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Mutagenicity of Combined Treatment with Sodium Nitrite and Ascorbic Acid in Bacterial and Mammalian Cells

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Simultaneous administration of sodium nitrite ($NaNO_2$) and ascorbic acid (AsA) induces weak oxidative DNA damage in forestomach epithelium and enhances forestomach carcinogenesis in F344 rats. To investigate the mutagenicity of the combination of $NaNO_2$ and AsA, we conducted reverse mutation assays in *E. coli* WP2*uvrA*/pKM101 and cytogenetic assays in cultured Chinese hamster lung CHL/IU cells without a metabolic activation system. When WP2*uvrA*/pKM101 was preincubated with a combination of 78.1–5000 μg/plate $NaNO_2$ and 5 mg/plate AsA in standard buffer (pH 7.4), the number of revertants slightly increased compared to treatment with $NaNO_2$ alone. Performing the same experiment using pH 6.0 buffer, in which the buffer decreased to about pH 4.9 due to the acidity of AsA, demonstrated that the number of revertants markedly increased. Additional experiments showed that the mutagenicity of $NaNO_2$ itself markedly increased at pH 5.0–5.5. These results suggest that the enhancement of the mutagenic activity of $NaNO_2$ in pH 6.0 buffer is likely due to a pH-lowering effect of AsA. In the cytogenetic assays, no substantial increase in chromosomally aberrant cells was observed in cultures treated with $NaNO_2$ or AsA alone for 3 h, but combined treatment with 5 mg/mL $NaNO_2$ and 1.5–2.5 mg/mL AsA significantly increased chromosome aberrations. We concluded that simultaneous treatment with $NaNO_2$ and AsA at high doses induced mutagenic or clastogenic damage to bacterial and mammalian cells and that such genotoxic damage probably contributed to forestomach carcinogenesis in rats treated with combined $NaNO_2$ and AsA.

Key words: sodium nitrite, ascorbic acid, combined treatment, reverse mutation assay, cytogenetic assay

Introduction

Nitrate can be found mostly in drinking water and vegetables. A part of the ingested nitrate is converted to nitrite by microbial reduction in the buccal cavity (1,2). Other ingested sources of nitrite are meat and fish that are treated with sodium nitrite ($NaNO_2$) as a food additive to improve their color and to prevent the growth of botulinum. $NaNO_2$ is mutagenic in bacteria such as *Salmonella typhimurium* TA1530, TA1535 and TA100, but inactive in frameshift-sensitive strains (3). $NaNO_2$ also has a clastogenic potential in cultured mammalian cells (4-6). In a two-year study of drinking water, there was no evidence that $NaNO_2$ had carcinogenic activity in F344/N rats or B6C3F1 mice, except that exposure to $NaNO_2$ resulted in increased incidence of epithelial hyperplasia in the forestomach of male and female rats and in the glandular stomach of male mice (7). It is well known that nitrite reacts with secondary amines in food under acidic conditions to produce mutagenic and carcinogenic *N*-nitroso compounds (8). Therefore, nitrite is classified as Group 2A (probably carcinogenic to humans) by the International Agency for Research on Cancer (IARC).

Ascorbic acid (AsA) is a known antioxidant, which has a potential to reduce chemicals. Although there are some reports that AsA is weakly mutagenic (9,10), it is in general regarded as a non-mutagenic substance (11). The formation of *N*-nitroso compounds can be inhibited by AsA (12–15), which means that AsA has antimutagenic or anticarcinogenic activity. Many studies have demonstrated the protective effects of AsA against mutagenic or carcinogenic damage induced by various mutagens *in vitro* and *in vivo* (16,17). However, Yoshida *et al.* (18) found unexpected effects of AsA in the long-term animal experiments when it was administered in combination with $NaNO_2$. When F344 male rats were treated with a combination of 0.3% $NaNO_2$ in their drinking water and 1.0% AsA in their diet for 51 weeks after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) initiation, forestomach carcinogenesis was strongly enhanced. Even in the rats without MNNG initiation, papilloma and severe hyperplasia were detected in 20% of them, indicating the carcinogenic potential of this

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combination; notably, papilloma was not detected in rats treated with $NaNO_2$ or AsA alone. In the early stage of carcinogenesis by the combination treatment, necrosis and inflammation as well as weak induction of 8-hydroxydeoxyguanosine (8-OHdG) were observed in the rat forestomach epithelium (19). This result allowed us to consider that the combination of $NaNO_2$ and AsA indirectly caused oxidative stress in the epithelium cells.

The aim of this study is to investigate the mutagenicity of the combination of $NaNO_2$ and AsA in the bacterial reverse mutation assays and *in vitro* cytogenetic assays. The World Health Organization (WHO) has been recommended the Nitrosation Assay Procedure (NAP test) to determine the nitrosatability of amine-containing drugs in intragastric conditions (20). In this test, following incubation of the drug with $NaNO_2$ in dilute acetic acid (pH 3.6), a sample of the reaction mixture is neutralized with sodium bicarbonate and then applied to the reverse mutation assay with *S. typhimurium* tester strain. Although the NAP test is certainly useful for detecting formation of mutagenic *N*-nitroso compounds, it is invalid for detecting unstable mutagenic products such as reactive oxygen species. In the present study, a bacterial strain and mammalian cells were directly exposed to $NaNO_2$ with or without AsA under weakly acidic conditions.

Materials and Methods

Chemicals: Sodium nitrite ($NaNO_2$) (CAS No. 7632–00–0, 98.5% purity) and L-ascorbic acid (AsA) (CAS No. 50–81–7, 99.5% purity) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Both chemicals were dissolved in physiological saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) immediately before use.

Bacterial strain and mammalian cells: *Escherichia coli* WP2*uvrA*/pKM101 (*trpE65*, *uvrA*, pKM101), which has an ability to detect oxidative mutagens (21), was used for the reverse mutation assays. The strain was cultured in 2.5% nutrient broth (Oxoid nutrient broth No. 2, Oxoid Ltd., Hampshire, UK) at 37°C with shaking. Chinese hamster CHL/IU cells (22) were used for the cytogenetic assays. They were grown in Eagle's MEM (GIBCO®, Invitrogen Corp., CA, USA) supplemented with 10% newborn calf serum (GIBCO®), 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5 % CO_2 at 37°C. The growth medium contained phenol red as a pH indicator. The doubling time of the cells was 15–16 h under these conditions.

Reverse mutation assay: The bacterial reverse mutation assays were conducted using a preincubation method (23). Aliquots of an overnight culture (0.1 mL) of the tester strain plus 0.05 or 0.1 mL of chemical solution(s) were added to 0.5 mL of 100 mM phosphate buffer (pH 7.4) or 100 mM acetate buffer (pH 5.0, 5.5, 6.0 or 7.0). After preincubation at 37°C for 20 min with shaking, 2 mL of molten top agar (0.6% agar and 0.5% NaCl) containing 0.05 mM L-tryptophan was added and overlaid on minimal glucose agar plates (AM-N plates, Oriental Yeast Co., Ltd., Tokyo, Japan). Trp^+ revertant colonies were counted after incubation at 37 °C for 2 days. Triplicate plates were used for each dose of the treatment. The assays were conducted in the absence of a metabolic activation system because the mutagenic activities of both $NaNO_2$ and AsA require no metabolic activation (3,9).

Cytogenetic assay: Exponentially growing cells were treated with several concentrations of $NaNO_2$ or AsA alone, or were simultaneously treated with a combination of $NaNO_2$ and AsA in the absence of a metabolic activation system. The pH of the medium of the cultures treated with AsA was measured immediately after addition. Three hours after the beginning of the treatment, all cultures were rinsed twice with PBS and cultured for a further 21 h in fresh medium. Colcemid® (Wako) was added to the cultures at a final concentration of 0.2 μg/mL 2 h prior to harvest to collect metaphase cells. A conventional air-drying method was used for chromosome preparations. Cells were detached with 0.25% trypsin and swollen with a hypotonic solution of 0.075 M KCl for approximately 10 min at room temperature. The cells were then fixed in methanol:acetic acid (3:1) and air-dried on glass slides. Chromosome preparations were all coded and stained with 2% Giemsa solution (Merck Ltd., Darmstadt, Germany) for 15 min at room temperature. For chromosomal aberration analysis, 200 well-spread metaphase cells per concentration were examined at 1000-fold magnification using a light microscope. The mitotic index was determined by counting 1000 cells for each concentration. The data were analyzed statistically using the chi-square test to compare each sample to the corresponding solvent control. A p-value of less than 0.05 was considered statistically significant.

Results

Reverse mutation assay: We examined the bacterial mutagenic activity of 78.1–5000 μg/plate $NaNO_2$ alone or in combination with 5 mg/plate AsA on WP2*uvrA*/pKM101 using pH 7.4 standard phosphate buffer (Fig. 1A). The number of Trp^+ revertants increased with the dose of $NaNO_2$ regardless of the presence of AsA, however, the combined treatment induced slightly more revertants than treatment with $NaNO_2$ alone. The treatment with 5 mg/plate AsA alone increased the number of revertants 1.7 times greater than the negative control, which indicated that AsA itself had a weakly mutagenic activity. We measured the pH of the reaction mixture (bacteria, chemicals

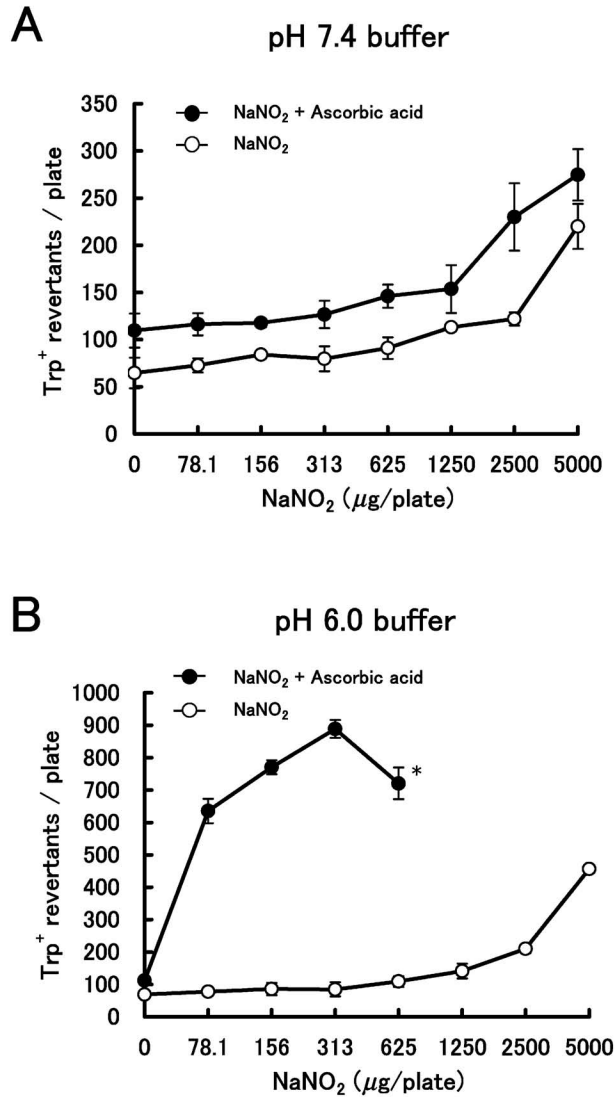

Fig. 1. Bacterial mutagenicity of the combined treatment with $NaNO_2$ and ascorbic acid (AsA) in pH 7.4 (A) and pH 6.0 (B) buffer solutions. WP2*uvrA*/pKM101 was treated simultaneously with $NaNO_2$ at the indicated concentration plus 5 mg/plate AsA. The asterisk indicates cytotoxicity. The error bars indicate standard deviation.

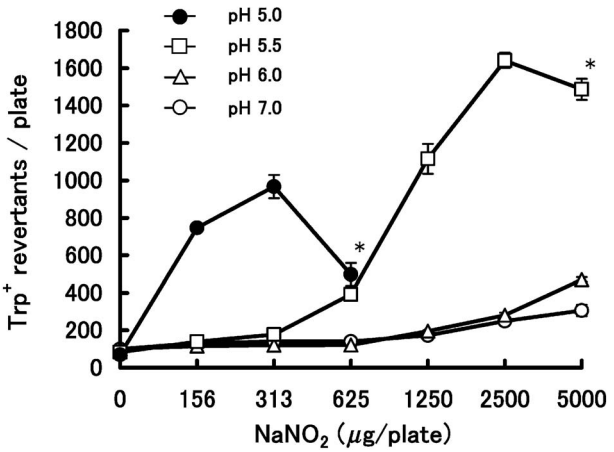

Fig. 2. The effect of buffer pH on the mutagenicity of $NaNO_2$. WP2*uvrA*/pKM101 was treated with $NaNO_2$ at the indicated concentration in pH 5.0-7.0 buffer. The asterisks indicate cytotoxicity. The error bars indicate standard deviation.

and buffer) during the preincubation period. The pH of cultures treated simultaneously with $NaNO_2$ and AsA decreased to about 6.4 due to the acidity of the AsA.

Next, we conducted the same experiment in pH 6.0 acetate buffer. The number of revertants increased markedly after treatment with a combination of $NaNO_2$ and AsA, with reduction of the background lawn at 625 μg/plate and severe cytotoxicity at 1250–5000 μg/plate $NaNO_2$ (Fig. 1B). The pH of reaction mixtures treated simultaneously with $NaNO_2$ and AsA decreased to about 4.9 whenever $NaNO_2$ was present at any dose during the preincubation period. Furthermore, we performed the same experiment using pH 5.0 acetate buffer, but the pH decreased to less than 4.0 in combination with AsA, and this decrease was lethal to the bacteria.

To examine the effect of pH on the mutagenicity of $NaNO_2$, an additional reverse mutation assay was carried out using pH 5.0–7.0 acetate buffer. The number of revertants dramatically increased as the pH of the buffer decreased from 6.0 to 5.0 (Fig. 2). The dose-response curve of combined treatment with $NaNO_2$ and AsA in pH 6.0 buffer (actual pH was 4.9) was almost identical to that of $NaNO_2$ alone in pH 5.0 buffer (Figs. 1B and 2).

Cytogenetic assay: A long-term treatment with $NaNO_2$ for 24 or 48 h induces chromosome aberrations in cultured mammalian cells such as CHL/IU and mouse FM3A cells (4–6). However, in the present study, no significant increase in the number of cells with chromosome aberrations was observed after a short-term treatment with $NaNO_2$ for 3 h (Table 1). The clastogenic activity of AsA is still inconclusive, as it has been reported to be both positive (24) and negative (25). In our assays, there was no significant increase in aberrant cells at 0.313–2.5 mg/mL, having a decrease in the pH of the medium at all AsA concentrations and severe cytotoxic damage at 5.0 mg/mL (Table 1).

In contrast, simultaneous treatment with a combination of $NaNO_2$ and AsA for 3 h resulted in a significant increase in the number of the aberrant cells at a concentration of 5.0 mg/mL $NaNO_2$ with 1.5, 2.0 and 2.5 mg/mL AsA (Table 2). At a lower concentration of $NaNO_2$, 0.5 mg/mL, a small increase in the incidence of aberrant cells was also observed with 1.5 and 2.5 mg/mL AsA (Table 2). The pH of the medium decreased to 6.1–6.3 in combination with 2.0 and 2.5 mg/mL AsA immediately after the addition of the AsA, but 30 min later it returned to nearly neutral pH in the

Table 1. No induction of chromosome aberrations in Chinese hamster CHL/IU cells treated with $NaNO_2$ or ascorbic acid alone

Treatment	Concentration (mg/mL)	No. of cells scored	Polyploid cells	No. of cells with structural chromosome aberrations									Mitotic index (%)	Relative mitotic index (%)	pH*
					Chromatid type		Chromosome type				Total				
				gap	ctb	cte	csb	cse	frg	others	+g	−g			
Physiological saline	1% (control)	200	0 (0)	3	3	1	0	0	0	0	7 (3.5)	4 (2.0)	6.8	100	8.1
$NaNO_2$	0.625	200	1 (0.5)	4	0	1	0	1	0	0	6 (3.0)	2 (1.0)	6.3	93	—
	1.25	200	2 (1.0)	2	1	0	2	2	0	0	7 (3.5)	5 (2.5)	5.6	82	—
	2.5	200	2 (1.0)	0	1	2	0	1	0	0	4 (2.0)	4 (2.0)	6.4	94	—
	5.0	200	2 (1.0)	0	0	8	3	0	0	0	11 (5.5)	11 (5.5)	5.6	82	—
Ascorbic acid	0.313	200	1 (0.5)	1	2	0	1	0	0	0	4 (2.0)	3 (1.5)	5.1	75	7.7
	0.625	200	0 (0)	4	0	2	0	1	0	0	7 (3.5)	3 (1.5)	5.5	81	7.5
	1.25	200	2 (1.0)	1	0	0	0	0	0	0	1 (0.5)	0 (0)	4.7	69	7.0
	2.5	200	3 (1.5)	0	3	4	1	0	0	0	8 (4.0)	8 (4.0)	4.8	71	6.4
	5.0	Toxic	—	—	—	—	—	—	—	—	—	—	—	—	4.9

ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; frg, fragmentations; others; multiple aberrations; +g, including gaps; −g, excluding gaps
The figures shown in parentheses are percentages.
*: pH of the culture medium immediately after treatment

Table 2. Induction of chromosome aberrations in Chinese hamster CHL/IU cells treated with a combination of $NaNO_2$ and ascorbic acid

Combined treatment		No. of cells scored	Polyploid cells	No. of cells with structural chromosome aberrations									Mitotic index (%)	Relative mitotic index (%)	pH*
$NaNO_2$ (mg/mL)	Ascorbic acid (mg/mL)				Chromatid type		Chromosome type				Total				
				gap	ctb	cte	csb	cse	frg	others	+g	−g			
0.5	0 (control)	200	1 (0.5)	2	2	0	2	1	0	0	7 (3.5)	5 (2.5)	7.2	100	8.0
	1.0	200	0 (0)	2	1	8	1	1	0	0	10 (5.0)	9 (4.5)	6.4	89	7.0
	1.5	200	1 (0.5)	4	4	8	6	2	1	0	18 (9.0)	15 (7.5)†	4.7	65	6.7
	2.0	200	1 (0.5)	4	2	7	2	1	0	1	14 (7.0)	11 (5.5)	4.8	67	6.3
	2.5	200	1 (0.5)	1	6	10	1	2	0	0	16 (8.0)	15 (7.5)†	5.0	69	6.2
5.0	0 (control)	200	1 (0.5)	2	3	5	2	1	0	0	11 (5.5)	10 (5.0)	6.7	100	8.3
	1.0	200	2 (1.0)	5	5	3	0	0	0	0	10 (5.0)	5 (2.5)	5.4	81	7.0
	1.5	200	3 (1.5)	10	8	21	6	1	0	3	39 (19.5)	33 (16.5)‡	5.1	76	6.8
	2.0	200	6 (3.0)	12	19	28	2	0	1	2	46 (23.0)	39 (19.5)‡	5.2	78	6.1
	2.5	200	1 (0.5)	13	25	38	6	2	1	1	66 (33.0)	57 (28.5)‡	2.3	34	6.1

ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; frg, fragmentations; others; multiple aberrations; +g, including gaps; −g, excluding gaps
The figures shown in parentheses are percentages.
*: pH of the culture medium immediately after treatment
†, ‡: Significantly different from the control at $p<0.05$ and $p<0.001$, respectively.

CO_2 incubator.

Discussion

The cytogenetic assays demonstrated that the incidences of metaphase cells with structural chromosome aberrations were significantly higher after treatment with $NaNO_2$ and AsA at pH 6.7 and 6.8 (Table 2). In such nearly neutral conditions, nitrite is largely stable but susceptible to reducing agents. On the other hand, AsA is unstable in the neutral pH and undergoes oxidization. Therefore, nitrite reacts with AsA generating nitric oxide (NO) and dehydroascorbic acid in neutral solution (26–29). There has been no evidence that dehydroascorbic acid is mutagenic, and hence NO was considered to contribute to the induction of chromosome aberrations in neutral conditions. NO is a free rad-

ical and reacts with superoxide to form peroxynitrite ($ONOO^-$), which can oxidize a variety of biomolecules either directly or *via* secondary radicals such as hydroxyl radicals (30–32). We propose that the hydroxyl radicals induced by NO play a key role in the mutagenicity of $NaNO_2$.

Chromosome damage was also observed in weakly acidic conditions of pH 6.1 and 6.2 (Table 2). It has been previously established that non-specific chromosome aberrations occur spontaneously when CHL/IU cells are cultured at pH 6.5 or below (33,34). Therefore, we cannot rule out the possibility that some part of chromosome aberrations induced by $NaNO_2$ in combination with 2.0 and 2.5 mg/mL AsA were a non-specific result of low pH.

The bacterial mutation assays using pH 7.4 buffer showed that the number of revertants induced by $NaNO_2$ was slightly increased in combination with AsA and that the increasing amount was practically constant at all doses of $NaNO_2$. This small additive increase may be due to the weakly mutagenic activity of AsA. Unlike the cytogenetic assays, mutation-enhancing effect was not observed, which seems that no nitrite reacts with AsA in this experimental condition. Most recently, however, Kuroiwa *et al.* have conducted bacterial reverse mutation assays in pH 7.4 buffer using WP2*mutM uvrA*/pKM101, which is a deficient strain in *mutM* genes encoding 8-OHdG DNA glycosylase, and found that the combination with 100 μg/plate $NaNO_2$ and 5 mg/plate AsA significantly increased mutation frequencies (unpublished observations). This result suggests that a small amount of oxidative damage actually occurs in the bacterial strains in neutral conditions, but it is eventually repaired by DNA repair enzymes. This is why we could not reveal the enhancing effect in the present bacterial experiment with pH 7.4 buffer.

In contrast, the bacterial mutagenic activity of $NaNO_2$ was markedly increased in combination with AsA in pH 6.0 buffer (Fig. 1B), where the pH of the reaction mixture decreased to about pH 4.9. Meanwhile, the mutagenicity of $NaNO_2$ itself was dramatically increased in acidic conditions of pH 5.0–5.5 (Fig. 2) and this mutagenic profile was almost identical to that obtained by the combined treatment in pH 6.0 buffer. These results suggest that mutagenic activity of the combined treatment depends on the pH of the reaction mixture and that AsA just contributes to lowering the pH. Although the etiology of the mutagenic enhancing effects of $NaNO_2$ at low pH is not well understood, it is probably associated with the NO formation by acidic conditions (35–37). Nitrite is unstable under acidic conditions, producing dinitrogen trioxide (N_2O_3) through free nitrous acid (HNO_2). N_2O_3 is a highly reactive compound and spontaneously decomposed into NO and nitrogen dioxide (NO_2). NO_2 does not have a directly mutagenic activity, since two molecules of NO_2 form a dimer naturally in aqueous solution and immediately reacts with H_2O, producing HNO_2 and nitric acid (HNO_3). Thus, NO is effectively generated from $NaNO_2$ in acidic environment. Also, NO could be formed by the reduction of nitrite in the presence of AsA because AsA is stable and has a high reducing ability at low pH. However, it seems that the reduction of nitrite by AsA does not proceed efficiently when compared to the decomposition of nitrite in acidic conditions with AsA.

AsA is an essential vitamin for humans, and it is recommended that adults consume 100 mg daily in the form of vegetables and fruits (38). Some beverages such as lemon juice and soda contain AsA at a concentration of around 2 mg/mL, and it is possible that consumption of such beverages could result in a temporary concentration of 1.5–2.5 mg/mL AsA in the stomach. On the other hand, the daily intake of nitrite is approximately 10 mg from saliva and 1.5 mg from exogenous sources such as water, vegetables and meats (1). Consequently, the concentration of nitrite in the stomach is usually under 50 μM (39), which concentration is much lower than the 0.5 or 5.0 mg/mL (7.2 or 72.0 mM) we used in our cytogenetic experiments. Interestingly, Okazaki *et al.* (19) recently demonstrated that NO is formed using a combination of 50 μM $NaNO_2$ and 10 mM (1.8 mg/mL) AsA at pH 1.5 in a test tube. Taken together, this finding and our results suggest that consuming high doses of AsA may cause mutagenic damage in the stomachs of humans.

In conclusion, the present study demonstrates that simultaneous treatment with $NaNO_2$ and AsA causes mutagenic damage to bacterial and mammalian cells. It is likely that such genotoxicity, which is most likely NO-associated oxidative DNA damage, also contributes to the carcinogenic effect of combined $NaNO_2$ and AsA on the forestomach of rats.

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