamous cell papilloma or well differentiated squamous cell carcinoma. In group 1 (4-NQO alone), 14 out of 24 rats (58%) developed tongue

Table I. Body, Liver, and Relative Liver Weights in Each Group

Group no.	Treatment	No. of rats examined	Body wt. (g)	Liver wt. (g)	Relative liver wt. (g/100 g body wt.)
1	4-NQO alone	24	320 ± 31[a]	12.3 ± 1.5	3.85 ± 0.31
2	4-NQO+0.01% ACA	20	329 ± 34	11.7 ± 1.5	3.57 ± 0.39[b]
3	4-NQO+0.05% ACA	23	314 ± 15	11.3 ± 1.1[c]	3.59 ± 0.32[c]
4	4-NQO→0.01% ACA	20	317 ± 25	12.1 ± 1.7	3.81 ± 0.39
5	4-NQO→0.05% ACA	23	326 ± 22	12.4 ± 1.7	3.79 ± 0.38
6	0.05% ACA	12	310 ± 22[d]	11.2 ± 1.3	3.62 ± 0.45
7	No treatment	12	334 ± 18	11.7 ± 0.9	3.51 ± 0.36

a) Mean±SD.
b, c) Significantly different from group 1 by Student's *t* test (*b*) $P<0.05$ and *c*) $P<0.01$).
d) Significantly different from group 7 by Student's *t* test ($P<0.01$).

Table II. Incidence of Tongue Neoplasms in Rats of Each Group

Group no.	Treatment	No. of rats examined	No. of rats with tongue neoplasms (%)		
			Total	Papilloma	Carcinoma
1	4-NQO alone	24	14 (58)	4 (17)	13 (54)
2	4-NQO+0.01% ACA	20	0[a]	0	0[a]
3	4-NQO+0.05% ACA	23	2 (9)[a]	2 (9)	1 (4)[a]
4	4-NQO→0.01% ACA	20	0[a]	0	0[a]
5	4-NQO→0.05% ACA	23	0[a]	0	0[a]
6	0.05% ACA	12	0	0	0
7	No treatment	12	0	0	0

a) Significantly different from group 1 by Fisher's exact probability test ($P<0.001$).

Table III. Incidence of Preneoplastic Lesions of Tongue in Rats of Each Group

Group no.	Treatment	No. of rats examined	No. of rats with neoplastic lesions (%)						
			Hyperplasia			Dysplasia			
			Total	Simple	Papillary	Total	Mild	Moderate	Severe
1	4-NQO alone	24	24 (100)	18 (75)	10 (42)	20 (83)	6 (25)	9 (38)	14 (58)
2	4-NQO+0.01% ACA	20	18 (90)	15 (75)	7 (35)	11 (55)[a]	2 (10)	8 (40)	1 (5)[b]
3	4-NQO+0.05% ACA	23	13 (57)[b]	13 (57)	4 (17)	10 (43)[c]	4 (17)	7 (30)	2 (9)[b]
4	4-NQO→0.01% ACA	20	10 (50)[b]	10 (50)	2 (10)[a]	14 (70)	4 (20)	7 (35)	3 (15)[c]
5	4-NQO→0.05% ACA	23	12 (52)[b]	12 (52)	2 (9)[a]	10 (43)[c]	5 (22)	3 (13)	2 (9)[b]
6	0.05% ACA	12	0	0	0	0	0	0	0
7	No treatment	12	0	0	0	0	0	0	0

a–c) Significantly different from group 1 by Fisher's exact probability test (*a*) $P<0.05$, *b*) $P<0.001$, and *c*) $P<0.01$).

neoplasms: 4 rats had papilloma (17%) and 13 rats had carcinoma (54%) (Table II). On the other hand, no tongue tumors developed in rats of groups 2, 4, and 5. In group 3, only 2 rats had tongue tumors (9%): 2 rats had papilloma (9%) and 1 rat had carcinoma (4%). In rats of groups 6 and 7, no neoplasms were present in any organs, including the oral cavity. Statistical analysis revealed a significant decrease in the incidence of tumors of rats in groups 2–5 ($P<0.001$). Besides these neoplasms, a number of preneoplastic lesions (squamous cell hyperplasia and dysplasia) were present in the tongue of rats in groups 1–5 (Table III). The incidence of rats with squamous cell hyperplasia in group 1 (4-NQO alone) was 100%. In rats fed ACA together with or after 4-NQO exposure (groups 2–5), the incidences of squamous cell hyperplasia were smaller than in group 1. Significant differences in the incidence of hyperplasia were found between group 1 and group 3, 4 or 5 ($P<0.001$). The

Table IV. Polyamine Levels of Tongue of Rats in Each Group

Group no.	Treatment	No. of rats examined	Polyamine levels (nmol/mg protein)			
			Diamine	Spermidine	Spermine	Total
1	4-NQO alone	24	0.25±0.36[a]	1.77±0.40[b]	2.01±0.30[b]	4.03±0.72[c]
2	4-NQO+0.01% ACA	5	0.08±0.03[g]	1.37±0.26[d]	1.17±0.29[f]	2.62±0.18[i]
3	4-NQO+0.05% ACA	5	0.04±0.01[h]	1.10±0.20[e]	1.38±0.32[f]	2.52±0.41[f]
4	4-NQO→0.01% ACA	5	0.07±0.02[g]	1.19±0.31[e]	1.17±0.25[f]	2.43±0.21[i]
5	4-NQO→0.05% ACA	5	0.05±0.01[g]	1.14±0.34[e]	1.25±0.21[f]	2.45±0.21[i]
6	0.05% ACA	5	0.06±0.04	1.22±0.18	1.18±0.34	2.45±0.35
7	No treatment	12	0.09±0.13	1.21±0.26	1.59±0.23	2.88±0.36

a) Mean±SD.
b) Significantly different from group 7 by Student's t test ($P<0.001$).
c) Significantly different from group 7 by Welch's t test ($P<0.001$).
d–f) Significantly different from group 1 by Student's t test (*d*) $P<0.05$, *e*) $P<0.01$, and *f*) $P<0.001$).
g–i) Significantly different from group 1 by Welch's t test (*g*) $P<0.05$, *h*) $P<0.01$, and *i*) $P<0.001$).

Table V. AgNORs Counting and BrdU-labeling Index of Non-lesional Areas of Tongue Squamous Epithelium

Group no.	Treatment	No. of rats examined	No. of AgNORs/nucleus	BrdU-labeling indices (%)
1	4-NQO alone	24	2.49±1.01[a, b]	11.7±3.7[c]
2	4-NQO+0.01% ACA	20	1.99±0.86	8.4±2.6[d]
3	4-NQO+0.05% ACA	23	1.78±0.75[d]	8.0±2.8[e]
4	4-NQO→0.01% ACA	20	2.06±0.81	9.0±2.6[d]
5	4-NQO→0.05% ACA	23	1.79±0.73[d]	8.0±3.3[e]
6	0.05% ACA	12	1.56±0.62	6.0±1.1
7	No treatment	12	1.54±0.78	6.5±1.2

a) Mean±SD.
b) Significantly different from group 7 by Student's t test ($P<0.01$).
c) Significantly different from group 7 by Welch's t test ($P<0.001$).
d, e) Significantly different from group 1 by Student's t test (*d*) $P<0.01$ and *e*) $P<0.001$).

incidence of dysplasia in group 1 was 83% (25% with mild, 38% with moderate, and 58% with severe dysplasia). In rats of groups 2–5, such incidences were lower than those in group 1. Significant differences in the incidence of severe dysplasia were present between group 1 and group 2, 3 or 5 ($P<0.05$–$P<0.001$).

Polyamine levels The results of polyamine assay of tongue epithelium are shown in Table IV. Total polyamine (diamine plus spermidine plus spermine), spermidine, and spermine levels in rats of group 1 were significantly greater than those of group 7 ($P<0.001$). Total polyamine, diamine, spermidine, and spermine levels in groups 2–5 were significantly smaller than those of group 1 ($P<0.001$, $P<0.01$ or $P<0.05$). These values, except spermidine content in group 6, were slightly lower than those of group 7.

Enumeration of AgNORs and BrdU-labeled cells The results of morphometric analysis of AgNORs and BrdU-labeling indices in the nonlesional squamous epithelium are shown in Table V. The mean number of AgNORs and the BrdU-labeling index in the tongue epithelium exposed to 4-NQO alone (group 1) were significantly higher than those of the untreated control (group 7) ($P<0.01$ and $P<0.001$). Dietary administration of ACA at a high dose (500 ppm) in groups 3 and 5 significantly decreased the mean number of AgNORs/nucleus ($P<0.01$). As for BrdU, ACA feeding significantly suppressed the labeling index in rats of groups 2–5 ($P<0.01$–$P<0.001$). The average number of AgNORs and the BrdU-labeling index in group 6 (500 ppm ACA alone) were similar to those of group 7 (untreated control).

DISCUSSION

The results in the present study indicated that dietary ACA administration during the initiation or postinitiation phase effectively suppressed oral carcinogenesis initiated with 4-NQO. In particular, ACA feeding at 100

ppm during the initiation phase and feeding of ACA at 100 ppm or 500 ppm during the postinitiation phase caused complete inhibition of tongue tumorigenesis.

Our results suggest that ACA may, in part, exert its inhibitory effect through inhibition of oxidative stress. In the present study, dietary ACA administration in either the initiation or the postinitiation stage significantly reduced cell proliferation activity 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, which have antioxidative properties and antitumor activity in oral carcinogenesis, also exert their antitumor effects conceivably through suppression of cell proliferation in the oral squamous epithelium.[38, 39] Similar findings were reported by Klaunig.[23] In his study, a catechin-containing green tea extract prevented the induction of hepatocyte cytolethality caused by oxygen radical-generating compounds, including xanthine oxidase, *in vivo* and significantly decreased hepatic cell proliferation. Recently, Cohen and Ellwein have highlighted the importance of increased cell proliferation in carcinogenesis.[40] Therefore, the powerful protective effect of ACA against 4-NQO-induced oral carcinogenesis suggests that this compound acts to reduce cell proliferation, although it could not be concluded whether the reduction in proliferation by ACA in the current study was a cause or a result of carcinogenesis inhibition.

As for the inhibitory effect of ACA when fed during the initiation phase, several other mechanisms may also operate. Like many other chemical carcinogens, 4-NQO requires metabolic activation to react with DNA. The first step of the metabolic activation is the reduction of 4-NQO to 4-hydroxyaminoquinoline 1-oxide (4-HAQO) by DT-diaphorase [NAD(P)H:dehydrogenase][41] and then adduct formation of 4-HAQO with DNA (N^2-guanine, C^8-guanine, and N^6-adenine adducts).[42] Possible mechanisms by which ACA feeding during the initiation stage might suppress tongue neoplasms include (a) inhibition of the conversion step of 4-NQO to 4-HAQO and (b) interference with adduct formation. Induction of detoxifying enzymes such as glutathione *S*-transferase and/or inhibition of ornithine decarboxylase resulting in a decreased tissue polyamines content might also be considered as possible mechanisms for the protective effect of ACA.

Since an important element in the evaluation of the possible role of chemopreventive compounds is the assessment of preclinical toxicity, the safety and toxicity of the possible chemopreventives deserve attention. In the current investigation, slight retardation of body weight gain was seen in rats fed ACA at a level of 500 ppm alone (group 6). However, no clinical or morphological signs of toxicity were recognized in these rats. Accordingly, the significant reduction in the incidence of tongue preneoplastic lesions induced by ACA feeding indicates that ACA could be applied for the prevention of second primary neoplasms in the oral cavity, and the development of local recurrence and second primary malignancies.[9]

In summary, dietary administration of ACA during the initiation and postinitiation phases effectively inhibited 4-NQO-induced oral carcinogenesis in rats, although the exact mechanisms by which ACA exerts its chemopreventive action are not clear. Additional studies on the mechanistic basis of ACA action in the inhibition of cancer development and on the inhibitory effects of this compound on carcinogenesis in other organs are under way.

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