

Dose-dependent promotion of rat forestomach carcinogenesis by combined treatment with sodium nitrite and ascorbic acid after initiation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine: Possible contribution of nitric oxide-associated oxidative DNA damage

Kazushi Okazaki,¹ Yuji Ishii,^{1,2} Yasuki Kitamura,¹ Satoshi Maruyama,² Takashi Umemura,¹ Makoto Miyauchi,¹ Megumi Yamagishi,¹ Takayoshi Imazawa,¹ Akiyoshi Nishikawa,¹ Yoshihiro Yoshimura,² Hiroyuki Nakazawa² and Masao Hirose^{1,3}

¹Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-Ku, Tokyo 158-8501, and ²Department of Analytical Chemistry, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan

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Dose-dependent promotion effects of combined treatment with sodium nitrite (NaNO₂) and ascorbic acid (AsA) on gastric carcinogenesis were examined in rats pretreated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Groups of 15 6-week-old F344 male rats were given 0.01% MNNG in their drinking water for 10 weeks to initiate carcinogenesis in the glandular stomach and a single intragastric administration of 100 mg/kg/bodyweight of MNNG by stomach tube at week 9 to initiate carcinogenesis in the forestomach. From week 11, they received either drinking water containing 0.05, 0.1 or 0.2% NaNO₂ and a diet supplemented with 0.1 or 0.2% AsA in combination, each individual chemical alone or a basal diet until the end of week 42. In the forestomach, the incidence of hyperplasia was increased dose dependently by the treatment with NaNO₂ alone. Incidences of neoplastic lesions were dramatically increased by the combined treatment with NaNO₂ and AsA in a dose-dependent manner, but AsA itself had no effect. In the glandular stomach, only toxicity and regenerative changes were increased by the high-dose combination. In a second short-term experiment conducted for sequential observation, necrosis and strong inflammation were found in the forestomach epithelium shortly after commencing combined treatment with 1.0% AsA and 0.2% NaNO₂, followed by hyperplasia, whereas there were no obvious effects in the glandular stomach. In addition, after a 4 h treatment with 1.0% AsA and 0.2% NaNO₂, a slight increase in the 8-hydroxy-deoxyguanosine levels in the forestomach epithelium was observed by high-performance liquid chromatography and an electrochemical detection system, albeit without statistical significance. *In vitro*, electron spin resonance demonstrated nitric oxide formation during incubation with NaNO₂ and AsA under acidic conditions. Thus, NaNO₂ was demonstrated to exert promoter action in the forestomach, with AsA acting as a strong copromoter through cytotoxicity and regenerative cell proliferation, possibly mediated by oxidative DNA damage, but the combined treatment with NaNO₂ and AsA had little influence on glandular stomach carcinogenesis. (*Cancer Sci* 2006; 97: 175–182)

Sodium nitrite is used widely as a food additive to preserve and tinge color-cured meat and fish. We intake nitrate from exogenous sources such as vegetables and water, and this is partly reduced to nitrite *in vivo* by microflora in the buccal cavity.^(1–3) It has been shown that sodium nitrite is a precursor of *N*-nitroso compounds with strong genotoxic and carcinogenic potential,⁽⁴⁾ and an increased incidence of tumors, such as forestomach papillomas and lymphoreticular tumors, has been found in rat carcinogenicity studies of NaNO₂.^(5,6) However, other studies failed to show any effects of nitrite administration on tumor incidence.^(7–9) Previously, we reported that when NaNO₂ was administered simultaneously with phenolic antioxidants, such as catechol, 3-methoxycatechol, hydroquinone, *tert*-butylhydroquinone, gallic acid and pyrogallol, the NaNO₂ induction of forestomach epithelial cell proliferation was augmented.^(10–13) After initiation with a carcinogen, NaNO₂ weakly enhanced forestomach carcinogenesis, and this was further increased by coadministration of antioxidants such as catechol, 3-methoxycatechol, *tert*-butylhydroquinone, α -tocopherol, propyl gallate and 1-*O*-hexyl-2,3,5-trimethylhydroquinone.^(11–15) Without carcinogen pretreatment, 0.2% NaNO₂ and 0.8% catechol in combination for 51 weeks induced squamous cell carcinomas in the rat forestomach.⁽¹²⁾

Ascorbic acid is a well-known vitamin with strong reducing as well as antioxidative activities, which inhibits formation of carcinogenic *N*-nitroso compounds *in vitro* and *in vivo* by blocking the reaction of nitrite and secondary amines.^(16,17) In contrast, similar to phenolic antioxidants, simultaneous administration of 1.0% AsA and 0.3% NaNO₂ has been

³To whom correspondence should be addressed. E-mail: m-hirose@nihs.go.jp
Abbreviations: AsA, ascorbic acid; BrdU, bromodeoxyuridine; DCTS, *N*-(dithiocarboxy)sarcosine; ESR, electron spin resonance; HPLC, high-performance liquid chromatography; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; NaNO₂, sodium nitrite; NO, nitric oxide; 8-OHdG, 8-hydroxy-deoxyguanosine.

reported to enhance forestomach epithelial cell proliferation in rats.⁽¹³⁾ Furthermore, combined treatment with 0.3% NaNO₂ and 1.0% AsA has been shown to promote MNNG-induced rat forestomach, but not glandular stomach, carcinogenesis, and the combination also induced forestomach papillomas without MNNG initiation.⁽¹³⁾ These results suggest that NaNO₂ is a weak promoter and AsA is a strong copromoter of rat forestomach carcinogenesis, and that combined treatment has carcinogenic potential. Although the mechanisms of promotion or carcinogenicity by the combined treatment with NaNO₂ and AsA are not well understood, the fact that AsA reduces nitrite into NO under acidic conditions^(17–20) indicates that NO in the stomach might be an important factor. Whereas at low levels NO acts as a key molecule in assisting in many physiological functions, data showing a contribution to carcinogenesis at high levels are accumulating.⁽²¹⁾

In our previous rat two-stage carcinogenesis model, a single intragastric administration of MNNG was used for the initiation. However, this procedure did not induce appreciable yields of neoplastic lesions in the glandular stomach corresponding to human gastric cancers.⁽¹⁴⁾ Therefore, initiation against glandular stomach may not be sufficient to demonstrate the weak promotion effect by the combination of NaNO₂ and AsA on glandular stomach. Generally, it requires at least 8–10 weeks of MNNG treatment in the drinking water to provide appropriate initiation of glandular stomach neoplasia.^(22,23) Therefore, we improved the initiation procedure by combining 100 mg/mL MNNG in the drinking water for 10 weeks with an intragastric administration of MNNG to investigate effects of NaNO₂ with or without AsA on both glandular stomach and forestomach in rats. In addition, we used a short-term study to examine whether oxidative stress might be involved in enhancement of carcinogenesis, and conducted an *in vitro* study to demonstrate NO formation.

Materials and Methods

Animals and chemicals for the *in vivo* animal studies

Male 5-week-old F344 rats were obtained from Charles River Japan (Atsugi, Japan) and housed five animals per polycarbonate cage under standard laboratory conditions: room temperature, 23 ± 2°C; relative humidity, 60 ± 5%; and a 12:12 h L:D cycle. After a 1-week acclimation period, the animals were used in the experiments. AsA and NaNO₂ were purchased from Wako Pure Chemical Industries (Osaka, Japan) and MNNG from Tokyo Kasei Kogyo (Tokyo, Japan). AsA was mixed with Oriental CRF-1 basal diet (Oriental Yeast, Tokyo, Japan) at various concentrations, replaced once a week, and stored at 4°C in the dark before use. NaNO₂ was dissolved in distilled water at various concentrations and replaced twice a week. Food and tap water were available *ad libitum*. The protocols for these studies were approved by the Animal Care and Utilization Committee for the National Institute of Health Sciences, Japan.

Two-stage carcinogenicity study

In the two-stage carcinogenicity study, as shown in Fig. 1, a total of 180 6-week-old rats were given 0.01% MNNG in drinking water for 10 weeks for the initiation of carcinogenesis in the glandular stomach. At week 9, they were then given 100 mg/kg bodyweight MNNG in vehicle (DMSO:water, 1:1) by stomach tube after 16 h starvation for initiation of carcinogenesis in the forestomach. From week 11, they were divided into 12 groups consisting of 15 animals each and treated as follows: group 1, basal diet; groups 2 and 3, 0.1 and 0.2% AsA in the diet; groups 4–6, 0.05, 0.1 and 0.2% NaNO₂, respectively, in the drinking water; groups 7–9, 0.1% AsA plus 0.05, 0.1 and 0.2% NaNO₂, respectively; and groups 10–12, 1.0% AsA plus 0.05, 0.1 and 0.2% NaNO₂, respectively. Bodyweight, food consumption and water intake were recorded

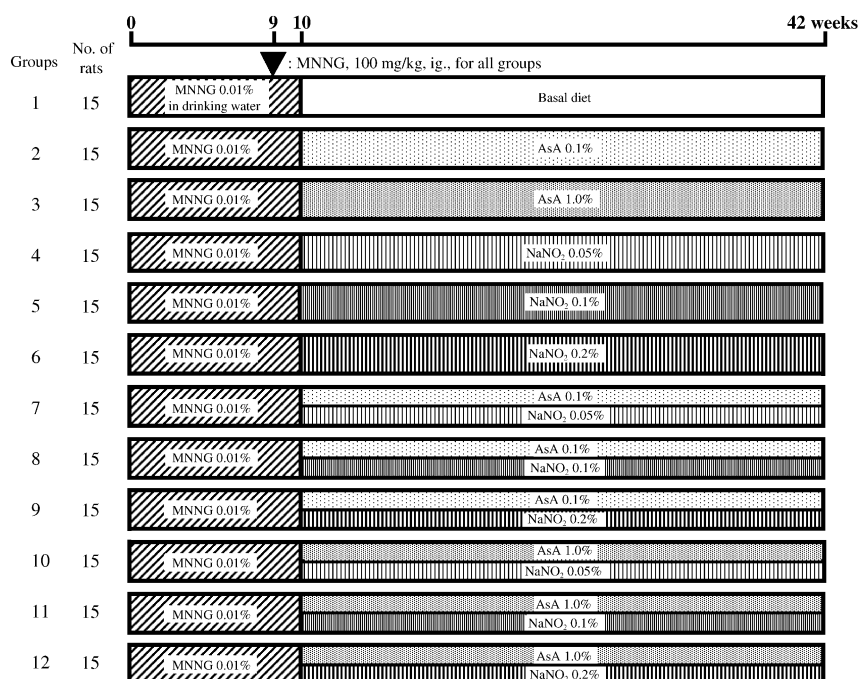


Fig. 1. Experimental design. F344 male rats were initiated with 0.01% *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in the drinking water for 10 weeks for carcinogenesis of the glandular stomach, and 100 mg/kg bodyweight at week 9 for carcinogenesis of the forestomach. From week 11, they received either drinking water containing 0.05, 0.1 or 0.2% sodium nitrite (NaNO₂) and diet supplemented with 0.1 or 0.2% ascorbic acid (AsA) in combination, each individual chemical alone or basal diet until the end of week 42.

at least once every 2–4 weeks and all survivors were killed under ether anesthesia at the end of week 42. The liver, kidneys, esophagus, stomach and intestines were excised, and the liver and kidneys were weighed and fixed in 10% buffered formalin solution. The esophagus, stomach and intestines were inflated with formalin solution and later opened. After fixation, the location and size of tumors were recorded, and six and three sections were cut from the forestomach and glandular stomach, respectively, and stained routinely with hematoxylin and eosin for histopathological assessment.

Short-term toxicity study

In the short-term toxicity study, a total of 36 rats at age 6 weeks were divided into four groups of nine animals each and treated as follows without MNNG initiation: group 1, basal diet; group 2, 1.0% AsA in the diet; group 3, 0.2% NaNO₂ in the drinking water; and group 4, 1.0% AsA plus 0.2% NaNO₂. Sub-groups of three animals each were killed after 1, 3 and 7 days of treatment. The stomachs were excised and treated in the same manner as described above.

Measurement of nuclear 8-OHdG in the forestomach epithelium

For measurement of nuclear 8-OHdG in the forestomach epithelium, a total of 60 6-week-old rats were divided into four groups of 15 animals each and treated as follows: group 1, basal diet; group 2, 1.0% AsA in the diet; group 3, 0.2% NaNO₂ in the drinking water; and group 4, 1.0% AsA plus 0.2% NaNO₂. They were fasted for 16 h before the chemical treatment and killed 4 h thereafter, when obvious histopathological changes were not observed in the forestomach epithelium. From excised stomachs, the forestomach epithelium was collected using a slide glass. Samples were frozen immediately in liquid nitrogen and stored at –80°C until measurement of 8-OHdG in nuclear DNA, according to the method of Nakae *et al.*⁽²⁴⁾ As the amount of forestomach tissue was small, forestomachs from three animals were combined as one sample. Nuclear DNA was extracted with a DNA Extractor WB Kit (Wako Pure Chemical Industries), digested to deoxynucleotides with nuclease P1 and alkaline phosphatase, and the levels of 8-OHdG (8-OHdG/10⁵ deoxyguanosine)

were assessed by HPLC with an electrochemical detection system (Coulouchem II; ESA, Bedford, MA, USA).

Nitric oxide measurement after reaction of NaNO₂ with AsA using ESR

Ascorbic acid, NaNO₂, ferrous sulfate and hydrochloric acid were purchased from Wako Pure Chemical Industries, and DTCS-Na was from Dojindo Laboratory (Kumamoto, Japan). Iron chelate Fe(DTCS)₂ was prepared freshly by mixing a stock solution of ferrous sulfate and DTCS-Na at a molar ratio of 1:5 in phosphate-buffered saline (pH 7.4, 100 mM) prior to the experiment. Fe²⁺ complexed with DTCS-Na was used as an NO-trapping agent and NO formation was determined by detecting the characteristic triplet ESR signal (*g* = 2.04) resulting from the reaction of the Fe(DTCS)₂ complex with NO, as reported previously.^(25,26) The ESR was measured with a JEOL JES-FR30 spectrometer (JEOL, Tokyo, Japan) under the following conditions: magnetic field, 330.7 ± 5 mT; power, 9.0 mW; frequency, 16 GHz; modulation width, 0.063 mT; gain, 500; sweep time, 0.5 min; and time constant, 0.03 s. The reaction procedure was as follows: 100 μL of 1 mM Fe(DTCS)₂ complex was added to pH 1.5 hydrochloric acid, followed by the addition of 100 μL of 50, 100 or 200 mM ascorbic acid, and/or 100 μL of 0.5 or 1 mM NaNO₂, at intervals of 15 s. This mixture was transferred to a quartz flat cell of 200 μL for ESR measurement after mixing for 30 s.

Statistical analysis

The Fisher's exact probability test and Dunnett's *t*-test were used for statistical analysis of the data.

Results

Two-stage carcinogenicity study

Final body and organ weights The final bodyweights were decreased significantly in the 1.0% AsA plus 0.1 or 0.2% NaNO₂ groups (groups 11 and 12) compared with the basal diet group (group 1). The relative kidney weights were generally increased in all groups and liver weights were slightly decreased in the 1.0% AsA plus 0.2% NaNO₂ group (group 12) compared with the basal diet group (group 1) (Table 1).

Table 1. Final body and organ weights

Group	Treatment		No. rats	Final bodyweight (g)	Organ weight (g/100 g bodyweight)	
	MNNG	Chemical			Liver	Kidneys
1	+	Basal diet	15	386 ± 22	2.88 ± 0.10	0.59 ± 0.04
2	+	0.1% AsA	14	408 ± 20 [†]	2.90 ± 0.09	0.64 ± 0.04 ^{††}
3	+	1.0% AsA	15	389 ± 21	2.87 ± 0.11	0.62 ± 0.05
4	+	0.05% NaNO ₂	15	381 ± 21	2.73 ± 0.23	0.63 ± 0.05 [†]
5	+	0.1% NaNO ₂	15	381 ± 20	2.80 ± 0.21	0.66 ± 0.05 ^{††}
6	+	0.2% NaNO ₂	13	372 ± 22	2.77 ± 0.11	0.63 ± 0.03 [†]
7	+	0.1% AsA + 0.05% NaNO ₂	14	385 ± 22 ^{††}	2.89 ± 0.11 [§]	0.64 ± 0.04 ^{††}
8	+	0.1% AsA + 0.1% NaNO ₂	15	389 ± 21 [†]	2.86 ± 0.11	0.65 ± 0.04 ^{††}
9	+	0.1% AsA + 0.2% NaNO ₂	15	372 ± 16 ^{††}	2.85 ± 0.10	0.66 ± 0.04 ^{††}
10	+	1.0% AsA + 0.05% NaNO ₂	15	376 ± 24	2.93 ± 0.09 ^{§§}	0.66 ± 0.03 ^{††,†}
11	+	1.0% AsA + 0.1% NaNO ₂	15	365 ± 21 ^{†,††}	2.88 ± 0.10	0.66 ± 0.03 ^{††,†}
12	+	1.0% AsA + 0.2% NaNO ₂	14	336 ± 18 ^{††,††,§§}	2.77 ± 0.15 [†]	0.69 ± 0.06 ^{††,††,§§}

[†]*P* < 0.05, ^{††}*P* < 0.01 compared to the basal diet group; [†]*P* < 0.05, ^{††}*P* < 0.01 compared to respective ascorbic acid (AsA) only groups; ^{§§}*P* < 0.05, ^{§§§}*P* < 0.01 compared to respective sodium nitrite (NaNO₂) only groups. MNNG, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine.

Table 2. Incidence of neoplastic lesions in the forestomach

Group	Treatment		No. rats	No. rats with (%)				
	MNNG	Chemical		Hyperplasia			Papilloma	SCC
				Mild	Moderate	Severe		
1	+	Basal diet	15	15 (100)	3 (20)	0 (0)	5 (33)	4 (27)
2	+	0.1% AsA	14	14 (100)	6 (43)	0 (0)	2 (14)	2 (14)
3	+	1.0% AsA	15	15 (100)	5 (33)	1 (7)	3 (20)	3 (20)
4	+	0.05% NaNO ₂	15	15 (100)	7 (47)	0 (0)	5 (33)	2 (13)
5	+	0.1% NaNO ₂	15	15 (100)	9 (60) [*]	2 (13)	8 (53)	5 (33)
6	+	0.2% NaNO ₂	13	13 (100)	11 (85) ^{††}	3 (23)	6 (46)	3 (23)
7	+	0.1% AsA + 0.05% NaNO ₂	14	14 (100)	4 (29)	0 (0)	5 (36)	4 (29)
8	+	0.1% AsA + 0.1% NaNO ₂	15	15 (100)	7 (47)	0 (0)	7 (47)	2 (13)
9	+	0.1% AsA + 0.2% NaNO ₂	15	15 (100)	15 (100) ^{††,††}	10 (67) ^{††,††,§}	11 (73) ^{†,††}	7 (47)
10	+	1.0% AsA + 0.05% NaNO ₂	15	15 (100)	13 (87) ^{††,††,§}	2 (13)	13 (87) ^{††,††,§§}	8 (53) [§]
11	+	1.0% AsA + 0.1% NaNO ₂	15	15 (100)	15 (100) ^{††,††,§§}	14 (93) ^{††,††,§§}	14 (93) ^{††,††,§}	9 (60) [‡]
12	+	1.0% AsA + 0.2% NaNO ₂	14	14 (100)	14 (100) ^{††,††}	13 (93) ^{††,††,§§}	14 (100) ^{††,††,§§}	10 (71) ^{†,††,§}

^{*}P < 0.05, ^{††}P < 0.01 compared to the basal diet group; ^{*}P < 0.05, ^{††}P < 0.01 compared to respective ascorbic acid (AsA) only groups; [§]P < 0.05, ^{§§}P < 0.01 compared to respective sodium nitrite (NaNO₂) only groups. MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; SCC, squamous cell carcinoma.

Table 3. Incidence of neoplastic lesions in the glandular stomach

Group	Treatment		No. rats	No. rats with (%) proliferative lesions				No. rats with (%) erosion or regeneration
	MNNG	Chemical		Hyperplasia	Adenoma	Adeno-carcinoma	Leiomyosarcoma	
1	+	Basal diet	15	0 (0)	3 (20)	0 (0)	0 (0)	1 (7)
2	+	0.1% AsA	14	0 (0)	0 (0)	0 (0)	0 (0)	2 (14)
3	+	1.0% AsA	15	0 (0)	0 (0)	0 (0)	0 (0)	3 (20)
4	+	0.05% NaNO ₂	15	0 (0)	0 (0)	0 (0)	1 (7)	4 (27)
5	+	0.1% NaNO ₂	15	1 (7)	0 (0)	1 (7)	0 (0)	4 (27)
6	+	0.2% NaNO ₂	13	1 (8)	0 (0)	1 (8)	0 (0)	2 (15)
7	+	0.1% AsA + 0.05% NaNO ₂	14	1 (7)	0 (0)	0 (0)	0 (0)	3 (21)
8	+	0.1% AsA + 0.1% NaNO ₂	15	0 (0)	2 (13)	0 (0)	0 (0)	2 (13)
9	+	0.1% AsA + 0.2% NaNO ₂	15	0 (0)	0 (0)	0 (0)	0 (0)	7 (47) [†]
10	+	1.0% AsA + 0.05% NaNO ₂	15	0 (0)	0 (0)	1 (7)	0 (0)	8 (53) ^{††}
11	+	1.0% AsA + 0.1% NaNO ₂	15	0 (0)	0 (0)	0 (0)	0 (0)	12 (80) ^{††,††,§§}
12	+	1.0% AsA + 0.2% NaNO ₂	14	1 (7)	0 (0)	1 (7)	0 (0)	9 (64) ^{††,†,§}

^{*}P < 0.05, ^{††}P < 0.01 compared to the basal diet group; ^{*}P < 0.05, ^{††}P < 0.01 compared to respective ascorbic acid (AsA) only groups; [§]P < 0.05, ^{§§}P < 0.01 compared to respective sodium nitrite (NaNO₂) only groups. MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.

Forestomach findings Grossly, a few small tumors were observed in the forestomach epithelium of animals treated with MNNG alone (group 1) and MNNG followed by 0.1 or 1.0% AsA (groups 2 and 3). However, the forestomachs of animals treated with MNNG followed by 0.1 or 1.0% AsA plus 0.2% NaNO₂ (groups 9 and 12) and 1.0% AsA plus 0.1% NaNO₂ (group 11) were thickened diffusely and filled with various-sized masses. Microscopically, the lesions observed in the forestomach were characterized as hyperplasias, papillomas and squamous cell carcinomas. Hyperplasias were further classified into mild, moderate and severe types as described previously.⁽¹¹⁾ The incidences of papillomas and carcinomas were increased dramatically in a dose-dependent manner in the AsA plus NaNO₂ groups, particularly with 1.0% AsA plus 0.1% NaNO₂ (group 11) and 1.0% AsA plus 0.2% NaNO₂ (group 12), compared with the basal diet (group 1), AsA (groups 2 and 3) and NaNO₂ (groups 4–6) cases. In the 0.1 and 0.2% NaNO₂ alone groups (groups 5 and 6), the grades of hyperplasias were much more severe than in the basal diet group (group 1). This was not the case with the 0.1 and 1.0% AsA alone groups (groups 2 and 3) (Table 2).

Glandular stomach findings Grossly, only few animals had macroscopic tumors in the glandular stomach. Microscopically, the majority of the lesions observed in the glandular stomach were characterized as hyperplasias, adenomas, adenocarcinomas and others, with no significant differences in the incidences of these lesions among MNNG-initiated groups (Table 3). In addition to the neoplastic lesions, erosion or regeneration were observed with high frequency especially with 1.0% AsA plus 0.05, 0.1 and 0.2% NaNO₂ and 0.1% AsA plus 0.2% NaNO₂ (Groups 9, 10, 11 and 12).

Regarding other organs, only one animal given 0.2% NaNO₂ plus 1.0% AsA had a duodenal tumor. No proliferative lesions were observed in the esophagus.

Short-term toxicity study

Microscopically, the forestomachs of rats treated with 1.0% AsA plus 0.2% NaNO₂ exhibited necrosis or erosion of the squamous epithelium accompanied by strong inflammatory changes with edema in the submucosa. These changes gradually became stronger from days 1–7 of treatment, and

Table 4. Histopathological findings for the epithelium of the forestomach in the short-term study

Group	No. rats/day	Necrosis or erosion			Ulcer			Hyperplasia		
		Day 1	Day 3	Day 7	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
Basal diet	3	-	-	-	-	-	-	-	-	-
1.0% AsA	3	-	-	-	-	-	-	-	-	-
0.2% NaNO ₂	3	-	-	-	-	-	-	-	-	-
1.0% AsA + 0.2% NaNO ₂	3	++	++	++	-	+	++	-	-	+

+, moderate; ++, severe; AsA, ascorbic acid; NaNO₂, sodium nitrite.

Table 5. Histopathological findings for the submucosa of the forestomach in the short-term study

Group	No. rats/day	Edema			Inflammatory cell infiltration			Granulation		
		Day 1	Day 3	Day 7	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
Basal diet	3	-	-	-	-	-	-	-	-	-
1.0% AsA	3	-	-	-	-	-	-	-	-	-
0.2% NaNO ₂	3	-	-	-	-	-	-	-	-	-
1.0% AsA + 0.2% NaNO ₂	3	+	++	++	±	+	++	-	-	+

±, mild; +, moderate; ++, severe; AsA, ascorbic acid; NaNO₂, sodium nitrite.

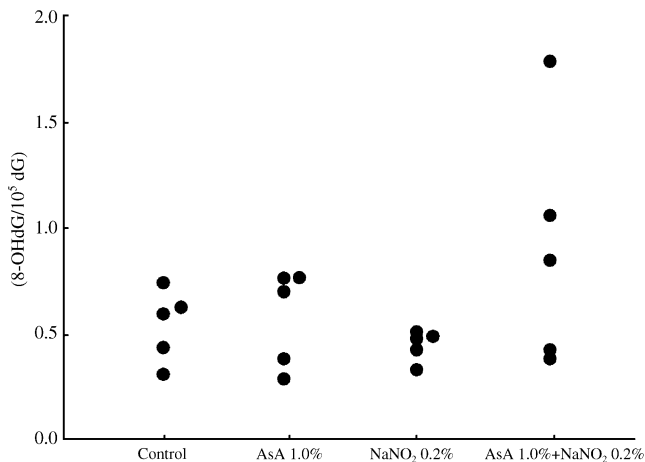


Fig. 2. 8-Hydroxy-deoxyguanosine (8-OHdG) levels in the rat forestomach epithelium 4 h after administration of 1.0% ascorbic acid (AsA) and/or 0.2% sodium nitrite (NaNO₂). Two samples given 1.0% AsA and 0.2% NaNO₂ demonstrated levels higher than in the controls. However, overall values were without statistical significance. Levels of 8-OHdG with AsA or NaNO₂ alone were comparable to control values.

regenerative hyperplasia eventually developed on day 7 (Tables 4, 5). In the 1.0% AsA and 0.2% NaNO₂ alone groups, there were no apparent changes. In the glandular stomach, toxicity as well as proliferative changes were not apparent in any of the groups.

Measurement of nuclear 8-OHdG in the forestomach epithelium

Data for 8-OHdG levels in the rat forestomach given 1.0% AsA and/or 0.2% NaNO₂ for 4 h are summarized in Fig. 2. At this stage, no apparent gross or microscopic changes were observed in the forestomach epithelium. Values for two samples given AsA and NaNO₂ simultaneously were higher than the range of control values, but the mean (mean ± SD,

0.89 ± 0.57/10⁵ dG) was not significantly different from the control value (0.54 ± 0.17/10⁵ dG). Values for the AsA (0.57 ± 0.23/10⁵ dG) and NaNO₂ (0.44 ± 0.07/10⁵ dG) alone groups also were not significantly different from the control value.

Nitric oxide measurement by the reaction of NaNO₂ and AsA using ESR

Figure 3a illustrates a typical ESR signal indicating NO formation. ESR signals were not observed after treatment with 0.05 or 0.1 mM NaNO₂, or with 5, 10 or 20 mM AsA alone under acidic conditions at pH 1.5 (Fig. 3b,c). However, formation of NO was clear after adding 5–20 mM AsA to 0.1 mM NaNO₂, the signal height being dependent on the AsA concentration (Fig. 3d). As shown in Fig. 4, the amount of NO generated also depended on the AsA and NaNO₂ doses.

Discussion

It has been reported that oral administration of 0.3% NaNO₂ in the drinking water over 1 year causes a significant increase in the incidence of forestomach papillomas in Wistar rats.⁽⁶⁾ We also reported that treatment with 0.3% NaNO₂ in drinking water for 51 weeks induces hyperplasia with a slight increase in the BrdU labeling index in the forestomach epithelium of F344 rats.⁽¹³⁾ Moreover, when 0.3% NaNO₂ was administered in drinking water after MNNG initiation, forestomach carcinogenesis was clearly enhanced in F344 rats.⁽¹³⁾ Our present data, showing that treatment with 0.1 or 0.2% NaNO₂ (but not 0.05% NaNO₂) increases hyperplastic lesions, are in agreement with the previous results. Therefore, it is suggested that NaNO₂ enhances forestomach tumorigenesis by increasing epithelial cell proliferation, depending on the dose. With combinations of NaNO₂ and 0.1 or 1.0% AsA, proliferative changes were further enhanced, again with a clear dose dependence. As AsA itself did not induce any proliferative lesions or enhance forestomach carcinogenesis after initiation,⁽¹³⁾ it can be concluded that NaNO₂ is a weak

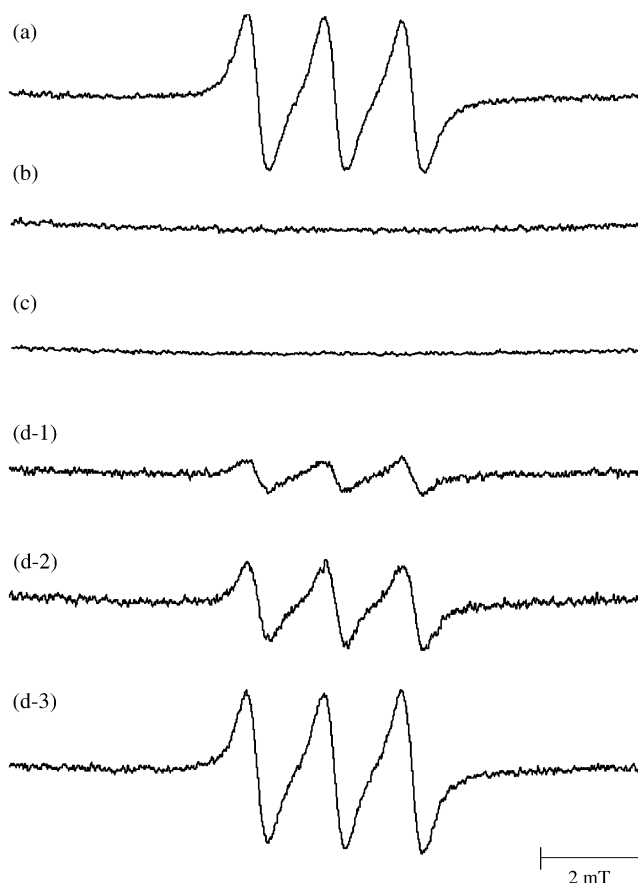


Fig. 3. Electron spin resonance (ESR) spectra for nitric oxide (NO)-Fe (*N*-[dithiocarboxy]sarcosine)₂ (DTCS). (a) NO-Fe (DTCS)₂ standard, (b) 20 mM ascorbic acid (AsA) + HCl (pH 1.5), (c) 0.1 mM sodium nitrite (NaNO₂) + HCl (pH 1.5), and (d) 5, 10 and 20 mM AsA + 0.1 mM NaNO₂ + HCl (pH 1.5). ESR signals were not observed during treatment with NaNO₂ or AsA alone under acidic conditions at pH 1.5, but formation of NO was apparent upon the addition AsA to NaNO₂, the signal height being dependent on the AsA concentration.

promoter of forestomach carcinogenesis and that AsA is a strong copromoter of forestomach carcinogenesis in the presence of NaNO₂. Combined treatment with 0.3% NaNO₂ in the drinking water and 1.0% AsA or sodium AsA in the diet for 51 weeks was earlier shown to induce forestomach papillomas in 20 and 53%, respectively, of animals without prior initiation treatment (accompanied by an increase in the BrdU labeling index), indicating carcinogenic potential with these combinations.⁽¹³⁾

Regarding the mechanisms underlying strong promotion of rat forestomach carcinogenesis by the combined treatment with NaNO₂ and AsA, erosion and/or necrosis of the forestomach squamous epithelium and strong inflammatory changes in the submucosa followed by regenerative proliferation of the epithelium might be crucial factors. It is well known that promotion activity and cell proliferation activity, as a result of early stage cytotoxicity, are well correlated with the enhancement of forestomach carcinogenesis, as are several compounds such as butylated hydroxyanisole, catechol and caffeic acid.^(11,27) The observed cytotoxicity followed by

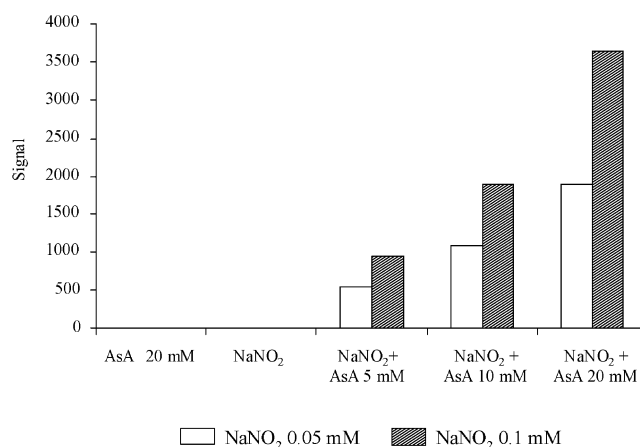


Fig. 4. Nitric oxide (NO) formation by the reaction of sodium nitrite (NaNO₂) with ascorbic acid (AsA) in HCl (pH 1.5). The amount of NO generated increased depending on both the AsA and NaNO₂ doses. NO was not generated by treatment with NaNO₂ or AsA alone under the same conditions.

cell proliferation in the present experiment thus provides an explanation for the initial step of the strong promotion of carcinogenesis by the combination of NaNO₂ and AsA. The etiology of the cytotoxicity remains uncertain, but the fact that AsA has the potential to reduce nitrite into NO under acidic conditions,⁽¹⁷⁻²⁰⁾ and the present *in vitro* finding of NO generation by the reaction of NaNO₂ and AsA at pH 1.5, point to a role for oxidative stress. It is generally accepted that NO reacts with superoxide to form peroxynitrite, which can oxidize a variety of biomolecules either by its direct reactions or via secondary radicals such as nitrogen dioxide and carbonate or hydroxyl radicals.⁽²⁸⁻³⁰⁾ Considering that NO and peroxynitrite promote tissue damage by free radical-mediated cytotoxicity,⁽³¹⁾ the production of NO resulting from the mixture of NaNO₂ with AsA might play a key role in cell injury. Furthermore, we speculate that chronic stimulation consequently causes activation of a variety of inflammatory cells, which induce various oxidant-generating enzymes such as NADPH oxidase and inducible NO synthase, subsequently generating high concentrations of diverse free radicals and oxidants in the forestomach.⁽³²⁾ Our present data, indicating a slight increase of 8-OHdG (albeit without statistical significance) in DNA of the forestomach of rats 4 h after cotreatment with NaNO₂ and AsA, suggest sustained oxidative stress even in the long term. Formation of oxidized bases also implies alteration of the intranuclear redox status, leading to the induction of cell cycle-dependent transcriptional events.^(33,34) Therefore, it is highly probable that the enhancement of tumor promotion observed in rats treated with the combination of NaNO₂ and AsA involves oxidative stress attributable to NO formation. However, we could not demonstrate immunohistochemical positivity for nitrotyrosine in the forestomach epithelium of these rats, probably due to a technical problem. We are now developing a new method for detecting nitroguanine using HPLC. In the glandular stomach, although ulceration and regenerative hyperplasia were not observed, erosion or regeneration was more frequent in animals given both NaNO₂ and AsA than with either

chemical alone. We can speculate that the same mechanisms are operating as in the forestomach. However, we could not detect any promotion effects of NaNO₂, with or without AsA, on glandular stomach tumor development under the present experimental conditions, despite effective initiation. The difference in the extent of injury between forestomach and glandular stomach may be due to differences in protective factors such as mucus formation, epithelial thickness and the concentrations of antioxidant enzymes (such as glutathione S-transferase and quinone reductase), which are active against external stress.^(35,36) In a rat multiorgan carcinogenesis model, simultaneous administration of 0.05% NaNO₂ and 0.25% AsA in the promotion stage demonstrated no apparent enhancement of carcinogenesis in any organs.⁽¹⁵⁾ Therefore, it can be concluded that the glandular stomach, which resembles the human stomach both morphologically and functionally, and other major organs, are not the targets of combined treatment with NaNO₂ and AsA, particularly at low dose levels.

Humans consume large amounts of AsA in foods such as vegetables and fruits, and daily intake is estimated to be approximately 50–200 mg per person.^(37,38) Some ascorbic acid derivatives, such as ascorbyl palmitate and ascorbyl stearate, have furthermore found application as food additives, and AsA and sodium AsA have been used for medical use and dietary supplements.^(39–41) AsA was shown to inhibit formation of endogenous *N*-nitroso compounds, which contribute to the etiology of human cancers.^(16,17) Treatment with 0.25% AsA inhibited combined 0.05% aminopyrene and 0.05% NaNO₂-induced rat hepatocarcinogenesis, but this dose level of AsA and NaNO₂ did not induce any forestomach lesions.⁽¹⁵⁾ However, combinations of higher doses of AsA and NaNO₂ were shown to promote forestomach carcinogenesis and induce forestomach tumors,⁽¹³⁾ as in the present study. NaNO₂ is also widely applied as a food addi-

tive and can be generated by a reduction in oral microflora of the entero-salivary recirculated dietary nitrate from meats, vegetables and tap water.^(1–3) The estimated daily intakes of NaNO₂ and sodium nitrate are approximately 0.1–10 and 30–400 mg per person, respectively.⁽²⁾ Furthermore, vegetarian intake of nitrite is several times higher than in non-vegetarians. However, the intakes of AsA and nitrite in the present experiment are estimated to be approximately 20–200^(39–41) and 5–30 times greater, respectively, than those in the human situation. Therefore, combined exposure of nitrite and AsA may not be a risk factor, at least in healthy people. Recently, a possible contribution of NO to the development of human adenocarcinomas in the gastro-esophageal junction in patients with reflux esophagitis has been suggested. A benchtop model indicated that when nitrite was added to the acidic luminal compartment containing AsA, a high concentration of NO was generated, and it rapidly diffused into the epithelial compartment. It could be that the combined exposure to low doses of AsA and NaNO₂ might enhance tumor induction in the junction through enhancement of NO production, and experiments to assess this possibility are now in progress in our laboratory.

In conclusion, the present study demonstrates that combined treatment with NaNO₂ and AsA causes initial toxicities, such as necrosis and inflammation, followed by regenerative hyperplasia, and such events enhance carcinogenesis in the rat forestomach, but not the glandular stomach. NO-associated oxidative stress may contribute to the observed toxicity and enhancement of forestomach carcinogenesis.

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