Effects of Antioxidant 1-O-Hexyl-2,3,5-trimethylhydroquinone or Ascorbic Acid on Carcinogenesis Induced by Administration of Aminopyrine and Sodium Nitrite in a Rat Multi-organ Carcinogenesis Model

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The effect of antioxidant, 0.25% 1-O-hexyl-2,3,5-trimethylhydroquinone (HTHQ) or 0.25% ascorbic acid (AsA), on carcinogenesis induced by administration of 0.05% aminopyrine (AP) and 0.05% sodium nitrite (NaNO2), was examined using a rat multi-organ carcinogenesis model. Groups of twenty F344 male rats were treated sequentially with an initiation regimen of N-diethylnitrosamine, N-methyl-N-nitrosourea, N-butyl-N-(4-hydroxybutyl)nitrosamine, N,N′-dimethyldihydrazine and 2,2′-dihydroxy-di-n-propynitrosamine during the first 4 weeks, followed by AP++++NaNO2, AP++++NaNO2++++HTHQ, AP++++NaNO2++++AsA, NaNO2++++HTHQ, NaNO2++++AsA, each of the individual chemicals alone or basal diet and tap water as a control. All surviving animals were killed at week 28, and major organs were examined histopathologically for development of preneoplastic and neoplastic lesions. In the AP++++NaNO2 group, the incidences of hepatocelluar adenomas and hemangiosarcomas were 95% and 35%, respectively. When HTHQ or AsA was simultaneously administered, the incidences decreased to 58% and 11%, or to 80% and 15%, respectively. On the other hand, in the AP++++NaNO2 group and the NaNO2-alone group, when HTHQ, but not AsA, was simultaneously administered, the incidence of carcinomas in the forestomach significantly increased. The results suggest that HTHQ can prevent tumor production induced by AP and NaNO2 more effectively than AsA. On the other hand, an enhancing or possible carcinogenic effect of simultaneous administration of HTHQ and NaNO2 only on the forestomach is suggested, while simultaneous treatment with the same dose of AsA and NaNO2 may not be carcinogenic to the forestomach or other organs.

Key words: Antioxidants — Aminopyrine — Sodium nitrite — Carcinogenesis — Rat

Many nitrosoamines are known to induce tumors in various organs.1) It was reported that some drugs with secondary or tertiary amino groups reacted with nitrite under acidic conditions to form nitrosamines in vitro and in vivo.2–5) In particular, aminopyrine (AP) is known to react with sodium nitrite (NaNO2) to form dimethylnitrosamine (DMN), which is a potent liver, lung and renal carcinogen.2–6) Feeding of AP and NaNO2 to rats has been shown to cause acute liver damage due to formation of DMN and eventually to cause liver tumors upon chronic treatment.2–7,8) It is also known that ascorbic acid (AsA), and phenolic antioxidants such as caffeic acid, ferulic acid, butylated hydroxyanisole (BHA) and propyl gallate, inhibit the formation of DMN in rats receiving AP and NaNO2.4,5) An inhibitory effect of caffeic acid on the endogenous formation of N-nitrosoproline was demonstrated in man.9) Kuening et al. suggest that dietary phenols may play an important role in the prevention of carcinogenesis by inhibiting the formation of nitrosamines.5)

On the other hand, it has been reported that mutagenic compounds were formed by the interaction of polyphenols with nitrite in vitro.10–11) It was also reported that when several phenolic antioxidants such as catechol, hydroquinone, gallic acid, t-butylhydroquinone (TBHQ) or AsA and 0.2% NaNO2 are simultaneously administered to rats, strong toxicity and cell proliferation are induced in the forestomach epithelium. Furthermore, continuous administration of AsA, sodium ascorbate, catechol or 3-methoxy-catechol and 0.2–0.3% NaNO2 for 24–51 weeks induced forestomach tumors, and also enhanced rat forestomach carcinogenesis initiated with N-methyl-N′-nitro-N-nitrosoguanidine (MNNNG).12–15) Such synergistic enhancing...
effects of carcinogenesis were limited to the forestomach epithelium in the case of catechol and 3-methoxy- catechol. However, the combined effects of AsA and NaNO₂ on other organs have not yet been evaluated.

In recent years, attention has been focused on a strong lipophilic phenolic antioxidant, 1-O-hexyl-2,3,5-trimethyl-
hydroquinone (HTHQ). This antioxidant possesses the
strongest potential for inhibiting lipid peroxidation in liver
microsomes among known antioxidants, including BHA,
butylated hydroxytoluene (BHT), propyl gallate, TBHQ,
and α-tocopherol. Its anti-mutagenic activity against 2-
amino-6-methyl-dipyrido[1,2-a:3′,2′-d]imidazole (Glup-P-1),
evaluated in the Ames assay, was also found to be
stronger than that of other antioxidants. In addition to
these in vitro effects, HTHQ potently inhibits Glu-P-1 or
2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline (MeIQx)-
induced hepatocarcinogenesis,23) 2-amino-1-methyl-6-phen-
ylimidazo[4,5-b]pyridine (PhIP)-induced mammary carcino-
genesis by simultaneous treatment,19) and 7,12-dimethyl-
benz(a)anthracene (DMBA)-initiated mammary carcino-
genesis in the post-initiation period20) in rats. On the other hand,
HTHQ was shown to enhance tongue and forestomach
carcinogenesis in rats pretreated with NaNO₂ and AP,
and also assessed the possibility of modification of car-
cinogenesis by blocking nitrosamine formation, and strongly
enhances forestomach carcinogenesis in the presence of
NaNO₂, like other phenolic antioxidants.

In the present experiment, we evaluated the inhibitory
effects of HTHQ or AsA on nitrosamine-induced carcino-
genesis by simultaneous administration of NaNO₂ and AP,
and also assessed the possibility of modification of car-
cinogenesis in major organs by simultaneous administra-
tion of HTHQ or AsA and NaNO₂, using a rat medium-
term multi-organ carcinogenesis model.

MATERIALS AND METHODS

Animals and chemicals Two hundred and fifty 5-week-
old male F344 rats were obtained from Charles River
Japan, Inc., Atsugi. They were randomly divided into
groups of five animals per cage with hard wood chips as
bedding in an air-conditioned room at 24±2°C and
55±5% humidity with a 12 h light/dark cycle. Food (Or-
iental MF, Oriental Yeast Co., Tokyo) and tap water were
available ad libitum. N-Diethylnitosamine (DEN), N,N′-
dimethylhydrazine (DMH) and N-butyl-N-(4-hydroxybut-
yl)nitrosamine (BNMN) were purchased from Tokyo Kasei
Kogyo Co., Ltd. (Tokyo), N-methyl-N-nitrosourea (MNU)
from Sigma-Aldrich (Tokyo) and 2,2′-dihydroxy-di-n-prop-
ylNitrosamine (DHPN) from Nakalai Tesque, Inc.
(Okayama). HTHQ was provided from Nippon Hypox Labo-
ratories, Inc. (Yamanashi), NaNO₂, AP and AsA from
Wako Pure Chemical Industries, Ltd. (Osaka).
Experimental design As shown in Fig. 1, at the age of 6
weeks, a total of 250 rats were divided into 20 groups.
Twenty animals each in groups 1–10 received the
DMBDD initiation treatment, composed of DEN (100 mg/
kg b.w., i.p., single dose at commencement), MNU (20
mg/kg b.w., i.p., on day 2, 5, 8 and 11), and DMH (40 mg/
kg b.w., s.c. on day 14, 17, 20 and 23). Animals were
simultaneously given BBN (0.05% in drinking water dur-
ing weeks 1 and 2) and DHPN (0.1% in drinking water
during weeks 3 and 4). Starting 1 week after this DMBDD
treatment, the animals were treated as follows: group 1,
AP+NaNO₂; group 2, AP+NaNO₂+HTHQ; group 3,
AP+NaNO₂+AsA; group 4, NaNO₂+HTHQ; group 5,
NaNO₂+AsA; group 6, AP; group 7, NaNO₂; group 8,
HTHQ; group 9, AsA; group 10, basal diet. Five animals
each in groups 11 to 20 were treated in the same way as
groups 1 to 10 without DMBDD treatment. AP and
NaNO₂ were dissolved in tap water at a dose of 0.05%,
and HTHQ and AsA were mixed in powdered basal diet at
a dose of 0.25%. Diet and water were prepared once every
1 to 2 weeks and stored in the dark until use. Body weight
and food consumption were measured once every 2 to 4
weeks during the administration periods. All survivors
were killed under ether anesthesia at the end of week 28
and subjected to complete autopsy. The liver, kidneys and
spleen were weighed. Ten-percent buffered formalin solu-
tion-fixed and paraffin-embedded sections of the liver,
kidneys, spleen, heart, thyroid, lungs, tongue, esophagus,
stomach, intestines, testes, urinary bladder and other tis-
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larger than 0.2 mm in diameter, including adenomas and carcinomas were measured with the aid of an image analyzer (VIP-21, Olympus Co., Tokyo).

**Statistical analysis** Student’s t test and Fisher’s exact probability test were used for statistical analysis of the data.

**RESULTS**

**Body and organ weights** The final body weights and relative organ weights of liver and kidneys are summarized in Table I. Regardless of DMBDD, HTHQ or AsA treatment, the final body weight of rats significantly decreased in all the AP+NaNO₂-treated groups as compared with the control group. Relative liver weights significantly increased in all test compound groups given DMBDD as compared with the control group. On the other hand, in the AP+NaNO₂ group without DMBDD treatment, a significant decrease was noted. In the AP+NaNO₂+HTHQ group, the value significantly increased. Relative kidney weights significantly increased in the AP+NaNO₂, AP+NaNO₂+HTHQ and HTHQ groups as compared with the control group. A significant increase was also found in the AP+NaNO₂ group without DMBDD treatment. The AP-alone group showed a significant decrease. Further, regardless of DMBDD, HTHQ or AsA treatment, relative spleen weights showed a significant increase in all the AP+NaNO₂-treated groups compared with the control group.

**GST-P-positive lesions** Quantitative data for numbers and areas of GST-P-positive lesions in the liver are summarized in Table II. In the AP+NaNO₂ group with DMBDD treatment, the number and area of GST-P-positive lesions significantly increased as compared with the control group, regardless of HTHQ or AsA administration. However, when HTHQ or AsA was administered simultaneously, the area of GST-P-positive lesions in the AP+NaNO₂+HTHQ group (9.64 mm²/cm², *P*<0.01) and that in the AP+NaNO₂+AsA group (11.35 mm²/cm², *P*<0.01) were significantly decreased in comparison with the AP+NaNO₂ group (33.6 mm²/cm²). In the AP-alone group, the number and area of GST-P-positive lesions significantly increased as compared with the control group. In the AP+NaNO₂ group without DMBDD treatment, both the number and area of GST-P-positive lesions significantly increased as compared with the control group. However, when HTHQ or AsA was administered simultaneously, both the number and area of GST-P-positive lesions decreased, though not with statistical significance.

**Histopathology** Quantitative data for neoplastic and pre-neoplastic lesions in major organs of the DMBDD-treated groups are summarized in Table III.

In the liver, the incidences of hepatocellular adenomas, carcinomas and hemangiosarcomas in the AP+NaNO₂

| Group | Treatment | No. of rats survived | Relative organ wt. (g/100 g body wt.) | Liver | Kidneys | Spleen |
|-------|-----------|----------------------|---------------------------------------|-------|---------|--------|
| 1     | + AP+NaNO₂ | 14                   | 2.95±0.42** 0.75±0.08** 0.29±0.10** |       |         |        |
| 2     | + AP+NaNO₂+HTHQ | 19         | 2.45±0.16** 0.70±0.08** 0.22±0.03** |       |         |        |
| 3     | + AP+NaNO₂+AsA | 20         | 2.32±0.09** 0.71±0.07** 0.22±0.02** |       |         |        |
| 4     | + NaNO₂+HTHQ | 20                   | 2.19±0.09** 0.68±0.19** 0.18±0.02 |       |         |        |
| 5     | + NaNO₂+AsA | 20                   | 2.05±0.06** 0.65±0.05** 0.18±0.02 |       |         |        |
| 6     | + AP       | 19                   | 2.19±0.14** 0.99±0.99 0.28±0.39  |       |         |        |
| 7     | + NaNO₂    | 20                   | 2.07±0.07** 0.63±0.09 0.18±0.01 |       |         |        |
| 8     | + HTHQ     | 20                   | 2.17±0.08** 0.65±0.08** 0.18±0.02 |       |         |        |
| 9     | + AsA      | 20                   | 2.07±0.08** 0.63±0.05** 0.18±0.01 |       |         |        |
| 10    | + Control  | 19                   | 1.99±0.06  0.59±0.03  0.18±0.01 |       |         |        |
| 11    | − AP+NaNO₂ | 5                    | 313±6** 1.98±0.03** 0.60±0.01** 0.19±0.02** |       |         |        |
| 12    | − AP+NaNO₂+HTHQ | 5         | 2.23±0.07  0.59±0.02  0.18±0.01** |       |         |        |
| 13    | − AP+NaNO₂+AsA | 5         | 2.09±0.07  0.57±0.02  0.17±0.01** |       |         |        |
| 14    | − NaNO₂+HTHQ | 5                  | 2.22±0.08  0.56±0.03  0.15±0.01 |       |         |        |
| 15    | − NaNO₂+AsA | 5                    | 2.05±0.05  0.58±0.01  0.15±0.01 |       |         |        |
| 16    | − AP       | 5                    | 2.04±0.06  0.55±0.02  0.15±0.01 |       |         |        |
| 17    | − NaNO₂    | 5                    | 1.94±0.03** 0.57±0.02  0.14±0.01 |       |         |        |
| 18    | − HTHQ     | 5                    | 2.19±0.08  0.56±0.02  0.14±0.00 |       |         |        |
| 19    | − AsA      | 5                    | 1.90±0.16  0.56±0.03  0.15±0.02 |       |         |        |
| 20    | − Control  | 5                    | 2.11±0.06  0.58±0.02  0.15±0.01 |       |         |        |

*a* Mean±SD.

**, ** Significantly different from group 10 or 20 at *P*<0.05, 0.01.
In the AP + NaNO₂ group, the incidences of squamous cell carcinoma were significantly increased in all HTHQ-treated groups. Fore- 

 stomach tumors were not found in these groups. The incidence of lung alveolar hyperplasias significantly increased in all HTHQ-treated groups. Fore- 

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Table III. Incidences of Preneoplastic and Neoplastic Lesions in Initiated Groups

| Group | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| DMBDD treatment | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| Test compounds   | AP +NaNO₂ | AP +NaNO₂ | AP +NaNO₂ | NaNO₂ +HTHQ | NaNO₂ +HTHQ | AsA | AP | NaNO₂ | HTHQ | AsA | Control |
| No. of rats examined | 20 | 19 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |

Liver

- Hepatocellular adenoma: 19 (95)** 11 (58)** 16 (80)** 4 (20) 2 (10) 2 (10) 1 (5) 1 (5) 1 (5) 1 (5) 1 (5)
- Hepatocellular carcinoma: 4 (20) 1 (5) 0 1 (5) 0 0 0 0 0 1 (5)
- Hepatocholangiocellular adenoma: 1 (5) 0 0 0 0 0 0 0 0 0
- Hepatocholangiocellular carcinoma: 1 (5) 0 0 0 0 0 0 0 0 0
- Hemangioma: 0 1 (5) 1 (5) 0 1 (5) 0 0 0 1 (5) 1 (5)
- Hemangiosarcoma: 7 (35)** 2 (11) 3 (15) 0 0 0 0 0 0 0

Forestomach

- Hyperplasia: 4 (20) 19 (100)** 5 (25) 20 (100)** 5 (25) 6 (30) 2 (10) 20 (100)** 4 (20) 4 (20)
- Papilloma: 2 (10) 8 (42)* 0 10 (50)** 1 (5) 2 (10) 4 (20) 13 (65)* 1 (5) 1 (5)
- Squamous cell carcinoma: 0 5 (26)* 0 8 (40)** 0 0 0 2 (10) 0 0

Kidney

- Renal cell tumor: 12 (60) 11 (58) 16 (80)** 9 (45) 11 (55) 10 (50) 11 (55) 8 (40) 13 (65)* 5 (25)
- Nephroblastosia: 15 (75) 13 (68) 16 (80) 9 (45) 10 (50) 13 (65) 10 (50) 9 (45) 9 (45) 12 (60)
- Transitional cell hyperplasia: 2 (10) 3 (16) 0 1 (5) 0 2 (10) 1 (5) 0 0 1 (5)
- Transitional cell carcinoma: 0 1 (5) 0 0 0 4 (20) 3 (15) 0 1 (5) 0

Thyroids

- Follicular adenoma: 6 (30) 10 (53) 11 (55) 7 (35) 6 (30) 12 (60) 8 (40) 9 (45) 7 (35) 7 (35)
- Follicular carcinoma: 4 (20) 3 (16) 5 (25) 8 (40) 6 (30) 9 (45) 7 (35) 11 (55) 3 (15) 6 (30)

Lung

- Adenoma: 8 (40) 8 (42) 12 (60) 9 (45) 6 (30) 9 (45) 9 (45) 9 (45) 7 (35) 9 (45) 8 (40)
- Carcinoma: 9 (45) 5 (25)* 8 (40) 10 (50) 7 (35) 5 (25)* 9 (45) 8 (40) 9 (45) 13 (65)

Tongue

- Hyperplasia: 0 4 (21) 0 9 (45)** 1 (5) 0 0 5 (25) 0 1 (5)
- Papilloma: 0 3 (16) 1 (5) 1 (5) 0 0 1 (5) 3 (15) 0 1 (5)

Esophagus

- Papilloma: 0 2 (11) 1 (5) 0 1 (5) 1 (5) 0 0 1 (5) 1 (5)
- Carcinoma: 0 0 0 1 (5) 0 0 0 1 (5) 0 0

Small intestine

- Adenoma: 0 0 0 0 1 (5) 3 (15) 6 (30) 0 0 4 (20)
- Adenocarcinoma: 0 0 0 0 1 (5) 1 (5) 0 0 2 (10) 0

Colon/Rectum

- Adenoma: 0 1 (5) 0 0 0 0 0 0 2 (10) 2 (10)
- Adenocarcinoma: 0 1 (5) 1 (5) 0 0 0 2 (10) 1 (5) 3 (15) 1 (5)

Urinary bladder

- Papilloma: 0 1 (5) 0 0 1 (5) 0 0 2 (10) 0 0
- Carcinoma: 1 (5) 1 (5) 0 1 (5) 1 (5) 0 2 (10) 0 2 (10) 1 (5)

Nasal cavity

- Papilloma: 1 (5) 0 0 3 (15) 5 (25) 1 (5) 1 (5) 3 (15) 1 (5) 1 (5)
- Carcinoma: 1 (5) 0 3 (15) 1 (5) 3 (15) 1 (5) 4 (20) 0 3 (15) 1 (5)

Abdominal cavity

- Schwannoma: 0 0 0 4 (20) 0 0 1 (5) 1 (5) 0 3 (15)
- Mesothelioma: 2 (10) 0* 0* 0* 6 (30) 1 (5) 5 (25) 0* 3 (15) 5 (25)

* Significant with respect to group 10 at P<0.05, 0.01.
** Significantly different from group 1 at P<0.01.

§§ Significantly different from group 1 at P<0.01.
DISCUSSION

In our present experiment, we evaluated the inhibitory effect of HTHQ or AsA on nitrosamine-induced carcinogenesis by simultaneous administration of NaNO₂ and AP, and also assessed the enhancing effects of combined treatment with HTHQ or AsA and NaNO₂ on major organ carcinogenesis including the forestomach, using a rat medium term multi-organ carcinogenesis model.

As expected, simultaneous administration of NaNO₂ and AP induced hepatocellular adenomas and hemangiosarcomas in the liver. When HTHQ was simultaneously given, these incidences remarkably decreased. Furthermore, the area of GST-P-positive lesions was also reduced. These results indicate that HTHQ inhibits nitrosamine-induced hepatocarcinogenesis induced by NaNO₂ and AP. On the other hand, when AsA was given with NaNO₂ and AP, the inhibitory effect was less potent than that of HTHQ. It was reported that AsA and sodium ascorbate (NaAsA) protected rats against liver tumor production or hepatotoxicity by simultaneous treatment with NaNO₂ and secondary amines such as AP.²²) AsA has been shown to inhibit the genotoxicity of nitrosoamines derived from NaNO₂ and secondary amines such as morpholine or proline in the Comet assay,²⁴) and is suggested to inhibit nitrosamine formation by reducing nitrous anhydride to nitric oxide, a non-nitrosating species, in the absence of catalysts.²⁵) Kuenzig et al.⁵) reported that caffeic acid and ferulic acid blocked the elevation of serum DMN level in rats receiving AP and NaNO₂. Caffeic acid and chlorogenic acid were able to scavenge the stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and inhibit nitrosamine formation by scavenging a nitrosating agent, nitrogen sesquinitoxine.²⁶) HTHQ has potent radical-scavenging activity, in addition to reducing activity, as evaluated using the stable free radical, DPPH²⁷) like caffeic acid and chlorogenic acid.²⁸) Such an effect may be partly responsible for the observed inhibition of hepatocarcinogenesis by HTHQ.

The number and area of GST-P-positive lesions significantly increased when AP alone was given after DMBDD treatment. It was reported that AP does not possess mutagenic or carcinogenic activity in mice.²⁹) Previously, we demonstrated that the number and area of GST-P-positive foci were increased by treatment with AP after initiation with DEN in the rat medium-term liver bioassay system.²⁹) The present results are in line with our previous results and suggest that AP is a weak promoter of hepatocarcinogenesis in rats.

It was shown that kidney and lung tumors were induced by combined oral treatment with AP and NaNO₂ at dose levels 0.1–0.2% AP and 0.1–0.2% NaNO₂.³,⁶) In the present study, however, tumor incidences were not significantly increased in the kidney and lung by the combined treatment with AP and NaNO₂. The reasons for the lack of enhancing effect may be the lower dose levels or strong initiation for these organs in view of the high incidences of tumors in groups without AP and NaNO₂. The incidence of lung carcinomas, but not adenomas, in the AP+NaNO₂+HTHQ group and the AP-alone group significantly decreased as compared with the control group. This was considered to be false-positive due to the high incidence in the control group.

In the upper digestive tracts, incidences of tongue hyperplasias, and forestomach hyperplasias and papillomas were increased in all the HTHQ-treated groups. The results are in line with our previous observations that 0.25% HTHQ weakly enhanced carcinogenesis in the

| Table IV: Incidences of Preneoplastic and Neoplastic Lesions in Non-initiated Groups |
| --- |
| **Group** | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| DMBDD treatment | − | − | − | − | − | − | − | − | − | − |
| Test compounds | AP | AP | AP | NaNO₂ | NaNO₂ | NaNO₂ | NaNO₂ | NaNO₂ | NaNO₂ | NaNO₂ |
| +HTHQ | +HTHQ | +HTHQ | +HTHQ | +HTHQ | +HTHQ | +HTHQ | +HTHQ | +HTHQ | +HTHQ |
| No. of rats examined | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Liver | | | | | | | | | | |
| Foci | 3 (60) | 3 (60) | 3 (60) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Hemangiosarcoma | 0 | 0 | 1 (20) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Forestomach | | | | | | | | | | |
| Hyperplasia | 0 | 5 (100)** | 0 | 5 (100)** | 0 | 0 | 0 | 5 (100)** | 0 | 0 |
| Lung | | | | | | | | | | |
| Alveolar hyperplasia | 4 (80)* | 3 (60) | 5 (100)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Adenocarcinoma | 0 | 1 (20) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* Significantly different from group 20 at $P<0.05$.

** Significantly different from group 20 at $P<0.01$. 

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tongue and forestomach, but not in the esophagus, in a rat medium term multi-organ model and in an N-ethylnitrosoarethene-initiated rat two-stage carcinogenesis model. Furthermore, incidence of forestomach carcinomas was further increased by additional treatment with NaNO₂. Although the data are not shown, the grade of hyperplasias in the HTHQ+NaNO₂ group was greater than that in the HTHQ-alone group in the present experiment. Although the carcinogenicity of these combination treatments was not demonstrated in long-term experiments, the present experiment confirms forestomach carcinogenicity of this combination treatment, because potential for promotion and cell proliferation activities in the forestomach parallels the potential for forestomach carcinogenicity. However, the strong enhancing or possible carcinogenic effect may be limited in the forestomach epithelium, because enhancement of proliferative lesions by the combination was not observed in any other organ, including the esophagus and other squamous cell epithelium.

Previously, we demonstrated that cell proliferation of forestomach mucosa was markedly increased when phenolic antioxidants such as TBHQ, gallic acid, catechol and hydroquinone, and NaNO₂ were simultaneously given to F344 male rat for 4 weeks. Continuous oral administration of catechol or 3-methoxy catechol and NaNO₂ for 24 weeks was demonstrated to increase the incidences of hyperplasias and/or papillomas in the forestomach. When catechol and NaNO₂ are given simultaneously to rats, DNA adducts were detected by 32P-postlabeling methods in the forestomach epithelium. Furthermore, reaction of catechol or phenol and nitrite produced o-benzoquinone and p-nitrosocatechol, and mutagenic diazonium compounds were produced after nitrosation under acidic conditions. Therefore, in simultaneous administration of phenolic compounds, including HTHQ, and NaNO₂, the production of these substances might have played a role in induction of cell proliferation and carcinogenesis.

AsA and NaAsA, at a dose of 1% in diet, have already been shown to promote forestomach carcinogenesis on simultaneous administration with 0.3% NaNO₂ in rats pretreated with MNNG, and they also induced forestomach tumors without prior MNNG treatment. In the present experiment, AsA did not show tumor promotion in any organ, including the forestomach, when administered simultaneously with 0.05% NaNO₂. Although the preventive effect of AsA against liver tumor production by NaNO₂ and AP was weaker than that of HTHQ, 0.25% AsA did not exert any harmful effect in any organ, even in the presence of 0.05% NaNO₂.

Humans ingest large amounts of NaAsA or AsA in foodstuffs such as vegetables and fruits, and the average daily intake is estimated to be 100 mg per person. The daily intake of nitrite is 6 to 10 mg from saliva and 1.5 mg from exogenous sources such as water, vegetables and meats. The intake increases with stimulation of salivary flow-rate, and in vegetarians, intake is several times higher than in non-vegetarians. Furthermore, intake of nitrate is much higher than that of nitrite, and the latter can be formed by reduction of nitrate in vivo. Therefore considerable amounts of nitrite can be formed in the human stomach. However, the amounts of NaAsA and AsA applied in the present experiment are more than 25 times the average exposure in human, and the figure for the amount of NaNO₂ may be more than 50 times. Therefore, low levels of exposure to AsA might not exert harmful effects in any human organs in the presence of low-dose NaNO₂.

In conclusion, HTHQ prevents tumor production induced by AP and NaNO₂ more effectively than AsA. A strong enhancing or possible carcinogenic effect only in the forestomach became clear when HTHQ and NaNO₂ were administered simultaneously. However, considering that humans do not possess a forestomach, HTHQ may be expected to be useful as a chemopreventive agent to inhibit nitrosamine-induced carcinogenesis caused by the administration of NaNO₂ and AP. Low-dose AsA weakly protected against carcinogenesis induced by AP and NaNO₂ without harmful effects in any other organs in the presence of low-dose NaNO₂.

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