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 gastric juice pH values above 2.4 versus below 2.4. Nitrite was detected more often and at higher levels among persons with later stage gastric lesions, especially when gastric pH was high. Of those with intestinal metaplasia, 17.5% had detectable levels of gastric nitrite, while this analyte was detected in only 7.2% of those with less advanced lesions. Relative to those with undetectable nitrite, the odds of intestinal metaplasia increased from 1.5 (95% confidence interval = 0.6-4.1) to 4.1 (95% confidence interval = 1.8-9.3) among those with low and high nitrite concentrations, respectively. Urinary acetaldehyde and formaldehyde levels also tended to be higher among those with more advanced pathology, particularly dysplasia. However, urinary excretion levels of total N-nitroso compounds and several nitrosamines acids differed little among those with chronic atrophic gastritis and intestinal metaplasia and dysplasia, consistent with findings from recent studies in the United Kingdom, France, and Colombia. The data from this high-risk population suggest that elevated levels of gastric nitrite, especially in a high pH environment, are associated with advanced precancerous gastric lesions, although specific N-nitroso compounds were not implicated.

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 with one of the world's highest rates of stomach cancer, we found several factors related to nitrosation to be associated with risk of this cancer (11). In particular, risk was elevated among those who consumed sour pancakes, in which mutagenic activity and NOC have been detected, and was lower among those with high intakes of allium vegetables, β-carotene, and vitamin C, which may inhibit the formation of NOC. Additional study has indicated that the prevalence of precancerous gastric lesions in this area is exceptionally high, with 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 antrum; 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,

The abbreviations used are: NOC, N-nitroso compounds; IM, intestinal metaplasia; SG, superficial gastritis; CAG, chronic atrophic gastritis; NPRO, N-nitrosopropionic; NMTCA, N-nitroso-2-methylthiazoline 4-carboxylic acid; NTCA, N-nitrosotoluamide 4-carboxylic acid; OR, odds ratio; CI, confidence interval.

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4 The abbreviations used are: NOC, N-nitroso compounds; IM, intestinal metaplasia; SG, superficial gastritis; CAG, chronic atrophic gastritis; NPRO, N-nitrosopropionic; NMTCA, N-nitroso-2-methylthiazoline 4-carboxylic acid; NTCA, N-nitrosotoluamide 4-carboxylic acid; OR, odds ratio; CI, confidence interval.
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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 tube, spiked with 25 μl of an aqueous internal standard solution for each participant for overnight urine collection. An aliquot of participants in this survey. A 500-ml plastic jar was provided (500 ml) or gastric juice (1000 ml) was diluted to 3 ml with 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 0.15–5 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. Photolytically released nitric oxide was swept through two cold traps (the first held near 0°C and the second at -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 that of N-nitrosoindigo 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 acetonitrile that had also been distilled from 2,4-dinitrophenylhydrazine. Aliquots of the acetonitrile extract were analyzed by high-pressure liquid chromatography on a Hewlett Packard Model 1090 chromatograph (Rockville, MD) equipped with a 250 mm × 4.6-mm column of Hypersil ODS (Hewlett Packard). The flow rate for the mobile phase (55:45 of acetonitrile: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 Einansor 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 acetone. After 40 s of reaction, the
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>0.05</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>0.00</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>NPRO</td>
<td>1.00</td>
<td>0.00</td>
<td>0.14</td>
</tr>
<tr>
<td>trans-NMTCA</td>
<td>1.00</td>
<td>0.28</td>
<td>0.24</td>
</tr>
<tr>
<td>cis-NMTCA</td>
<td>1.00</td>
<td>0.29</td>
<td>0.22</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>0.01</td>
<td>0.14</td>
</tr>
<tr>
<td>CH₃CHO</td>
<td>1.00</td>
<td>0.00</td>
<td>0.03</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>0.02</td>
<td>0.22</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>0.00</td>
<td>0.07</td>
</tr>
<tr>
<td>β-carotene</td>
<td>1.00</td>
<td>0.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

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

**P < 0.01.

***P < 0.05.

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 1M 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 nitrosamino 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 2 shows geometric mean values of the compounds tested according to gastric pathology. Because there was little difference in the means for IM with or without accompanying dysplasia, we compared values for those with IM versus SG/CAG and did not separately consider dysplasia. Gastric juice nitrite concentrations were above the detection level (2 μM) for 7% of those with SG/CAG versus 17% for those with IM (P < 0.01). Furthermore, the average detectable level was twice as high (P = 0.06) among those with IM as with SG/CAG. Urinary formaldehyde tended to be detected somewhat more often and at higher levels, whereas urinary nitrate occurred generally at lower levels, among those with IM. However, these trends were not statistically significant nor were the differences in nitrosamino acid or sodium levels in urine between the two histological categories.

Differences in mean levels of most analytes with respect to sex, age, and cigarette smoking status were generally small,
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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>SG/CAG</th>
<th>IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>% detectable</td>
<td>Mean±95% CI</td>
</tr>
<tr>
<td>Gastric nitrite</td>
<td>209</td>
<td>7.2 ± 0.4</td>
</tr>
<tr>
<td>Nitrate</td>
<td>256</td>
<td>100.0 ± 281</td>
</tr>
<tr>
<td>Total NOC</td>
<td>147</td>
<td>95.9 ± 2.0</td>
</tr>
<tr>
<td>NPRO</td>
<td>255</td>
<td>83.9 ± 7.9</td>
</tr>
<tr>
<td>trans-NMTCA</td>
<td>255</td>
<td>48.2 ± 6.1</td>
</tr>
<tr>
<td>cis-NMTCA</td>
<td>255</td>
<td>36.6 ± 4.5</td>
</tr>
<tr>
<td>NTCA</td>
<td>255</td>
<td>85.1 ± 9.7</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>243</td>
<td>11.1 ± 0.4</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>243</td>
<td>54.3 ± 0.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>98</td>
<td>99.0 ± 31.6</td>
</tr>
<tr>
<td>Proline</td>
<td>98</td>
<td>85.9 ± 11.9</td>
</tr>
<tr>
<td>Sodium</td>
<td>256</td>
<td>100.0 ± 3549</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).
* Mean values among persons with detectable levels.

Table 3: Geometric mean gastric juice nitrite according to gastric juice pH and gastric pathology

<table>
<thead>
<tr>
<th>Gastric pathology</th>
<th>Low (≤2.4)</th>
<th>High (&gt;2.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>% detectable</td>
<td>Mean±95% CI</td>
</tr>
<tr>
<td>SG/CAG</td>
<td>126</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>IM</td>
<td>95</td>
<td>10.5 ± 0.7</td>
</tr>
</tbody>
</table>

* Mean (ng/ml) among those with detectable levels.

although total NOC concentrations were higher for females than males (2.5 versus 1.7 µg/ml; P < 0.05). Nitrite levels, however, were significantly (P < 0.01) higher among those with gastric pH>2.4 than among those with pH ≤ 2.4. There was a significantly (P < 0.01) higher percentage of detectable levels of nitrite among those with IM versus SG/CAG only among the subjects with high pH (Table 3).

Table 4 shows ORs of IM compared to SG/CAG, according to categorical levels of exposure to the gastric nitrite and urinary compounds. Increased risks of IM with rising exposure levels were found for gastric nitrite and urinary formaldehyde, but not other compounds, although only the elevated risk of IM among those with high nitrite concentration was statistically significant. For dysplasia, elevated risks were associated with above-median levels of both acetaldehyde and formaldehyde; the ORs were 1.5 (95% CI = 0.9-5.7) and 2.5 (95% CI = 1.1-5.7), respectively. For the other variables in Table 4, ORs for IM with dysplasia were generally not higher than the ORs for IM without dysplasia.

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 nitrosamine acids, 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-
Adjusted for sex, age, and smoking.

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 nitrosamines. Because of the weak associations, we could not determine whether an antiinflammation 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-nitroso compounds, and that it may inhibit ω-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 been shown to be 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 gastroscopic 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.

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