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Effects of Combined Treatment with Phenolic Compounds and Sodium Nitrite on Two-stage Carcinogenesis and Cell Proliferation in the Rat Stomach

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The effects of combined treatment with $NaNO_2$ and phenolic compounds on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) stomach carcinogenesis were investigated in F344 rats. In the first experiment, groups of 15–20 male rats were treated with an intragastric dose of 150 mg/kg body weight of MNNG, and starting 1 wk later, were given 2.0% butylated hydroxyanisole, 0.8% catechol, 2.0% 3-methoxycatechol or basal diet either alone or in combination with 0.2% $NaNO_2$ in the drinking water until they were killed at week 52. All three antioxidants significantly enhanced forestomach carcinogenesis without any effect of additional $NaNO_2$ treatment. However, in the absence of MNNG pretreatment, the grade of forestomach hyperplasia in the catechol and 3-methoxycatechol groups was significantly increased by the combined treatment with $NaNO_2$. In a second experiment, the combined effects of various phenolic compounds and $NaNO_2$ on cell proliferation in the upper digestive tract were examined. Groups of 5 rats were given one of 24 phenolic compounds or basal diet either alone or in combination with 0.3% $NaNO_2$ for 4 weeks and then killed. Particularly strong enhancing effects in terms of thickness of the forestomach mucosa were seen with *t*-butylhydroquinone (TBHQ), catechol, gallic acid, 1,2,4-benzenetriol, *dl*-3-(3,4-dihydroxyphenyl)-alanine and hydroquinone in combination with $NaNO_2$. In the glandular stomach, similar enhancing effects were evident in 11 cases, and in the esophagus with phenol, TBHQ and gallic acid. These results demonstrate that $NaNO_2$ can augment cell proliferation induced in the stomach epithelium by various phenolic compounds.

Key words: Phenolic compound — Sodium nitrite — Rat forestomach — Promotion effect — Cell proliferation

Nitrate and nitrite are contained in foods such as cured meat products, bread and vegetables, and nitrate is also converted to nitrite by microbial reduction in the buccal cavity.[1,2] This may be of significance, since nitrite has been shown to be mutagenic in various test systems,[3] and in rat carcinogenesis studies, it was found to increase the incidence of lymphoreticular tumors and forestomach papillomas.[4,5] However, its potential tumorigenicity in rats and mice is still a matter of discussion.[6-8]

Nitrite has been demonstrated to react with secondary amines under the acidic conditions in the stomach to produce N-nitroso compounds.[9] In addition, reaction mixtures of nitrite and phenolic antioxidants such as phenol, 3-methoxycatechol, catechol and vanillin under acidic conditions exert direct-acting genotoxicity in the SOS Chromotest and in *Salmonella typhimurium* strains TA 98 and TA 100.[10,11] Although BHA[2] itself does not show genotoxic activity under acidic conditions after nitrosation in the SOS Chromotest,[10] t-BQ and the dimer of t-BQ, which are the reaction products of BHA and nitrite, proved positive in Ames and Rec mutation assays.[12] Some synthetic and naturally occurring phenolic compounds have already been demonstrated to have carcinogenic and/or promotion activity in the forestomach, glandular stomach or other organs.[13-23] Thus, the effects of nitrite and phenolic antioxidants in combination might be of considerable importance in stomach carcinogenesis. Recently, it was reported that combined treatment with catechol or 3-methoxycatechol and $NaNO_2$ enhanced cell proliferation in the forestomach.[24] Furthermore, in the multi-organ carcinogenesis model, additional exposure to $NaNO_2$ can modify phenolic antioxidant-induced carcinogenesis, particularly in the upper digestive tracts.[25]

The present study was performed to confirm the effects of combined treatment with BHA, catechol or 3-methoxycatechol and $NaNO_2$ in the two-stage MNNG rat stomach model, which targets simply forestomach and glandular stomach, and to ascertain whether 4 weeks' exposure to a number of phenolic compounds and $NaNO_2$ can induce cell proliferation in the upper digestive tract.

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[2] The abbreviations used are: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; dl-dopa, *dl*-3-(3,4-dihydroxyphenyl)alanine; HSC, Hickory-smoke concentrate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; $NaNO_2$ sodium nitrite; PTBC, 4-*t*-butylcatechol; TBHQ, *t*-butylhydroquinone; t-BQ, 2-*t*-butyl-*p*-quinone.

MATERIALS AND METHODS

Animals A total of 530 five-week-old male F344 rats were obtained from Charles River Japan, Inc., Atsugi. They were housed 5 to a plastic cage on hardwood chip bedding in an air-conditioned room at a temperature of 22±2°C with a 12-h light, 12-h dark cycle. They were maintained on Oriental MF basal diet (Oriental Yeast Co., Tokyo) and tap water *ad libutum*.

Chemicals MNNG, catechol (purity >99.0%), 3-methoxycatechol (purity >98.0%), TBHQ (purity >98.0%), 3-methylcatechol (purity >99.0%), gallic acid (purity >98.0%), protocatechuic acid (purity >96.0%), caffeic acid (purity >98.0%), 1,2,4-benzenetriol (purity >98.0%), ferulic acid (purity >98.0%), dl-dopa (purity >98.0%), vanillic acid (purity >98.0%) and vanillin (purity >97.0%) were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, $NaNO_2$, BHA (purity >98.0%), phenol (purity >98.0%), resorcinol (purity >99.8%), BHT (purity >98.0%), 4-methoxyphenol (purity >99.0%), PTBC (purity >99.0%), hydroquinone (purity >99.0%), pyrogallol (purity >98.0%) and guaiacol (purity >98.0%) were from Wako Pure Chemical Ind., Ltd., Osaka, 4-methylphenol (purity >98.0%) and eugenol (purity >90.0%) were from Katayama Chemical Ind., Co., Ltd., Osaka, 4-methylcatechol (purity >99.0%) was from Aldrich Chemical Co., Inc., Milwaukee, WI, sesamol (purity >98.0%) was from Fluka Chemie, AG., Switzerland and HSC was from Nabisco Brands, Inc., East Hanover, NJ.

Two-stage stomach carcinogenesis study One hundred and sixty animals were given 150 mg/kg body weight of MNNG in dimethyl sulfoxide through a stomach tube. Starting 1 wk later, 8 groups of 20 rats each were treated with 2.0% BHA, 0.8% catechol, 2.0% 3-methoxycatechol or no supplement in the diet, with or without 0.2% $NaNO_2$ in the drinking water, for 51 weeks. A further 8 groups of 15 rats received the same compounds without MNNG pretreatment. Body weights were measured once every 2 weeks until 14 weeks and thereafter once every 4 weeks.

Rats that became moribund were killed for autopsy, and all surviving animals were killed under ether anesthesia at the end of 52 weeks. The stomach, esophagus, liver, kidneys and macroscopic lesions were removed and fixed in 10% buffered formalin solution, the liver and kidneys first being weighed. In the cases of the esophagus and stomach, 10% formalin solution was injected. The organs were subsequently opened via an incision along the greater curvature and about 3 sections were cut in each case from the forestomach and glandular stomach, and 1 from the esophagus. Tissues were processed routinely for histopathological examination of hematoxylin and eosin-stained sections.

Short-term cell proliferation study Groups of five animals were treated with 2.0% phenol, 2.0% resorcinol, 0.7% BHT, 2.0% TBHQ, 2.0% 4-methoxyphenol, 2.0% sesamol, 0.8% catechol, 2.0% 4-methylcatechol, 2.0% 3-methylcatechol, 2.0% gallic acid, 2.0% PTBC, 2.0% protocatechuic acid, 2.0% caffeic acid, 2.0% 1,2,4-benzenetriol, 2.0% ferulic acid, 2.0% dl-dopa, 2.0% hydroquinone, 2.0% vanillic acid, 2.0% vanillin, 2.0% pyrogallol, 2.0% guaiacol, 2.0% 4-methylphenol, 2.0% eugenol, 2.0% HSC or no supplement in the diet, with or without 0.3% $NaNO_2$ in the drinking water, for 4 weeks and then killed under ether anesthesia. They received a single ip injection of 20 mg/kg body weight bromodeoxyuridine (BrdU) one hour before killing. Stomachs and esophagi were removed and injected with 10% buffered formalin solution. After opening along the greater curvature, six strips each were cut from the anterior and posterior walls of the forestomach and 6 strips were cut from the pyloric region of the glandular stomach. Tissues were processed routinely and sections were stained with hematoxylin and eosin and used for demonstration of anti-BrdU binding.

The forestomach was divided into prefundic and mid regions, and mucosal thickness was measured and expressed as the mean±SD of data for 6 different areas within each region. Mucosal thickness of the glandular stomach was similarly expressed as the mean±SD of findings for 6 different areas of the pyloric region. In the esophagus, thickness was measured at the upper, middle and lower regions. For the analysis of BrdU labeling indices, 100 basal cells each in the upper, middle and lower esophagus, and 30 crypts in the pyloric region of glandular stomach were counted and the data were expressed as number of labeled cells/100 basal cells and number of labeled cells/crypt, respectively.

Statistical analysis The significance of differences between groups in body and organ weights, height of mucosa and BrdU labeling indices was analyzed using Student's *t* test according to Welch. The significance of differences in lesion incidences between different groups was examined using Fisher's exact probability test.

RESULTS

Two-stage stomach carcinogenesis study Table I summarizes final body and relative organ weights and average food consumption data. In the groups receiving simultaneous treatment with $NaNO_2$ and phenolic compounds, except for catechol, after MNNG initiation, final body weights were significantly decreased as compared with phenolic compound alone values. A statistically significant reduction in relative liver weights was noted in rats treated with $NaNO_2$ and 3-methoxycatechol. Relative kidney weights in all groups given combined treatment

Table I. Final Body Weight, Relative Organ Weight and Average Food Consumption Data

Treatment			No. of rats	Final body wt. (g)	Relative organ weights (%)		Food consumption (g/rat/day)
MNNG	$NaNO_2$	Chemical			Liver	Kidneys	
+	+	BHA	18	268±32[b]	3.01±0.12[a]	0.81±0.05[b]	13.0
+	−	BHA	18	365±23	3.14±0.44	0.66±0.02	14.6
+	+	Catechol	14	318±20	2.97±0.30	0.73±0.04[b]	12.0
+	−	Catechol	16	333±29	3.01±0.30	0.66±0.04	12.5
+	+	3-Methoxycatechol	18	323±44[b]	2.77±0.54	0.69±0.08[b]	13.1
+	−	3-Methoxycatechol	17	408±33	2.64±0.21	0.60±0.04	14.4
+	+	Basal diet	20	397±42[b]	2.53±0.65	0.61±0.07[b]	13.6
+	−	Basal diet	18	452±21	2.30±0.10	0.54±0.03	14.8
−	+	BHA	15	309±22[b]	2.97±0.14	0.74±0.06[b]	13.3
−	−	BHA	15	390±17	3.00±0.12	0.61±0.03	15.2
−	+	Catechol	15	350±24[b]	2.56±0.11	0.69±0.03[b]	13.1
−	−	Catechol	15	402±28	2.56±0.12	0.59±0.03	13.8
−	+	3-Methoxycatechol	15	369±20[b]	2.33±0.08[b]	0.61±0.03[b]	14.3
−	−	3-Methoxycatechol	15	435±20	2.47±0.11	0.57±0.03	15.7
−	+	Basal diet	15	407±16[b]	2.25±0.08	0.58±0.03[b]	13.8
−	−	Basal diet	15	449±18	2.31±0.16	0.54±0.03	15.1

a) Data are mean±SD values.
b) Significantly different from the values for the treated groups without $NaNO_2$ at $P<0.01$.

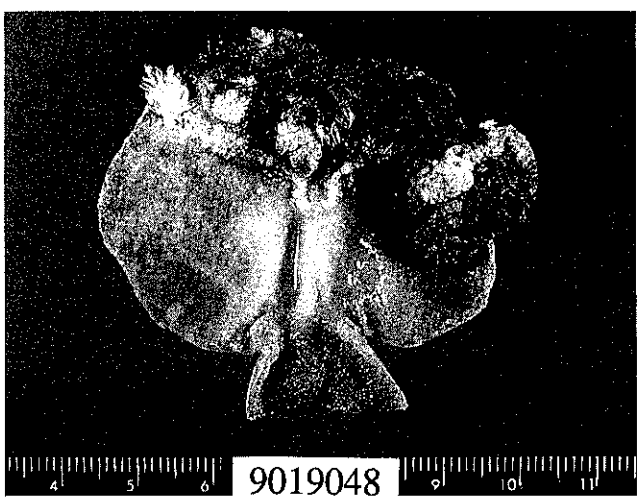

Fig. 1. Macroscopic appearance of the stomach of a rat treated with $NaNO_2$ and 3-methoxycatechol after MNNG initiation. Blackened nodular lesions are evident in the forestomach epithelium.

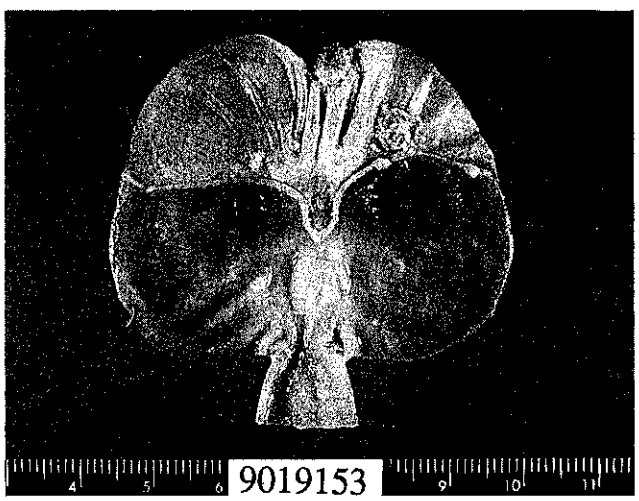

Fig. 2. Macroscopic appearance of the stomach of a rat treated with MNNG only. Small nodules are evident in the forestomach.

were higher than the individual phenolic compound alone group values. Food consumption tended to be decreased in the combination groups.

Grossly, the forestomachs of rats treated simultaneously with $NaNO_2$ and phenolic compounds after MNNG initiation were filled with nodules or masses, and the surfaces of lesions induced by $NaNO_2$ and catechol or 3-methoxycatechol were brown to black in color (Fig. 1). Treatment with MNNG alone resulted in only small nodules in the forestomach (Fig. 2). In rats treated with phenolic compounds without carcinogen, small nodules or thickening were also observed in the forestomach epithelium, and lesions were more pronounced with additional $NaNO_2$, being associated generally with blacken-

ing, except in the BHA group (Fig. 3). In the pyloric region of the glandular stomach, diffuse thickening or polypoid lesions were found in rats given catechol or 3-methoxycatechol irrespective of MNNG or $NaNO_2$ treatment.

Findings for histopathological forestomach changes are summarized in Tables II and III. Forestomach lesions were classified into hyperplasia, papilloma and squamous cell carcinoma (SCC). Hyperplasia was further classified into mild, moderate and severe, depending on the thickness of the mucosa. Invasion of SCCs was divided into four degrees. Incidences of papillomas and/or SCCs were significantly increased by the treatment with antioxidants. Combined treatment with $NaNO_2$ did not further modify tumor incidences in the BHA, catechol and 3-methoxycatechol groups, but significantly inhibited the degree of invasion of SCC ($P<0.01$) in the BHA group. In the non-MNNG treated groups, BHA, catechol or 3-methoxycatechol alone was associated with significant development of hyperplasias. Incidences of papillomas showed a non-significant tendency for increase with 3-methoxycatechol treatment. Additional exposure to $NaNO_2$ further increased the degree of hyperplasia and papilloma development in the catechol and 3-methoxycatechol groups. In contrast, the incidence of severe hyperplasia in the group given $NaNO_2$ and BHA was reduced as compared with the BHA alone case ($P<0.05$). The incidence of SCC also tended to decrease but this was not statistically significant. Treatment with $NaNO_2$ alone did not induce hyperplasia or neoplastic lesions in the forestomach.

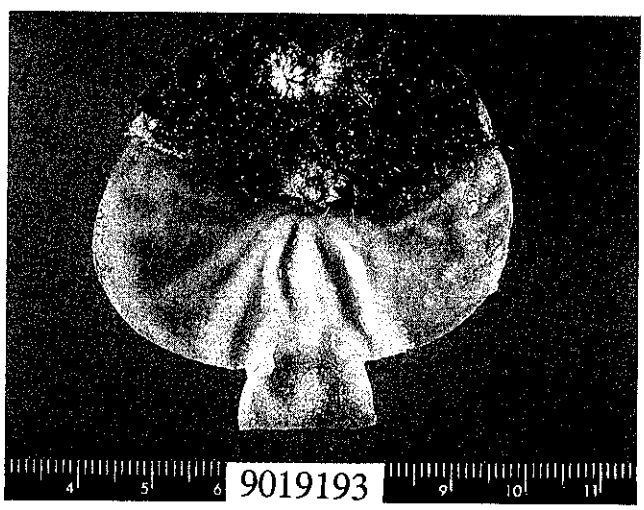

Fig. 3. Macroscopic appearance of the stomach of a rat treated with $NaNO_2$ and 3-methoxycatechol without MNNG initiation. Forestomach nodules are brown to black in color.

Data for lesions observed in the glandular stomach are summarized in Table IV and V. Lesions were classified into three types, submucosal hyperplasia, adenomas and adenocarcinomas. After MNNG initiation, catechol enhanced the incidence of adenocarcinomas ($P<0.01$) but additional treatment with $NaNO_2$ did not further enhance lesion development. 3-Methoxycatechol also increased the incidence of adenocarcinomas ($P<0.05$), but additional treatment with $NaNO_2$ inhibited the induction of adenomas ($P<0.01$). In the non-MNNG treated case, catechol alone induced submucosal hyperplasia (27%),

Table II. Lesions of the Forestomach Epithelium in Rats Given Phenolic Antioxidants and $NaNO_2$ with MNNG Pretreatment

Treatment			Effective no. of rats	No. of rats with (%)						
							Invasion			
MNNG	$NaNO_2$	Chemical		Hyperplasia	Papilloma	SCC	m[a]	sm[b]	pm[c]	s[d]
+	+	BHA	19	18 (95)	14 (74)	19 (100)[g]	0	15[e]	2	2[e]
+	−	BHA	20	18 (90)	17 (85)[f]	20 (100)[g]	0	7	2	11
+	+	Catechol	20	14 (70)	15 (75)	19 (95)[g]	0	10	3	6
+	−	Catechol	20	19 (95)	18 (90)[g]	17 (85)[g]	0	7	6	4
+	+	3-Methoxycatechol	20	18 (90)	17 (85)[f]	10 (50)	0	7	2	1
+	−	3-Methoxycatechol	19	18 (95)	18 (95)[g]	8 (42)	1	3	2	2
+	+	Basal diet	20	20 (100)	15 (75)	5 (25)	0	4	1	0
+	−	Basal diet	18	18 (100)	9 (50)	6 (33)	4	1	1	0

a) m: mucosa.
b) sm: submucosa.
c) pm: muscular layer.
d) s: serosa.
e) Significantly different from the values for the treated group without $NaNO_2$ at $P<0.01$.
f) Significantly different from the value for the basal diet group without $NaNO_2$ at $P<0.05$.
g) Significantly different from the values for the basal diet group without $NaNO_2$ at $P<0.01$.

Table III. Lesions of the Forestomach Epithelium in Rats Given Phenolic Antioxidants and $NaNO_2$ without MNNG Pretreatment

Treatment			Effective no. of rats	No. of rats with (%)					
				Hyperplasia				Tumor	
MNNG	$NaNO_2$	Chemical		Mild	Moderate	Severe	Total	Papilloma	SCC
−	+	BHA	15	15[d]	15[d]	2[a]	15 (100)[d]	3 (20)	0
−	−	BHA	15	15[d]	15[d]	9[d]	15 (100)[d]	3 (20)	3 (20)
−	+	Catechol	15	15[b, d]	15[b, d]	5[a, c]	15 (100)[b, d]	4 (27)	1 (7)
−	−	Catechol	15	6[c]	0	0	6 (40)[c]	0	0
−	+	3-Methoxycatechol	15	15[d]	15[b, d]	7[b, d]	15 (100)[d]	10 (67)[d]	0
−	−	3-Methoxycatechol	15	14[d]	0	0	14 (93)[d]	4 (27)	0
−	+	Basal diet	15	0	0	0	0	0	0
−	−	Basal diet	15	0	0	0	0	0	0

a) Significantly different from the value for the treated group without $NaNO_2$ at $P<0.05$.
b) Significantly different from the values for the treated groups without $NaNO_2$ at $P<0.01$.
c) Significantly different from the values for the basal diet group without $NaNO_2$ at $P<0.05$.
d) Significantly different from the values for the basal diet group without $NaNO_2$ at $P<0.01$.

Table IV. Lesions of the Glandular Stomach Epithelium in Rats Given Phenolic Antioxidants and $NaNO_2$ with MNNG Pretreatment

Treatment			Effective no. of rats	No. of rats with (%)			
MNNG	$NaNO_2$	Chemical		Submucosal hyperplasia	Adenoma	Adenocarcinoma	Sarcoma
+	+	BHA	19	3 (16)	1 (5)	0	0
+	−	BHA	20	1 (5)	0	0	0
+	+	Catechol	20	10 (50)[c]	15 (75)[c]	15 (75)[c]	0
+	−	Catechol	20	8 (40)[c]	15 (85)[c]	15 (75)[c]	1 (5)
+	+	3-Methoxycatechol	20	9 (45)[c]	6 (30)[a, b]	6 (30)[b]	2 (10)
+	−	3-Methoxycatechol	19	11 (58)[c]	17 (89)[c]	6 (32)[b]	0
+	+	Basal diet	20	0	3 (15)	0	0
+	−	Basal diet	18	0	0	0	0

a) Significantly different from the value for the treated group without $NaNO_2$ at $P<0.01$.
b) Significantly different from the values for the basal diet group without $NaNO_2$ at $P<0.05$.
c) Significantly different from the values for the basal diet group without $NaNO_2$ at $P<0.01$.

adenomas (100%) and adenocarcinomas (33%), but no influence of $NaNO_2$ was found. 3-Methoxycatechol alone also induced submucosal hyperplasia (73%) and adenomas (93%), being significantly decreased by the additional $NaNO_2$ treatment (40%) ($P<0.01$).

Esophageal hyperplasias were sporadically observed, but no significant induction was found for any of the phenolic compounds. In the other organs, no histopathological findings suggestive of influence of phenolic compounds and $NaNO_2$ were evident.

Short-term cell proliferation study The body weights of animals treated with phenolic compounds or $NaNO_2$ individually were 10 to 33% less than the basal diet alone values except in the resorcinol, 4-methoxyphenol, sesamol, gallic acid, caffeic acid, ferulic acid, vanillic acid, pyrogallol and HSC cases, for which the reduction was less than 10%. Additional treatment with $NaNO_2$ was associated with a further reduction of 8 to 36% as compared with individual phenolic compound alone group values. Relative liver and kidney weights were increased by the phenolic compounds given alone. Liver weights were reduced and kidney weights were further increased by the additional treatment with $NaNO_2$ (data not shown).

Data for mucosal thickness and labeling indices of forestomach, glandular stomach and esophagus are shown in Table VI. Significant increase in the thickness of the forestomach mucosa in the prefundic or mid regions was observed in rats treated with 12 of the phenolic compounds, these being TBHQ, 4-methoxyphenol, sesamol, catechol, 4-methylcatechol, 3-methylcatechol, PTBC, caffeic acid, pyrogallol, guaiacol, 4-

Table V. Lesions of the Glandular Stomach Epithelium in Rats Given Phenolic Antioxidants and $NaNO_2$ without MNNG Pretreatment

Treatment			Effective no. of rats	No. of rats with (%)		
MNNG	$NaNO_2$	Chemical		Submucosal hyperplasia	Adenoma	Adenocarcinoma
−	+	BHA	15	0	0	0
−	−	BHA	15	0	0	0
−	+	Catechol	15	4 (27)	15 (100)[c]	2 (13)
−	−	Catechol	15	4 (27)	15 (100)[c]	5 (33)[b]
−	+	3-Methoxycatechol	15	6 (40)[b]	6 (40)[a, b]	0
−	−	3-Methoxycatechol	15	11 (73)[c]	14 (93)[c]	0
−	+	Basal diet	15	0	0	0
−	−	Basal diet	15	0	0	0

a) Significantly different from the value for the treated group without $NaNO_2$ at $P<0.01$.
b) Significantly different from the values for the basal diet group without $NaNO_2$ at $P<0.05$.
c) Significantly different from the values for the basal diet group without $NaNO_2$ at $P<0.01$.

methylphenol and eugenol, as well as $NaNO_2$. Effects of 4-methoxyphenol, sesamol, 4-methylcatechol, 3-methylcatechol or caffeic acid were more pronounced than other chemicals.

Additional treatment with $NaNO_2$ markedly enhanced (to more than 10 times the antioxidant alone value) forestomach cell proliferation in rats receiving TBHQ, catechol, gallic acid, 1,2,4-benzenetriol, dl-dopa and hydroquinone. Less marked, but significant, enhancement was observed in rats treated with phenol, BHT, 4-methoxyphenol, sesamol, 4-methylcatechol, 3-methylcatechol, PTBC, protocatechuic acid, ferulic acid, vanillic acid, vanillin, pyrogallol, guaiacol, 4-methylphenol, eugenol or HSC. On the other hand, $NaNO_2$ was not effective in enhancing cell proliferation in rats treated with resorcinol. In the caffeic acid case, mid-region thickness was significantly reduced by the combined treatment with $NaNO_2$.

In the glandular stomach, either thickness or labeling indices were significantly increased in all groups given phenolic compounds alone, except phenol, resorcinol, gallic acid, protocatechuic acid, pyrogallol, eugenol and HSC. Significant enhancing effects in terms of either mucosal thickness or labeling indices were observed for $NaNO_2$ given along with phenol, resorcinol, TBHQ, gallic acid, 1,2,4-benzenetriol, hydroquinone, vanillic acid, pyrogallol, guaiacol, 4-methylphenol and HSC.

In the esophagus, either mucosal thickness or labeling index was increased in rats treated with 4-methoxyphenol, sesamol, catechol, 4-methylcatechol, 3-methylcatechol, PTBC, 1,2,4-benzenetriol, hydroquinone, guaiacol or 4-methylphenol. Of these chemicals, 4-methoxyphenol, PTBC or 4-methylphenol significantly increased both thickness and labeling indices. Additional treatment with $NaNO_2$ increased either thickness or labeling index in animals treated with phenol, TBHQ or gallic acid.

DISCUSSION

We earlier found, using a multi-organ carcinogenesis model, that additional treatment with $NaNO_2$ did not further enhance 3-methoxycatechol promotion of forestomach carcinogenesis, despite strongly enhancing the degree of forestomach hyperplasia.[25] This discrepancy between cell proliferation and promotion of carcinogenesis in the forestomach epithelium was also observed in the present experiment, and therefore it clearly does not depend on the carcinogen used for the initiation step. The lack of any increase in the catechol case might be partly due to the induced incidence of forestomach carcinomas being too high for any additional effect to be evaluable. 4-Methoxyphenol is a non-genotoxic forestomach carcinogen,[26] and its carcinogenic activity is stronger than that of BHA at the same dietary concentration. However, it was found not to promote forestomach carcinogenesis in rats initiated with MNNG.[27] One possible interpretation is that 4-methoxyphenol kills the initiated cells induced by MNNG, since it causes extensive forestomach damage one week after treatment.[28] Similar extensive forestomach damage was observed in rats simultaneously treated with $NaNO_2$ and catechol for one week (unpublished observation), raising the possibility that the observed discrepancy between cell proliferation and promotion of forestomach carcinogenesis induced by combined treatment with 3-methoxycatechol and $NaNO_2$ might be due to strong cytotoxicity.

In the glandular stomach, catechol and 3-methoxycatechol promoted MNNG–induced carcinogenesis and themselves also caused development of hyperplasia, adenomas or adenocarcinomas. However, the promoting effect of 3-methoxycatechol was inhibited by combined treatment with $NaNO_2$. This could have been partly due to suppression of food intake.

Table VI. Mucosal Thickness and Proliferative Indices in the Upper Digestive Tract of Rats Given Phenolic Compounds and $NaNO_2$

Treatment		No. of rats	Height of mucosa ($\times 10^{-2}$ mm)				No. of rats	Labeling indices	
			Forestomach		Gl. stomach	Esophagus		Gl. stomach (No. of cells /crypt)	Esophagus (No. of cells /100 cells)
$NaNO_2$	Chemical		Prefundic	Mid					
+	Phenol	5	4.0±1.3[a,b]	1.7±0.3	24.1±6.0[b]	1.4±0.3[b]	5	2.6±0.3[b]	13.8±2.8
−	Phenol	5	2.1±0.1	1.5±0.3	16.3±2.7	1.0±0.1	5	1.7±0.5	16.4±8.5
+	Resorcinol	5	2.9±0.3	1.3±0.3	22.9±3.1	1.3±0.2	5	3.4±0.9[b]	15.0±6.4
−	Resorcinol	5	2.8±1.3	2.9±1.9	17.7±4.0	1.4±0.2	5	2.2±0.4	18.0±3.1
+	BHT	5	5.0±1.3[c]	3.1±1.2[b]	15.9±3.9	1.0±0	3	3.0±0.1	11.0±8.7
−	BHT	5	1.6±0.4	1.1±0.2	17.7±4.0	0.9±0.2	5	2.6±0.6[d]	12.4±7.1
+	TBHQ	5	40.6±10.5[c]	15.2±2.8	23.8±3.9[b]	2.5±0.4[c]	4	5.4±1.1[c]	14.0±4.3
−	TBHQ	5	3.6±0.8[e]	8.2±8.0	19.0±2.2[e]	1.5±0	3	3.1±0.2[c]	10.4±3.1
+	4-Methoxyphenol	5	45.9±4.8[c]	30.3±8.1	24.5±5.7	1.6±0.3	4	7.8±2.8	13.8±2.2
−	4-Methoxyphenol	5	12.6±9.6	24.9±3.0[e]	18.3±2.3[d]	2.5±1.0[d]	5	6.5±0.6[c]	21.3±6.0[d]
+	Sesamol	5	40.4±8.3[c]	38.1±7.0[c]	20.6±3.1	1.1±0.2	2	6.5	19.5±3.5
−	Sesamol	5	7.4±3.9[d]	21.5±5.9[e]	22.8±5.9[d]	1.4±0.3	5	6.2±0.6[e]	26.2±8.8[d]
+	Catechol	5	34.9±2.7[c]	12.8±4.3[c]	26.3±2.2	1.0±0.2	5	3.5±0.7	14.8±4.5
−	Catechol	5	2.3±0.4	2.4±0.7[d]	21.6±8.6	1.1±0.1	5	4.0±1.3[e]	23.4±4.8[e]
+	4-Methylcatechol	5	45.3±11.6[c]	22.7±7.3[b]	30.5±8.3	1.4±0.2	4	6.1±2.4	17.8±10.3
−	4-Methylcatechol	5	13.7±2.6[e]	11.9±3.7[e]	33.5±5.8[c]	1.9±0.2[e]	4	3.8±1.1[e]	19.4±5.2
+	3-Methylcatechol	5	48.4±4.2[c]	27.9±6.9	29.6±3.0	1.5±0.3	3	5.4±1.8	13.7±4.5
−	3-Methylcatechol	5	24.2±4.6[e]	19.0±6.0[e]	36.9±6.0[c]	1.5±0.2[d]	4	4.9±1.7[d]	21.3±6.9
+	Gallic acid	5	39.1±6.4[c]	38.3±7.2[c]	19.8±2.4[b]	1.1±0.2[b]	4	4.5±1.1[c]	9.0±5.7
−	Gallic acid	5	2.2±0.4	1.6±0.3	14.0±3.9	0.7±0.2	4	1.8±0.4	14.8±5.7
+	PTBC	5	56.0±8.4[c]	23.8±6.2[c]	27.3±2.5	2.0±0.4	2	4.7	13.5±2.1
−	PTBC	5	10.1±3.2[e]	8.0±3.4[d]	21.3±7.1[d]	1.9±0.2[e]	3	4.0±0.6[e]	22.5±3.1[e]
+	Protocatechuic acid	5	20.2±2.1[c]	3.9±1.6[b]	21.1±4.6	0.8±0.1	4	3.0±1.6	16.0±6.4
−	Protocatechuic acid	5	2.1±0.3	1.6±0.3	16.6±2.3	1.2±0.1	4	2.5±0.8	12.0±2.0
+	Caffeic acid	5	22.6±5.0	3.8±1.0[c]	18.3±4.2	0.9±0.1	4	3.6±1.5	13.5±1.9
−	Caffeic acid	5	22.8±4.3[c]	33.7±13.4[e]	19.7±2.5[e]	1.0±0	5	3.1±0.8[c]	16.0±1.4
+	1,2,4-Benzenetriol	5	27.4±7.3[c]	9.7±3.5[c]	22.1±3.5[b]	0.9±0.2	2	4.1[c]	17.5±2.1
−	1,2,4-Benzenetriol	5	1.9±0.4	1.5±0.3	15.8±3.1	1.7±0.4[d]	4	2.5±0.6[d]	13.0±5.2
+	Ferulic acid	5	5.4±2.1[b]	2.6±0.5[c]	17.6±2.1	0.8±0.3	5	3.3±0.8	12.8±1.8
−	Ferulic acid	5	1.9±0.5	1.5±0.4	15.1±3.3	0.8±0.1	5	4.4±1.0[e]	12.3±1.5
+	dl-dopa	5	23.2±1.2[c]	22.8±8.1[c]	15.6±2.2	0.8±0.4	4	3.5±1.0	15.0±2.8
−	dl-dopa	5	2.1±0.5	2.0±0.9	21.4±6.7[d]	0.9±0.3	5	2.9±1.2	14.6±2.8
+	Hydroquinone	5	40.7±5.9[c]	19.4±3.5[c]	22.7±2.7	1.0±0.4	5	5.2±1.0[b]	15.6±8.1
−	Hydroquinone	5	2.0±0.3	1.5±0.5	21.9±1.5[c]	1.4±0.4	5	3.4±0.8[c]	19.0±3.4[d]
+	Vanillic acid	5	11.8±4.7[c]	1.4±0.3	22.9±2.6[c]	1.4±0.8	5	4.9±1.7[b]	18.8±7.4
−	Vanillic acid	5	1.9±0.3	1.4±0.3	15.1±3.0	1.1±0.5	5	2.7±0.6[d]	17.4±3.3
+	Vanillin	5	5.2±0.8[c]	3.0±1.0	21.6±3.6	1.1±0.2	5	4.4±0.9	12.2±6.1
−	Vanillin	5	2.1±0.3	1.8±0.6	19.4±3.5[d]	1.4±0.7	5	3.4±0.8[e]	11.8±4.4
+	Pyrogallol	5	51.8±10.0[c]	55.7±15.1[c]	20.1±2.2	0.8±0.4	5	3.9±0.7[c]	14.8±4.3
−	Pyrogallol	5	5.3±1.2[e]	5.6±1.5[e]	17.2±4.8	1.6±0.6	4	2.4±0.6	15.6±4.2
+	Guaiacol	5	19.7±7.1[c]	18.5±10.8[b]	20.6±2.3	1.0±0.2	5	3.9±1.3[b]	16.4±4.9
−	Guaiacol	5	3.4±1.3	2.9±0.5[e]	19.5±1.7[c]	1.8±0.3[e]	5	2.2±0.7	15.3±3.9
+	4-Methylphenol	5	9.4±2.4[c]	15.0±8.7[b]	20.4±2.4[b]	1.8±0.3	4	4.1±0.4[c]	12.5±6.5
−	4-Methylphenol	5	3.2±0.3[e]	2.5±0.3[e]	16.9±2.2[d]	2.2±0.6[e]	5	2.6±0.8[d]	19.4±2.1[d]
+	Eugenol	5	16.0±2.8[c]	18.2±7.1[c]	17.2±3.0	1.0±0.2	5	2.9±1.2	16.2±2.6
−	Eugenol	5	2.1±0.4	2.5±0.7[d]	15.9±6.0	1.3±0.3	5	1.5±0.9	13.8±7.5
+	HSC	5	3.2±0.7[c]	2.7±1.2	16.5±1.0	1.1±0.2	5	2.8±0.9[b]	13.2±2.4
−	HSC	5	1.8±0.5	1.6±0.5	14.4±2.7	1.0±0.2	5	1.7±0.5	14.6±1.7
+	Basal diet	5	3.4±0.6[e]	2.3±0.3[e]	18.4±4.4	1.2±0.4	5	2.2±0.6	14.8±6.6
−	Basal diet	5	2.3±0.4	1.4±0.2	13.5±2.4	1.0±0.4	5	1.6±0.5	14.2±2.9

a) Data are mean±SD values.
b) Significantly different from the values for the treated groups without $NaNO_2$ at $P<0.05$.
c) Significantly different from the values for the treated groups without $NaNO_2$ at $P<0.01$.
d) Significantly different from the values for the basal diet group without $NaNO_2$ at $P<0.05$.
e) Significantly different from the values for the basal diet group without $NaNO_2$ at $P<0.01$.

Catechol, 3-methoxycatechol, phenol, resorcinol, 4-methylcatechol, caffeic acid, hydroquinone, vanillin, pyrogallol and guaiacol all exhibit direct-acting genotoxicity in the SOS Chromotest after nitrosation by incubation with $NaNO_2$ under acidic conditions.[10] Mutagenic metabolites could be quinones, nitrosophenols or diazonium compounds. The reaction of phenol with nitrite can produce mutagenic *p*-diazoquinone in *Salmonella typhimurium* strains TA 98 and TA 100.[11] A mixture of BHA and $NaNO_2$ under acidic conditions did not demonstrate genotoxicity in the SOS Chromotest[10] although it produced mutagenic t-BQ and the dimer of t-BQ.[12] A single intragastric administration of t-BQ was shown to induce DNA damage in the rat forestomach epithelium.[29] In addition, treatment with BHA alone for 2 weeks induces very small amounts of DNA adducts in the rat forestomach epithelium, with the same spots in TLC plates being increased by combined treatment with $NaNO_2$.[30] DNA adducts were also detected on TLC plates by ^{32}P-postlabeling methods in the forestomach epithelium of rats treated for 2 weeks with $NaNO_2$ and catechol, but not with catechol alone. While combined treatment with catechol and $NaNO_2$ induced strong forestomach cell proliferation, phenol together with $NaNO_2$ was without such an effect. The fact that $NaNO_2$ did not enhance BHA-induced cell proliferation further indicates that mutagenic activity and cell proliferation do not necessarily correlate. The data also suggest that different metabolites may be responsible for genotoxicity and cell proliferation.

In the caffeic acid case, simultaneous treatment with $NaNO_2$ proved exceptional in reducing cell proliferation in the forestomach mucosa. The reasons remain to be elucidated.

BHA, catechol, sesamol and caffeic acid have all been reported to be carcinogenic for the rat stomach.[13–15, 17] Recently, 4-methoxyphenol and 4-methylcatechol were also demonstrated to exert carcinogenicity for the forestomach and/or glandular stomach in a rat long-term experiment.[26] BHA, catechol, 4-methylcatechol, PTBC, caffeic acid, 3-methoxycatechol, 4-methoxyphenol, sesamol and 1,2,4-benzenetriol, which were used in this study, have also all been shown to exert promoting and/or cell proliferation stimulating effects in the rat forestomach, glandular stomach or esophagus.[20–24, 27, 31–33] Most of these phenolic compounds exist in our environment, and some of them are integral components of the human diet. Although the major target of combined treatment with phenolic compounds and $NaNO_2$ is the forestomach, which humans lack, the possibility of modification in the other upper digestive organs should be clarified for human bazard estimation. In conclusion, the present study demonstrated that $NaNO_2$ can stimulate phenolic compound-induced cell proliferation in the upper digestive tract, particularly in the forestomach epithelium.

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