Nitrite, N-Nitroso Compounds, and Other Analytes in Physiological Fluids in Relation to Precancerous Gastric Lesions


Abstract

Levels of gastric juice nitrite, several urinary N-nitroso compounds, and other analytes were examined among nearly 600 residents in an area of Shandong, China, where precancerous gastric lesions are common and rates of stomach cancer are among the world’s highest. Gastric juice nitrite levels were considerably higher among those with chronic atrophic gastritis than among those with normal or dysplastic mucosa, and were higher among those with more advanced pathology. Unfortunately, urinary nitrite concentrations were not determined in this study.

Introduction

NOC, especially nitrosamides such as N-methyl-N'-nitro-N-nitrosoguanidine, have long been suspected as gastric carcinogens, perhaps affecting early stages of the carcinogenic process (1, 2). This hypothesis has stimulated several investigations of intragastric nitrosation in individuals with precancerous gastric lesions, but results have been mixed (3-10). In an epidemiological study in Linqu, a rural area in China’s Shandong province, nearly one-half of the residents ages 35-64 years diagnosed with IM and 20% with gastric dysplasia (12). Herein we report results of a quantitative analysis of gastric juice nitrite and urinary NOC excretion among persons with precancerous lesions.

Materials and Methods

Individuals in the present study participated in a gastroscopic screening survey during 1989-1990 in 14 villages in Linqu County. Details are described in an earlier report (12). In brief, a total of 3433 adults, representing 83% of eligible residents ages 35-64 years, received a physical and endoscopic examination. The gastric mucosa was observed visually by a gastroenterologist, and seven biopsies were taken from the following standard locations: two from the body; one from the angulus; and four from the antrum of the stomach. In two of the villages, an eighth biopsy was taken from within 2 cm of the cardia along the lesser curvature.

Biopsy diagnoses were based on criteria proposed by the Chinese Gastric Pathology Association after review by experts on stomach pathology in both China and the United States (13). Each slide was reviewed by three senior pathologists at the Beijing Institute for Cancer Research (Beijing, China), and a consensus diagnosis was made. The presence or absence of SG,

Received 6/1/95; revised 8/25/95; accepted 9/5/95.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This study was supported in part by National Cancer Institute contracts NO1-CP-15620, NO1-CP-05631, NO1-CP-21009, and NO1-CP-33041.
2 To whom requests for reprints (China) should be addressed, at Beijing Institute for Cancer Research and School of Oncology, Beijing Medical University, Beijing 100034, China.
3 To whom requests for reprints should be addressed, at National Cancer Institute, EPN Room 543, Bethesda, MD 20892.

The abbreviations used are: NOC, N-nitroso compounds; IM, intestinal metaplasia; SG, superficial gastritis; CAG, chronic atrophic gastritis; NPRO, N-nitrosopropylamine; NMTCA, N-nitroso-2-methyl-thiazolidine 4-carboxylic acid; NTCA, N-nitrosodihyrdzoline 4-carboxylic acid; OR, odds ratio; CI, confidence interval.
CAG, IM, or dysplasia was recorded for each biopsy, and each subject was given an overall diagnosis based on the most severe histology. Almost always IM occurred in the presence of CAG, and dysplasia almost always occurred in the presence of IM in the same or a different biopsy (12).

During the physical examination, fasting gastric juice and overnight urine specimens were collected from approximately 600 subjects selected at random, representing nearly 20% of the participants in this survey. A 1500-ml plastic jar was provided for each participant for overnight urine collection. An aliquot of 50 ml of urine from each subject was transferred to a centrifuge tube, spiked with 25 µl of an aqueous internal standard solution of 0.15 mm in N-methyl-N-nitroso-β-alanine and 1.5 mm in piperidine 2-carboxylic acid, and immediately frozen on alcohol-dry ice. Fasting gastric juice was collected through a plastic tube from the individuals during the endoscopic examination. The urine and gastric juice specimens were stored at −20°C and then moved into a −70°C freezer within 2 or 3 days. The urine and gastric juice specimens, packed with dry ice, were shipped to the National Cancer Institute’s Frederick Cancer Research and Development Center (Frederick, MD) for analysis of NOC and other substances.

The nitrosamino acids, NPRO, cis- and trans-NMTCA, and NTCA, were quantified by the method of Ohshima and Bartsch (14), adapted as follows. Fifteen ml of urine were mixed with 5 g of sodium chloride and 2 ml of 20% ammonium sulfamate in 1.8 ml sulfuric acid. The resulting solution was extracted three times with 25 ml of 10% methanol in dichloromethane. The combined extracts were dried over anhydrous sodium sulfate, evaporated to dryness, dissolved in 0.5 ml of diethyl ether, and derivatized with treatment with a diazomethane-ether solution. The latter was prepared by dropping a solution of diazomethane in 2-propanol to a solution of N-nitrosopiperazine (Diazald; Aldrich Chemical Co., Milwaukee, WI) in diethyl ether into a warm, stirring mixture of 5 g of potassium hydroxide with 20 ml of 50% aqueous ethanol and distilling out the diazomethane-ether solution, 2 ml of which were added to the sample. The ether was evaporated, and the residue was dissolved in 0.1 ml of dichloromethane. The resulting solution was analyzed by gas chromatography on a capillary column coated with a 1-µm layer of DB-FFAP (J & W Scientific, Folsom, CA) held at 75°C for 2 min and then programmed at 10°C/min to 200°C, where it was held for 10 min. Helium flow rate was 10 ml/min. Temperature of the injector was 250°C, whereas that of the transfer line connecting the column to the nitrosamine-specific chemiluminescence detector (Tera Analyser 610 Nitrogen analyzer; Thermedics, Inc., Woburn, MA) was 225°C. The integrals of the peaks, the retention times of which correspond to those of integrated authentic NPRO, NTCA, and cis- and trans-NMTCA standards were compared to that of the internal standard, N-methyl-N-nitroso-β-alanine, to infer the concentration of each analyte in the urine. Standard curves prepared by analyzing spiked urine revealed that response was linear to >20 ng/ml for each compound, with detection limits of 0.1–0.2 ng/ml each. Detection of N-nitrosopiperazine 2-carboxylic acid was taken as evidence for artificial N-nitrosation after the sample was collected; <1% of the samples contained detectable N-nitrosopiperazine 2-carboxylic acid, and the highest value seen was 2 ng/ml.

Nitrate was determined by ion chromatography. Urine (500 µl) or gastric juice (100 µl) was diluted to 3 ml with deionized water. The resulting mixture was first passed through a Burdick and Jackson (Baxter Healthcare Corp., Muskegon, MI) Solid Phase System 200-mg Octadecyl (C18) column, then through an Alltech Associates (Deerfield, IL) IC-Ag® cartridge column for cleanup. The nitrite ion in the eluate was discarded, and 30 µl of the subsequent eluate was injected onto a Dionex (Sunnyvale, CA) IonPac AS5 column in a Dionex ion chromatography apparatus. By using a mobile phase prepared by dissolving 240 mg of sodium carbonate and 470 mg of sodium bicarbonate in 1 liter of deionized water (flow rate 1.5 ml/min), the nitrate signal was quantified by integrating the conductivity detector response and comparing the result to those obtained for separately injected standard nitrate solutions.

Nitrite was assayed using the colorimetric Griess reagent (15). A solution of 10 g of sulfanilamide/liter of 5% phosphoric acid was mixed with an equal volume of a second solution containing 1 g of N-(1-naphthyl) ethylenediamine dihydrochloride/liter of water. Two ml of the resulting solution were mixed with urine or gastric juice (diluted as necessary to 1 ml) in a disposable cuvette, and the absorbance at 546 nm was measured 5 min later. Absorbance was converted to nitrite concentration via a calibration curve prepared from standards containing authentic nitrite at concentrations of 0.1–5 µg/ml.

Total NOC values were determined by the method of Janini et al. (16). Aliquots (1 ml) of urine or gastric juice were passed through a 0.45-µm nylon filter, treated with 250 µl of 20% aqueous sulfamic acid, and filtered through a 0.2-µm nylon filter. A 50-µl aliquot of the resulting solution was introduced into a Nitrolite photolysis unit (Thermedics, Inc.), where it was irradiated with a mercury vapor lamp. Photometrically released nitric oxide was swept through two cold traps (the first held near 0°C and the second ≤−80°C) into the chemiluminescence chamber of a Model 502A Thermal Energy Analyzer (Thermedics, Inc.) by flushing the photolysis unit with helium at the rate of 20 ml/min. The integral of the nitric oxide signal was compared to those of N-nitrosopiperidine 4-carboxylic acid standards to provide a measure of the total NOC concentration in the sample.

Formaldehyde and acetaldehyde were analyzed by the method of Farrelly (17). Briefly, urine was diluted as necessary to 1 ml and mixed with 1.25 mg of 2,4-dinitrophenylhydrazine dissolved in 0.5 ml of 6 M hydrochloric acid. The resulting solution was extracted with 5 ml of isooctane that had been purified by distillation from a pot containing 2,4-dinitrophenylhydrazine. The isooctane layer was extracted in turn with 1 ml of acetoniitrile that had also been distilled from 2,4-dinitrophenylhydrazine. Aliquots of the acetoniitrile extract were analyzed by high-pressure liquid chromatography on a Hewlett Packard Model 1090 chromatograph (Rockville, MD) equipped with a set of 4.6-mm columns of Hypersil ODS (Hewlett Packard). The flow rate for the mobile phase (55:45 of acetoniitrile:water) was 1 ml/min, with detection at 340 nm. Peak areas were compared to those of standards of known composition to obtain the concentrations of the aldehydes in urine and gastric juice.

Sodium ion concentrations were measured with the aid of a Perkin Elmer Cetus Model 5000 Atomic Absorption unit (Norwalk, CT) at 589 nm, with power set to 7 mA. Urine was diluted 104-fold with deionized water before analysis, and sodium ion levels were read from a response curve prepared by plotting concentration versus response for various dilutions of the 1000 ppm Fisher Scientific Co. (Pittsburgh, PA) standard solution.

Proline and arginine were determined by the method of Einanson et al. (18). Four-hundred µl of urine (diluted 1:100) were mixed with 100 µl of 1 M boric acid (adjusted to pH 6.2 with 5 M sodium hydroxide) and 500 µl of 5 mM 9-fluorenylmethylchloroformate in acetonitrile. After 40 s of reaction, the

Downloaded from cbep.aacrjournals.org on October 14, 2017. © 1996 American Association for Cancer Research.
resulting solution was extracted twice with 3 ml of 4:1 pentane:ethyl acetate. The aqueous phase was analyzed on the same high-pressure liquid chromatographic system described above for the aldehyde analysis, except that the column was 20 cm long, and detection was by fluorescence at 340 nm with excitation at 260 nm. The mobile phase for the first 12 min after injection was 50:40:10 acetic acid buffer:methanol:acetonitrile and 50:50 acetic acid buffer:acetonitrile thereafter. The acetic acid buffer was prepared by adding 3 ml of acetic acid and 1 ml of triethylamine to 1 liter of distilled water and adjusting the pH to 4.2 with sodium hydroxide. Flow rate was 0.8 ml/min. Quantitation was accomplished via a calibration curve.

Creatinine (19) was determined by mixing 40 l of urine, 400 l of 1 M sodium hydroxide, and 2 ml of saturated aqueous picric acid solution and then diluting it 10 min later with water to 10 ml. The absorbance at 530 nm was measured 15 min after dilution and compared to a standard curve to obtain the creatinine concentration.

In the gastric juice samples, nitrite, total NOC, and nitrosoamino acids were measured, but only nitrite levels are presented because levels of the NOC were very low or undetectable in the large majority of samples. Serum levels of ascorbic acid and β-carotene, assayed by high-pressure liquid chromatographic methods, were also available for most of these individuals (20).

Spearman correlation coefficients were calculated as measures of association between pairs of analytes. Geometric means and SDs were calculated for each compound, the distributions of which tended to be skewed. The means were determined for strata defined by sex, age, gastric juice pH, cigarette smoking status (determined during standardized interviews that accompanied the screening examinations), and gastric mucosa histology. Most analyses examined two histology categories, the first combining SG and CAG and the second being IM with or without accompanying dysplasia. There were no individuals with normal mucosa in every biopsy, and only about 2% presented with normal mucosa or SG on every biopsy. Thus, the category of normal mucosa/SG could not be used as a reference group. Instead, ORs were computed as measures of association between the compounds and risk of IM with or without accompanying dysplasia relative to the risk of SG/CAG, adjusting for sex, age, and smoking status (risk factors for IM/dysplasia; Ref. 21) in logistic regression models (22). We also calculated ORs separately for dysplasia and for IM without dysplasia, but because these ORs tended to be similar we do not report them, except for several analytes where the patterns for dysplasia differed from those for IM.

### Results

Data were available on individual gastric juice, urinary, or serum compounds for up to 583 persons (312 males and 271 females). Among these study subjects, none had all normal biopsies, 8 had SG as the most severe histological diagnosis, 200 had IM without dysplasia, and 127 had IM with dysplasia. Table 1 shows the pairwise correlations among the compounds tested. Gastric juice nitrite was strongly correlated with urinary total NOC (r = 0.87; P < 0.01), less strongly but significantly correlated with trans- and cis-NMTCA and NTCA (r = 0.10–0.41; P < 0.01) and not significantly associated with urinary nitrate, amino acids or aldehydes or serum nutrients. Urinary nitrate was significantly correlated with NPRO and NTCA, whereas the four nitrosamino acids were significantly correlated with one another. Serum vitamin C showed a small negative correlation with most of the NOC, but a positive association with nitrate and sodium, whereas β-carotene was not related with any of the compounds.

### Table 1 Correlation coefficients among gastric juice nitrite, the urinary compounds, and serum micronutrients

<table>
<thead>
<tr>
<th></th>
<th>Gastric</th>
<th>Urinary</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO₂</td>
<td>NO₃</td>
<td>Total NOC</td>
</tr>
<tr>
<td>NO₂</td>
<td>1.00</td>
<td>0.00</td>
<td>0.05</td>
</tr>
<tr>
<td>NO₃</td>
<td>1.00</td>
<td>0.05</td>
<td>0.13</td>
</tr>
<tr>
<td>Total NOC</td>
<td>1.00</td>
<td>0.18</td>
<td>0.32</td>
</tr>
<tr>
<td>NPRO</td>
<td>1.00</td>
<td>0.13</td>
<td>0.11</td>
</tr>
<tr>
<td>trans-NMTCA</td>
<td>1.00</td>
<td>0.98</td>
<td>0.28</td>
</tr>
<tr>
<td>cis-NMTCA</td>
<td>1.00</td>
<td>0.29</td>
<td>0.11</td>
</tr>
<tr>
<td>NTCA</td>
<td>1.00</td>
<td>0.02</td>
<td>0.11</td>
</tr>
<tr>
<td>CH₂O</td>
<td>1.00</td>
<td>0.12</td>
<td>-0.04</td>
</tr>
<tr>
<td>CH₃CHO</td>
<td>1.00</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.00</td>
<td>0.14</td>
<td>0.21</td>
</tr>
<tr>
<td>Proline</td>
<td>1.00</td>
<td>0.23</td>
<td>0.04</td>
</tr>
<tr>
<td>Na⁺</td>
<td>1.00</td>
<td>0.12</td>
<td>-0.01</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>1.00</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>β-carotene</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Correlations computed among persons with detectable levels of each pair of compounds.

*b* P < 0.01.

*c* P < 0.05.
Nitrosation as a Risk Factor in Gastric Lesions

Table 2: Geometric mean levels and 95% CI of gastric juice nitrite and the urinary compounds among those with IM versus SG/CAG

<table>
<thead>
<tr>
<th>Analyte</th>
<th>n</th>
<th>% detectable</th>
<th>Mean (ng/ml)</th>
<th>95% CI</th>
<th>n</th>
<th>% detectable</th>
<th>Mean (ng/ml)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>256</td>
<td>100.0</td>
<td>281</td>
<td>254-312</td>
<td>327</td>
<td>99.4</td>
<td>242</td>
<td>223-263</td>
</tr>
<tr>
<td>Total NOC</td>
<td>147</td>
<td>95.9</td>
<td>2.0</td>
<td>1.8-2.3</td>
<td>206</td>
<td>96.6</td>
<td>2.0</td>
<td>1.8-2.3</td>
</tr>
<tr>
<td>NPRO</td>
<td>255</td>
<td>83.9</td>
<td>7.9</td>
<td>6.7-9.1</td>
<td>327</td>
<td>83.5</td>
<td>7.0</td>
<td>6.3-7.9</td>
</tr>
<tr>
<td>cis-NMTCA</td>
<td>255</td>
<td>48.2</td>
<td>6.1</td>
<td>5.1-7.2</td>
<td>327</td>
<td>46.6</td>
<td>5.8</td>
<td>4.8-6.8</td>
</tr>
<tr>
<td>NTCA</td>
<td>255</td>
<td>85.1</td>
<td>9.7</td>
<td>8.5-11.1</td>
<td>327</td>
<td>82.9</td>
<td>10.1</td>
<td>8.9-11.3</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>243</td>
<td>11.1</td>
<td>0.4</td>
<td>0.3-0.6</td>
<td>311</td>
<td>15.1</td>
<td>0.5</td>
<td>0.3-0.7</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>243</td>
<td>54.3</td>
<td>0.4</td>
<td>0.3-0.5</td>
<td>311</td>
<td>58.8</td>
<td>0.4</td>
<td>0.3-0.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>98</td>
<td>99.0</td>
<td>31.6</td>
<td>25.6-38.7</td>
<td>116</td>
<td>94.0</td>
<td>34.9</td>
<td>27.6-44.3</td>
</tr>
<tr>
<td>Proline</td>
<td>98</td>
<td>85.9</td>
<td>11.9</td>
<td>8.9-16.0</td>
<td>116</td>
<td>78.4</td>
<td>10.1</td>
<td>7.1-14.4</td>
</tr>
<tr>
<td>Sodium</td>
<td>256</td>
<td>100.0</td>
<td>3549</td>
<td>3281-3839</td>
<td>327</td>
<td>100.0</td>
<td>3537</td>
<td>3297-3794</td>
</tr>
</tbody>
</table>

*All values are given in units of ng/ml, except that those for total NOC and sodium ion are expressed in units of μg/ml. Total NOC concentrations were determined as N-nitrosopiperidine 4-carboxylic acid (M, 158).

Discussion

In an area of China where precancerous gastric lesions are very common, this gastroscopy-based study in the general population revealed a significantly increased risk of IM among subjects with elevated levels of gastric nitrite. Detectable nitrite levels were found more often and at higher levels among those with IM versus SG/CAG when gastric pH was high. The excesses were not reflected, however, in urinary excretion levels of total NOC or individual nitrosamines, with small differences seen for NPRO, trans-NMTCA, cis-NMTCA, or NTCA according to gastric histology. The findings add to ongoing research that has implicated certain fermented foods (sour pancakes) and other dietary items, cigarette smoking, and infection with Helicobacter pylori in the process of gastric carcinogenesis in this high-risk population (11, 12, 20–21).

Elsewhere in China it has been shown that endogenous formation of NOC is greater in areas of high versus low risk of esophageal-gastric cancer (8). Previous studies evaluating the relationship between NOC (mostly studies of NPRO) and precancerous gastric lesions, however, have yielded contradictory findings. Few have examined total NOC, but similar to our study, some have failed to show elevated NPRO levels among subjects with more advanced gastric lesions (7, 23, 24). Part of the difficulty in assessing differences between histology groups may have stemmed from the relatively large individual variation in the urinary NOC levels, hindering detection of small differences between groups.

Sex differences in analyte concentrations generally were not large. Males have about a 20% higher prevalence of IM, a 60% higher prevalence of gastric dysplasia, and >3-fold excess of gastric cancer compared with females in this area (12), but higher NOC concentrations were not found among men. At least part of the male excess in the prevalence of IM, dysplasia, and gastric cancer is related to cigarette smoking (11, 21), a habit practiced primarily by men in Linqu, but smoking was unrelated to most of the compounds analyzed, although gastric nitrite and total urinary NOC levels were nonsignificantly higher among smokers. Others have reported increased NPRO formation in cigarette smokers (25, 26), perhaps because of higher concentrations of thiocyanate, a catalyst of nitrosation in the gastric juice (27). A recent study in Colombia showed a higher concentration of urinary 7-methylguanine, a metabolic product of certain methylating agents, among smokers (9).

Levels of serum vitamin C, urinary nitrate, and urinary sodium were significantly correlated with one another, suggesting that salty and vitamin C containing foods (such as vegeta-
NPRO were significantly reduced after ingestion of vitamin C with proline (31). We have reported separately that the odds of IM are 50% lower among those with the highest tertile levels of serum vitamin C, and that β-carotene also is protective (20). In the present study, serum β-carotene showed a weak negative correlation with gastric nitrite and total urinary NOC but was not significantly associated with these compounds or any of the nitrosamino acids. Because of the weak associations, we could not determine whether an antinitrosation effect might be greater for vitamin C or β-carotene or whether vitamin C and β-carotene may have different protective mechanisms as suggested by others (28, 29).

Formaldehyde has been found to be mutagenic in bacterial systems and carcinogenic in some animal studies (32, 33). Although mechanisms are unclear, it has been suggested that formaldehyde is a mutagenically active intermediate formed during metabolism of N-nitrodimethylamine, and that it may inhibit O2-alkylguanine-DNA alkyltransferase activity (33, 34). Thus, it is of interest that the urinary levels of formaldehyde and acetaldehyde were high among subjects with dysplasia, suggesting that these compounds might promote the later stages of gastric carcinogenesis in this high-risk area. Acetaldehyde has shown a carcinogenic potential in animal experiments (35). It is a major metabolite of ethanol, although consumption of alcoholic beverages has not been associated generally with risk of gastric cancer or precancerous lesions in China or other regions of the world (11, 21).

In summary, by gastroscope screening of a high-risk population for stomach cancer in China, relationships emerged between advanced precancerous gastric lesions (IM with or without dysplasia) and level of gastric nitrite and, to a lesser extent, urinary formaldehyde and acetaldehyde. The findings of this study suggest that nitrite is a marker for and/or a compound involved in gastric carcinogenesis (36). Although we had hypothesized that NOC might play key roles, specific NOCs were not implicated. Thus, the possibility is raised that other or additional mechanisms might be involved (37), but evaluation of these is beyond the scope of our analysis.

Acknowledgments

We thank Drs. Mao-lin Jin and Bo-qin Yang for endoscopical examinations; Drs. Ji-you Li, Sen Hu, and Yu-quan Xie for histological review; and Dr. Jason Hu for computer programming support.

References

Nitrosation as a Risk Factor in Gastric Lesions


Nitrite, N-nitroso compounds, and other analytes in physiological fluids in relation to precancerous gastric lesions.

W C You, L Zhang, C S Yang, et al.


Updated version
Access the most recent version of this article at:
http://cebp.aacrjournals.org/content/5/1/47

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.