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Co-carcinogenic Effect of Retinyl Acetate on Forestomach Carcinogenesis of Male F344 Rats Induced with Butylated Hydroxyanisole

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The potential modifying effect of retinyl acetate (RA) on butylated hydroxyanisole (BHA)-induced rat forestomach tumorigenesis was examined. Male F344 rats, 5 weeks of age, were maintained on diet containing 1% or 2% BHA by weight and simultaneously on drinking water supplemented with RA at various concentrations (w/v) for 52 weeks. In groups given 2% BHA, although marked hyperplastic changes of the forestomach epithelium were observed in all animals, co-administration of 0.25% RA significantly ($P<0.05$) increased the incidence of forestomach tumors (squamous cell papilloma and carcinoma) to 60% (9/15, 2 rats with carcinoma) from 15% (3/20, one rat with carcinoma) in the group given RA-free water. In rats given 1% BHA, RA co-administered at a dose of 0.05, 0.1, 0.2 or 0.25% showed a dose-dependent enhancing effect on the development of the BHA-induced epithelial hyperplasia. Tumors, all papillomas, were induced in 3 rats (17%) with 0.25% RA and in one rat (10%) with 0.05% RA co-administration. RA alone did not induce hyperplastic changes in the forestomach. These findings indicate that RA acted as a co-carcinogen in the BHA forestomach carcinogenesis of the rat.

Key words: Butylated hydroxyanisole — Retinyl acetate — Co-carcinogenicity — Forestomach — Rat

Butylated hydroxyanisole (BHA) is an antioxidant that has been widely used as a food additive to prevent oxygen-induced lipid peroxidation,[1] and has been found to reduce the carcinogenicity in rats and mice of various chemicals when administered before or together with them.[1,2] However, Ito *et al.* have reported carcinogenic activity of BHA for the forestomach of rats[3] and hamsters.[4] Moreover, enhancing or promoting effects of BHA and other phenolic compounds have been demonstrated in several experimental carcinogenesis models.[5–7]

Retinoids, the family of molecules comprising both natural and synthetic analogues of vitamin A, have been demonstrated to be potent agents for control of cellular differentiation and proliferation, especially in epithelial cells,[8,9] and they have been extensively examined for chemopreventive and anti-neoplastic activities *in vivo*[10,11] and *in vitro*.[12,13] Inhibitory or preventive effects of retinoids in experimental carcinogenesis have been reported in skin, lung, mammary gland, urinary bladder and digestive tract and associated organs.[10,11,14–16] Currently retinoids are being evaluated in clinical chemoprevention trials.[11,17] However, recent reports indicate that retinoids can also enhance tumorigenesis in certain animal tumor models[11,18–21] or the risk of certain cancers in humans.[22,23]

The forestomach of the rodent is lined with stratified squamous cell epithelium. As previously reported, retinoids mainly affect epithelial tissues, and deficiency results in squamous metaplasia in several organs.[9,24] In the present experiment, the modifying effect of excess retinyl acetate (RA) in the diet on forestomach carcinogenesis induced by BHA was examined in male F344 rats.

Materials and Methods

Chemicals RA (CAS: 127-47-9; dry vitamin A acetate, stabilized with tocopherol, type 325L; 325,000 IU/g) was supplied by Hoffmann-La Roche & Co. Ltd., Basel, Switzerland. RA was in the form of stable gelatinized beadlets. Since the stability of a 1% solution of RA in distilled water was 100% in 96 hr when shielded from light at room temperature,[25] RA-containing drinking water was renewed twice weekly and was administered from light-shielded bottles. The dose levels of 0.05–0.25% were chosen on the basis of a

previous subchronic experiment.[25] BHA (lot 83-18) was obtained from Nikki Universal Co., Kanagawa, and added to the powdered diet (CRF-1, Charles River Japan, Inc., Kanagawa) at levels of 1% or 2% by weight. The purity of the BHA was greater than 99.5%.

Animals and Housing Conditions Male F344/DuCrj rats (specific pathogen-free), 4 weeks old, were purchased from Charles River Japan, Inc., and housed in plastic cages, 5 rats per cage, with sterilized softwood chips as bedding in a barrier-sustained animal room at 24±1° and 55±5% humidity with a 12 hr light and 12 hr dark daily cycle.

Treatment A total of 130 rats were randomly divided into 9 groups as shown in Table II. Groups 1, 2, 3, and 7 consisted of 20 animals each, and the other groups consisted of 10 animals each. The experiment was started when the animals were 5 weeks old, and terminated 52 weeks later. Diet and water were available *ad libitum*. Body weight was measured periodically. Necropsies were performed on all rats which died or were sacrificed upon becoming moribund. At week 52 of the experiment, all surviving rats were sacrificed under light ether anesthesia for necropsy, and the stomach, esophagus, kidney, liver, adrenal and all macroscopic lesions were processed for histologic examination. All organs removed were fixed in 10% phosphate-buffered formalin solution.

Histological Examination of Stomach The stomachs were inflated *in situ* with 10% phosphate-buffered formalin and removed. About 10 min later, the stomachs were opened along the greater curvature, extended on paper, and placed in formalin solution for further fixation. Step sections at 2–3 mm intervals were made longitudinally to the curvature and processed routinely for staining with hematoxylin and eosin (H-E). Special stains, including PAS and Alcian blue, were employed when necessary.

Statistical Analysis Statistical analysis was performed using Student's *t*-test and the Cochran *t*-test in combination with the *F* test for variability for differences between means and the Fisher exact test for differences between data of proportion.

Results

Body and Organ Weights Several rats died before termination of the experiment, and most of these rats showed retarded body weight gain. Rats surviving throughout the experimental period were taken into the effective number of animals. As shown in Table I, the body weight of rats given 2% BHA and 0.25% RA remained at an extremely low level, and a dose-related retardation of body weight gain was apparent for both BHA and RA treatments.

Table I. Body and Stomach Weight of Rats after 52 Weeks of Treatment

Group	No. of rats	Body weight (g)	Stomach weight (g)
1	15	212±15[a]	2.50±0.25[b]
2	20	334±39	2.67±0.12
3	18	319±16[c]	2.31±0.17[c]
4	10	332±21[c]	2.29±0.17[c]
5	10	368±16[d]	2.13±0.12
6	10	382±23	2.31±0.12[c]
7	20	386±20	2.08±0.13
8	10	409±25	1.95±0.13
9	9	460±23	2.05±0.16

Mean±SD.
a, b) Significantly different from group 2 at $P<0.001$ or $P<0.05$, respectively.
c, d) Significantly different from group 7 at $P<0.001$ or $P<0.05$, respectively.

Food and water consumptions were not periodically recorded. Food intake determined several times showed no statistically significant difference between groups (11.8–13.3 g/rat/day). Water intake was almost the same in all groups, but exact RA intake was not determined.

Gross Appearance Grossly, focal thickening of the forestomach epithelium with or without ulcer formation was observed in rats given BHA, sometimes covered with a dense grayish-white material. In a dose-related manner, co-administration of RA extended the distribution of the lesions, and the epithelium was more thickened. Papillary protuberances were observed in the forestomach of several rats, especially those treated with the highest dose of BHA and the highest dose of RA. Stomach weight measured after fixation correlated with the dose levels of RA (Table I). The esophagus and other organs showed no gross changes except for the kidneys. The surface of the kidneys was granular and calcification in the medulla and papilla was found.

Histological Findings

Stomach Histological findings of the stomach are summarized in Table II. Hyperplasia of the forestomach was classified into two types, focal and diffuse, based on the size of the lesion, and each type was further classified into two categories, slight and severe, based

Table II. Effects of Retinyl Acetate on BHA Carcinogenesis of the Rat Forestomach

Group	Treatment		Effective no. of rats	Hyperplasia[a]				Neoplasia		
				Focal		Diffuse		Papilloma	SCC	Total
	BHA (%)	RA (%)		+	++	+	++			
1	2	0.25	15				15 (100)[b]	7[c]	2	9 (60)[c]
2	2	—	20				20 (100)	2	1	3 (15)
3	1	0.25	18		15	3 (17)		3		3 (17)
4	1	0.2	10		9	1 (10)				0
5	1	0.1	10		10 (100)					0
6	1	0.05	10		10 (100)			1		1 (10)
7	1	—	20	2	18 (90)					0
8	—	0.25	10							0
9	—	—	9							0

a) Each rat is placed in the column corresponding to the most advanced lesion present in each animal.
b) Numbers in parentheses are percentages of rats included in the effective number in the group.
c) Significantly different from group 2 at $P<0.05$.
Symbols and abbreviations: +; slight, ++; moderate, SCC; squamous cell carcinoma.

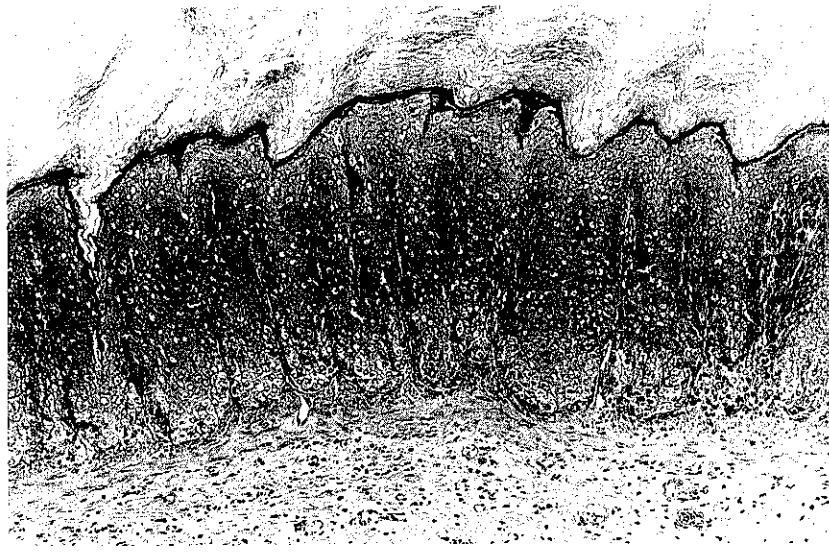

Fig. 1. Severe hyperplasia of the forestomach epithelium with hyperkeratosis and inflammatory change in the lamina propria. This rat had received 2% BHA and 0.25% RA for 52 weeks. H-E, ×300.

on the thickness of the epithelium (Fig. 1). Focal hyperplasia was always observed around the area of the transition between the esophagus and the forestomach. Papilloma was a lesion in which the epithelium showed papillary upward growth with finger-like processes of connective tissue covered with hyperplastic epithelium (Fig. 2). Carcinoma was usually observed as a downward growth of atypical squamous cells in cords or small groups of dark-staining anaplastic epithelial cells (Fig. 3).

Marked hyperplastic changes were induced in all rats treated with 2% BHA irrespective

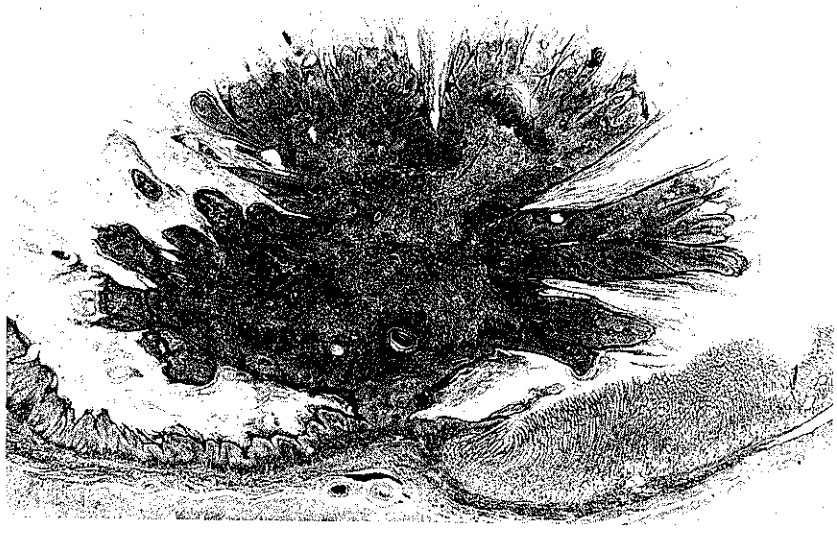

Fig. 2. Papilloma showing upward growth with finger-like processes of connective tissue covered with hyperplastic epithelium. H-E, ×40.

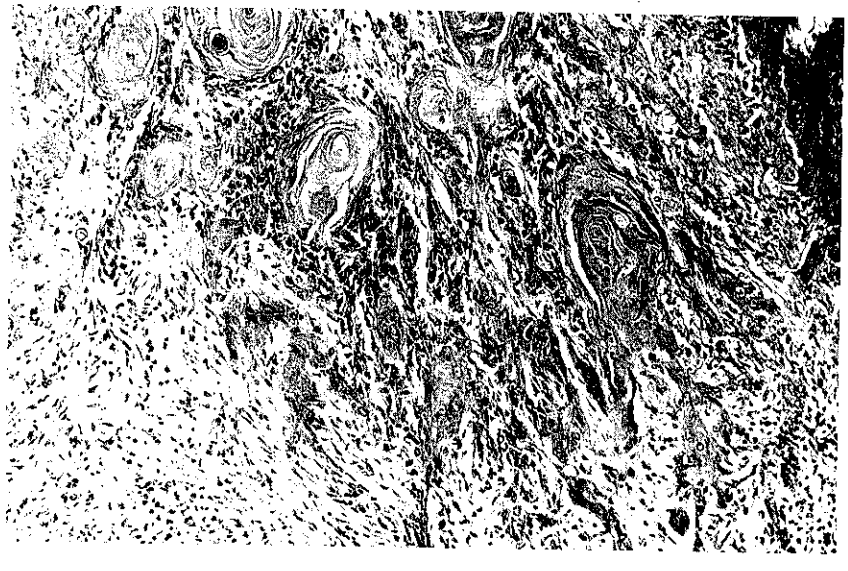

Fig. 3. Squamous cell carcinoma of the forestomach showing downward growth of atypical squamous cells. This rat had received both 2% BHA and 0.25% RA for 52 weeks. H-E, ×300.

of the RA treatment. The incidence of papilloma, however, was significantly increased by co-administration of RA, and squamous cell carcinoma was observed in 2 rats in group 1 and one rat in group 2. The total number of rats bearing papilloma or carcinoma was significantly increased by co-administration of RA. In groups given 1% BHA, 3 of 20 rats

Table III. Non-neoplastic Lesions in the Stomach of Rats Treated with BHA and/or Retinyl Acetate

Group	Effective no. of rats	Forestomach				Fundic stomach
		Ulcer formation	Keratinizing cyst			Squamous metaplasia
			Slight	Moderate	Total	
1	15	9 (60)[a]	3	11	14 (93)	13 (87)
2	20	5 (25)	13	7	20 (100)	11 (55)
3	18	1 (6)	7	7	14 (78)[b]	2 (11)
4	10	0	6	2	8 (80)[b]	3 (30)[c]
5	10	0	6	0	6 (60)[b]	1 (10)
6	10	0	0	0	0	0
7	20	1 (5)	0	0	0	0
8	10	0	0	0	0	0
9	9	0	0	0	0	0

a) Numbers in parentheses are percentages of rats included in the effective number in the group.
b, c) Significantly different from group 7 at $P<0.001$ or $P<0.05$, respectively.

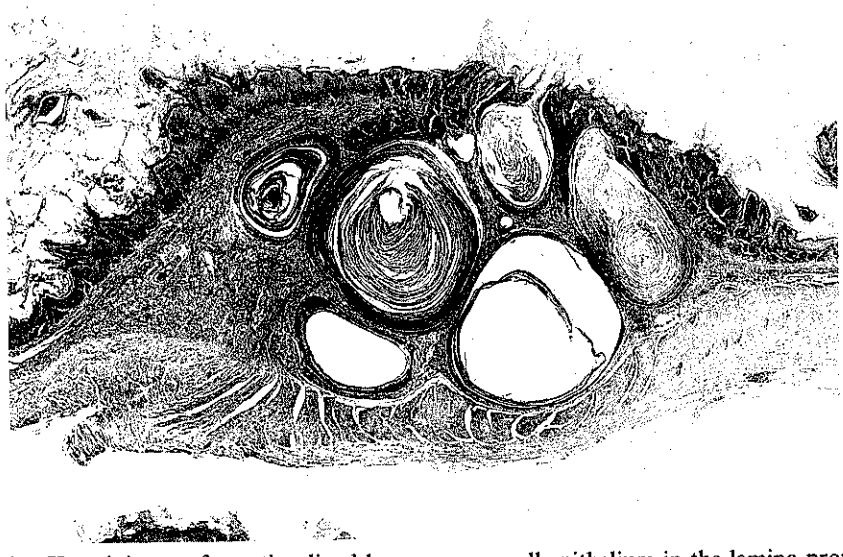

Fig. 4. Keratinic cyst formation lined by squamous cell epithelium in the lamina propria. Marked inflammatory cell infiltration is observed in the surrounding connective tissue. This rat had received 1% BHA and 0.25% RA. H-E, ×50.

co-administered 0.25% RA and one rat given 0.05% RA developed papilloma, whereas 1% BHA alone did not induce tumors in 20 rats. Hyperplastic changes of the forestomach were RA dose-related.

Other histopathological findings in the stomach are shown in Table III. Ulcer formation was observed particularly in group 1, and cysts lined with keratinized squamous cell epithelium were found in many animals treated with BHA and the highest dose of RA (Fig. 4). The lesion consisting of stratified squamous epithelium in the superficial layer of the glandular mucosa adjacent to the ridge between the forestomach and the glandular stomach, but without any findings suggesting a direct extension of the forestomach epithelium, was termed squamous metaplasia. Squamous metaplasia was frequently observed in rats in group 1.

Other organs In the kidney, papillary necrosis associated with calcinosis was frequently ob-

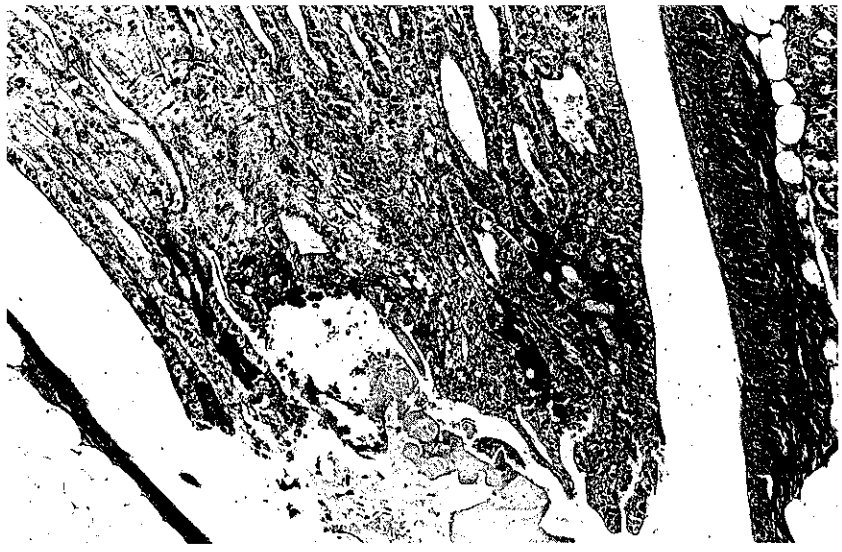

Fig. 5. Papillary necrosis associated with moderate calcinosis in a rat treated with 2% BHA and 0.25% RA for 52 weeks. H-E, ×300.

served in rats treated with the highest dose of RA and BHA. In group 1, slight or moderate papillary necrosis was observed in all rats and 11 rats (73%) showed associated papillary calcinosis (Fig. 5). Bilateral calcification was observed in 6 rats (40%). In group 2, 12 of 20 rats (60%) developed slight to moderate papillary necrosis but none showed associated calcification. In group 3, 7 rats (39%) developed slight papillary necrosis and 6 showed associated mineral deposition. Bilateral calcinosis was induced in only one rat. In the other groups, these lesions were not observed. Tubulo-interstitial nephritis characterized by tubular atrophy associated with small round cell infiltration and fibrosis in the interstitial space of the cortex was more severe in rats of group 1.

Medullary hyperplasia of the adrenal glands, and vacuolization of Kupffer cells or Ito cells of the liver were selectively observed in rats given RA irrespective of other treatment.

Discussion

BHA was recently found to be carcinogenic in the forestomach of rats and hamsters.[3,4] Although a few studies with negative results cannot completely rule out mutagenic and clastogenic potential, BHA has repeatedly been shown to be non-mutagenic in the Ames test and in eukaryote tests (see ref. 1). Furthermore, Hirose *et al.*[26] could not detect binding of orally administered ^{14}C-BHA to DNA and other macromolecules of the forestomach epithelial cells. Based on these findings, BHA is generally recognized as a non-genotoxic carcinogen. Recently, however, we demonstrated tumor-initiating activity of BHA in the two-stage mouse skin carcinogenesis model.[27]

In a recent review on forestomach carcinogenesis, Kroes and Wester[28] listed diallyl phthalate, allyl chloride, propionic acid and sodium saccharin, in addition to BHA, as non-genotoxic forestomach carcinogens and concluded that the carcinogenic activity of non-genotoxic substances may be due to their strong irritative and hyperplastogenic properties, although the exact nature of the proliferative stimuli has not been elucidated. Compounds structurally related to BHA which induce proliferative lesions in the forestomach were suggested to have possible carcinogenic potency in the forestomach in rats[29] and in hamsters.[30] Enhancement of

forestomach tumorigenesis induced by a variety of carcinogens has been reported for histamine, aspirin, sodium chloride and a zinc-deficient diet, all of which produce hyperplasia, hyperkeratosis, and/or parakeratosis in the forestomach by themselves (see ref. 28). Thus induction of proliferative events seems to be closely related to the development of forestomach tumors.

Retinoids have been extensively examined for anti-tumorigenic activity,[10-16] and currently they are being evaluated in clinical chemoprevention trials.[11, 17] Vitamin A has a role in maintaining the normal differentiation of epithelial tissues,[9, 24, 31] and this effect has been demonstrated in the gastrointestinal tract (esophagus, stomach, small bowel, pancreatic ducts) as well as in extradigestive organs (trachea, bronchi, uterus, kidney, bladder, testicle, prostate, and skin). Early investigators observed proliferative lesions in the forestomach induced by vitamin A deficiency. The first experimental observations in rats were reported by Pappenheimer and Larimore in 1924 and confirmed by Wolbach and Howe (see ref. 31). Tanaka *et al.*[32] recently reported that vitamin A deficiency enhanced betel quid-induced hyperplasia in the forestomach epithelium of rats. Prevention of the occurrence of carcinoma in hamsters receiving 7, 12 - dimethylbenz[*a*]anthracene (DMBA) or benzo[*a*]pyrene has been reported with retinyl palmitate.[33, 34]

In contrast to earlier studies showing inhibition of carcinogenesis by retinoids, several recent reports indicate that retinoids can also enhance tumorigenesis in animals[11, 18-21] or increase the risk of certain cancers in humans.[22, 23] The present experiment demonstrated co-carcinogenic activity for RA in the forestomach of rats when administered in the drinking water in combination with BHA given in the diet. However, it is uncertain whether RA exerted the co-carcinogenic activity through a hyperplastogenic activity, since RA alone did not induce any proliferative lesions in the forestomach. It apparently enhanced the inflammatory and proliferative changes in the forestomach induced by BHA. RA administered to F344 rats for 2 years did not induce any histopathological lesion in the forestomach, but it induced pheochromocytomas in the adrenal glands.[35]

Of the organs demonstrating enhancing effects by retinoids, the epidermis is histologically similar to the forestomach, being composed of a stratified squamous cell epithelium. In mouse skin models, Verma[18] showed that retinoic acid failed to inhibit ornithine decarboxylase induction and formation of skin papillomas by repeated applications of small doses of DMBA. In some experiments retinoic acid actually potentiated the number of papillomas per mouse. Retinoic acid has also been shown to enhance UV-induced skin carcinogenesis in hairless mouse.[36] Of the possible mechanisms involved in the enhancing effects of retinoids on skin carcinogenesis, hyperplastogenic activity for the epidermis[37] and enhancement of an early step in the transformation of mouse epidermal cells[19] have been proposed.

It is of interest that in the experimental carinogenesis model of the respiratory system using hamsters, lung carcinomas induced by repeated intragastric intubations of retinyl acetate after exposure to benzo[*a*]pyrene were of a squamous or mixed squamous-mucinous type.[38] It was noted in relation to that study that the toxicity of high dose of RA and an increase in respiratory infections of the animals receiving retinoids affected the induction of lung tumors. Again, these represent chronic irritating and proliferative stimuli.

The combination of butylated hydroxytoluene (BHT), another phenolic antioxidant, and RA was reported by McCormick *et al.*[39] to increase anticarcinogenic and hepatotoxic activity in liver carcinogenesis induced by DMBA compared to BHT or RA alone. Nothing was reported about the forestomach. Similarly, a synergistic enhancement of the pathological effects of BHA and RA was observed in the kidney in the present experiment. Severe papillary necrosis and calcinosis were frequently observed in rats treated with the combination of 2% BHA and 0.25% RA. It is uncertain whether the co-carcinogenic effect in the forestomach and enhancing effect in kidney calcification by BHA and RA are exerted independently by different mechanisms.

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