Urinary Excretion of Nitrate, \(N\)-Nitrosopropylamine, 3-Methyladenine, and 7-Methylguanine in a Colombian Population at High Risk for Stomach Cancer


Division of Toxicology [W. G. S., J. G., H-X. X., J. S. W., S. R. T.] and Department of Chemistry [S. R. T.], Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; Department of Pathology, Louisiana State University, Medical Center, New Orleans, Louisiana 70112 [D. Z., P. C.] and Departamento de Patología, Facultad de Medicina, Universidad del Valle, Cali, Colombia [G. M.]

ABSTRACT

Urinary excretion levels of nitrate and \(N\)-nitrosopropylamine were determined in 160 individuals in a Colombian population at high risk for gastric cancer. In 156 of these subjects urinary levels of 3-methyladenine and 7-methylguanine were determined. Gastric biopsy specimens were obtained from 118 individuals and were histologically characterized according to pathological criteria into the following groups: normal, superficial gastricitis, chronic atrophic gastritis, chronic atrophic gastritis with intestinal metaplasia, and dysplastic. The histological changes were correlated with the four variables listed above. There were no significant differences in the excretion of nitrate, 3-methyladenine, and 7-methylguanine in subjects with different pathological changes. A statistically significant correlation was present between nitrate and \(N\)-nitrosopropylamine excretion in the total population group \((r = 0.297, P = 0.0001)\). A highly significant correlation \((r = 0.56, P = 0.0002)\) was noted for urinary nitrate and \(N\)-nitrosopropylamine excretion in individuals with intestinal metaplasia and dysplasia. An increase in the urinary excretion of 3-methyladenine and 7-methylguanine was associated with tobacco smoking in the total population group.

INTRODUCTION

Gastric cancer is highly prevalent in the inhabitants of rural areas in the Nariño region of Colombia. A series of histopathological changes of the gastric mucosa has been identified in this population, apparently representing stages of a continuum of cancer precursor lesions (1). These changes constitute the multifocal chronic atrophic gastritis complex and are characterized in order of severity by superficial gastritis, atrophy, intestinal metaplasia, and dysplasia (2). The pathological changes are accompanied by profound alterations in the microecology of the gastric mucosa, including elevated gastric pH, altered enzyme levels, and increased gastric nitrite and nitrate concentrations (3, 4). Intragastric nitrosation of nitrogen compounds has been postulated as a source of locally active carcinogens (5). The \(in vivo\) nitrosation hypothesis of gastric carcinogenesis has been subjected to test by measuring urinary NPRO\(^3\) excretion (6), with inconsistent results. Nitrate intake and excretion is an important determinant of \(in vivo\) nitrosation. This report studies the relationship between the urinary excretion of nitrate and that of nitrosopropylamine, as well as the methylated purines 3-methyladenine and 7-methylguanine (which have been proposed as biological markers of exposure to \(in vivo\) methylation by nitrosatable agents) in the Colombian population of Nariño.

MATERIALS AND METHODS

Chemicals and Reagents. Standards of NPRO and \(N\)-nitrosopropionic acid were synthesized according to the previously described procedures (7). Boron trifluoride-methanol and 3-methyladenine were purchased from Sigma Chemical Co. (St. Louis, MO). \(N\)-(tert)Butylidimethylsilyl-\(N\)-methyltrifluoroacetamide was purchased from Pierce Chemical Co. (Rockford, IL). Samples of \(3\-{ }^3\text{H}\)methyladenine and \(7\-{ }^3\text{H}\)methylguanine were provided by Dr. D. E. G. Shuker (IARC, Lyon, France). The organic solvents were "distilled in glass" grade and, for the 3-methyladenine assay, were redistilled before use.

Study Population. The study group of 160 individuals was randomly selected from the town of Tuquerres in Nariño. This is a rural, homogeneous, and somewhat closed population of predominantly Indian ancestry with a high incidence of gastric carcinoma. History of smoking and consumption of alcoholic beverages was collected by direct interview. A random sample of the population was based on a household survey in which volunteers were requested to participate in the study. Twenty-five men and 25 women for each of the four decades covering ages 20–59 were invited.

Determination of Urinary Nitrate. Twenty-four-h urine samples were collected from the individuals in the study. Boric acid (10 g) was placed into each of the 500-ml collecting jars provided to the patient. The total collection of urine was mixed, and an aliquot was taken and placed in a −20°C freezer. The samples were shipped in dry ice to the central laboratory within 2 wk of collection. The total amount of urine excreted over a 24-h period was recorded. Nitrate was measured by an automated procedure previously described by Green et al. (8). In brief, the samples were passed through a column of copper-plated cadmium, thereby reducing nitrate to nitrite. Nitrite was measured following reaction with the Griess reagent \((1\% \text{ sulfanilamide/0.1\% \text{naphthylethylenediamine dihydrochloride/2.5\% } \text{H}_3\text{PO}_4)\), which forms a chromophore absorbing at 540 nm. The absorbance was detected by a flow-through UV/visible spectrometer connected to a Hewlett-Packard 3390 A integrator. The urine samples were run in duplicate and aqueous standards of sodium nitrate ranging from 5.0 to 50 \(\mu\)M were analyzed daily to check column efficiency. Stock urine samples were also analyzed periodically to check for system variability.

Determination of Urinary \(N\)-Nitrosopropylamine. An aliquot of urine (12 ml) was mixed with the internal standard (50 ng of \(N\)-nitrosopropionic acid in a glass vial containing 6 g of sodium chloride). After addition of 4 ml of a 20% solution of ammonium sulfate in 3.6 \(\text{N}\) \(\text{H}_2\text{SO}_4\), the sample was mixed and poured onto a Clin-Elut column (Analytichem International, Harbor City, CA). After standing for 2 min the tube was eluted with 15 ml of a methanol: dichloromethane solution (8:92, by volume), and the effluent was collected. This step was repeated 3 times and the combined extract was then concentrated to dryness under vacuum. Dichloromethane (2 × 3 ml) was added to the flask and the sample was transferred to a test tube. After evaporation under nitrogen at room temperature, 1 ml of 14% boron trifluoride-methanol was
EXCRETION OF NITRATE, NPRO, 3-METHYLADENINE, AND 7-METHYL GUANINE

added. The solution was heated to 65° for 50 min. After cooling, the sample was mixed with 6 ml of water. One ml of dichloromethane was added and the tube contents were mixed well. The dichloromethane layer was collected and the extraction was repeated with another 1 ml of dichloromethane. The organic layers were dried over sodium sulfate and evaporated under nitrogen to approximately 300 µl. The sample was then analyzed by gas chromatography using a thermal energy analyzer (Thermo Electron Corp., Waltham, MA). A 5% methylsilicone fused-silica capillary column (25 m x 0.53 mm inside diameter; 2.0-µm film thickness; Quadrex, New Haven, CT) was used in the temperature-programmed analysis.

Practical limits of measurability were of the order of 0.2 ng NPRO/ml of urine. In several cases (n = 11) in the study the NPRO excretion was below the analytical limit of detection. These collections of urine samples were arbitrarily given an excreted value of 0 ng of NPRO per subject for 24 h.

Determination of Urinary 3-Methyladenine and 7-Methylguanine. The analysis of 3-methyladenine and 7-methylguanine in urine was carried out as previously described (9). Briefly, a 10-ml aliquot of urine, spiked with 500 ng of 3-[3H]methyladenine and 12 µg of 7-[3H]methylguanine, was applied to a column of Amberlite XAD-2 resin (Aldrich Chemical Co., Milwaukee, WI). The column was eluted with 0.001 N HCl and the fraction containing 3-methyladenine and 7-methylguanine was collected and evaporated to dryness. Further purification was accomplished by bonded-phase extraction column chromatography using carboxylic acid carboxylic acid carboxylic acid (Supelco, Bellefonte, PA) with helium as carrier gas. Quantification of 3-methyladenine was done by selected ion monitoring for the M−/C5H11 fragment ion at m/z 206 for the d6-compound and m/z 209 for the d7-analogue used as internal standard. Quantification of 7-methylguanine in urine was carried out in the same analysis. Combined gas chromatography/mass spectrometry was performed using a Hewlett-Packard Model 5996 system operated in the electron ionization mode. The column used was a fused-silica capillary (30-m x 0.25-mm SPB-1; Supelco, Bellefonte, PA) with helium as carrier gas. Quantification of 3-methyladenine was done by selected ion monitoring for the M−/C5H11 fragment ion at m/z 206 for the d6-compound and m/z 209 for the d7-analogue used as internal standard. Quantification of 7-methylguanine in urine was carried out in the same analysis. In this case the ion at m/z 336 and m/z 339 for the di-tert-butylidemethylsilyl derivatives of the d6 and d7-compound, respectively, were monitored.

Histopathology. The gastric biopsy specimens were prepared as previously described (10). Histological diagnosis was made by the pathologist without previous knowledge of gastroscopic or clinical findings. Histologically, the specimens were classified into the following groups: group 1, normal; group 2, superficial gastritis; group 3, chronic atrophic gastritis; group 4, chronic atrophic gastritis with intestinal metaplasia; and group 5, dysplastic.

Statistical Analysis. Linear regression analysis was carried out to study the correlation among the data sets tested. The correlation coefficients and P values were determined using the SAS statistical package. Linear regression analysis was performed on all combinations of data sets using the total data sets and certain subsets of data, for example, different biopsy subgroups. The two-sample test was used to determine differences between the mean excretion values of the title compounds for smokers versus nonsmokers and for drinkers versus nondrinkers.

RESULTS

The gastric pathology results obtained from the biopsy specimens of 118 of the subjects are shown in Table 1. Due to a lack of normal subjects the results from the one normal individual have been deleted in the following analyses and the data from the 3 individuals diagnosed with dysplasia have been combined with those from patients with intestinal metaplasia to give a subtotal of three different pathology groups.

Comparison of Urinary Nitrates, N-Nitrosoproline, 3-Methyladenine, and 7-Methylguanine Excretion versus Pathogenic Change. A comparison of the excreted levels of nitrate, NPRO, 3-methyladenine, and 7-methylguanine in the total population and by histological diagnosis was made. These results are summarized in Table 2. There was essentially no difference among the groups in the mean and median values of urinary nitrate excretion. No significant relationship was found between pathological finding and NPRO excretion. The mean and median values were similar in the three groups.

A lack of correlation between gastric pathology and 3-methyladenine excretion in the three subsets was also noted. The mean and median values showed no significant change with the degree of pathology. No significant difference in excretion of 7-methylguanine was observed among the histopathology groups.

Regression Analysis of Urinary Nitrate and N-Nitrosoproline in the Total Population and in the Three Diagnostic Groups. In the present study group there were 79 men (average age, 38.2, with a range of 18 to 59 yr) and 81 women (average age, 39.5, with a range of 20 to 58 yr). Regression analysis of urinary nitrate excretion versus age in the total population showed no correlation between the two parameters (r = 0.16, P = 0.04).

The results of linear regression analysis of the excretion of NPRO as a function of nitrate excretion are shown in Tables 3 and 4 and Figs. 1–3. A moderate correlation of borderline significance was detected in patients with superficial gastritis or chronic atrophic gastritis. In patients with intestinal metaplasia and dysplasia, a strong (r = 0.56) and highly significant correlation (P = 0.0002) was found. When one outlier is excluded from this group, an even stronger correlation (r = 0.6) is found and a plot of the linear regression line (Fig. 3) shows a steep slope (B = 0.64). The slopes of the regression lines for subjects with superficial gastritis and chronic atrophic gastritis are much less steep (B = 0.26 and 0.24, respectively).

Regression Analysis of 3-Methyladenine and 7-Methylguanine versus Nitrate and NPRO. Regression analysis of the excretion of 3-methyladenine with nitrate in the total population and the three subset groups showed no significant correlations for the parameters tested. Regression analysis data of the excretion of 3-methyladenine versus NPRO in the total population are shown in Table 3. A moderate correlation between NPRO excretion and 3-methyladenine was found (r = 0.19, P = 0.02). Regression analysis of nitrate versus 7-methylguanine excretion showed a somewhat weaker correlation (r = 0.14, P = 0.07).

The effect of smoking and alcohol consumption on the excretion of the title compounds was also studied. There were no differences between drinkers and nondrinkers in excretion of any of the title compounds (data not shown). An increase in the median value of urinary 3-methyladenine excretion was observed for smokers (7.3 µg/subject/24 h, n = 40) when compared to nonsmokers (5.5 µg/subject/24 h; n = 116). When calculating the means three outliers (nonsmokers) were removed and a value of 7.05 ± 5.7 (SD) µg/subject/24 h was obtained for nonsmokers. For smokers a mean value of 9.4 ± 5.2 µg/subject/24 h was obtained. A comparison of the two means using a 2-sided t test showed a significant difference (P = 0.025). These mean values are within the range reported for the healthy population.
EXCRETION OF NITRATE, NPRO, 3-METHYLADENINE, AND 7-METHYLGUANINE

Table 2: Means and medians for the amounts of nitrate, NPRO, 3-methyladenine, and 7-methylguanine excreted in urine

<table>
<thead>
<tr>
<th>Group</th>
<th>Nitrate (µmol/person/24 h)</th>
<th>NPRO (ng/person/24 h)</th>
<th>3-Methyladenine (µg/person/24 h)</th>
<th>7-Methylguanine (mg/person/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total population</td>
<td>1000.9 ± 53.3*</td>
<td>929.7 ± 41.1</td>
<td>9.4 ± 1.2</td>
<td>6.9 ± 0.2</td>
</tr>
<tr>
<td>2—superficial gastritis</td>
<td>951.3 ± 122.0</td>
<td>843.2 ± 84.7</td>
<td>7.3 ± 1.0</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>3—chronic atrophic gastritis</td>
<td>1083.8 ± 126.4</td>
<td>979.3 ± 97.3</td>
<td>10.4 ± 2.9</td>
<td>7.8 ± 0.4</td>
</tr>
<tr>
<td>4 + 5—intestinal metaplasia + dysplasia</td>
<td>947.9 ± 101.6</td>
<td>1005.8 ± 78.1</td>
<td>9.3 ± 1.5</td>
<td>6.5 ± 0.5</td>
</tr>
</tbody>
</table>

* Mean ± SE.

Table 3: Linear regression analysis data of urinary excretion levels of nitrate, NPRO, 3-methyladenine, and 7-methylguanine

<table>
<thead>
<tr>
<th>Variable</th>
<th>Correlation coefficient</th>
<th>P</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPRO vs. nitrate</td>
<td>0.30</td>
<td>0.0001</td>
<td>160</td>
</tr>
<tr>
<td>NPRO vs. 3-methyladenine</td>
<td>0.19</td>
<td>0.02</td>
<td>156</td>
</tr>
<tr>
<td>NPRO vs. 7-methylguanine</td>
<td>0.08</td>
<td>0.30</td>
<td>156</td>
</tr>
<tr>
<td>Nitrate vs. 3-methyladenine</td>
<td>0.06</td>
<td>0.46</td>
<td>156</td>
</tr>
<tr>
<td>Nitrate vs. 7-methylguanine</td>
<td>0.14</td>
<td>0.07</td>
<td>156</td>
</tr>
<tr>
<td>3-Methyladenine vs. 7-methylguanine</td>
<td>-0.02</td>
<td>0.77</td>
<td>156</td>
</tr>
</tbody>
</table>

Table 4: Linear regression analysis of urinary nitrate versus NPRO excretion

<table>
<thead>
<tr>
<th>Group</th>
<th>Slope estimate (β)</th>
<th>Correlation coefficient (r)</th>
<th>P</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total population</td>
<td>0.29</td>
<td>0.30</td>
<td>0.0001</td>
<td>160</td>
</tr>
<tr>
<td>Group 2 (superficial gastritis)</td>
<td>0.26</td>
<td>0.37</td>
<td>0.03</td>
<td>35</td>
</tr>
<tr>
<td>Group 3 (chronic atrophic gastritis)</td>
<td>0.24</td>
<td>0.32</td>
<td>0.04</td>
<td>44</td>
</tr>
<tr>
<td>Group 4 + 5 (intestinal metaplasia + dysplasia)</td>
<td>0.43</td>
<td>0.56</td>
<td>0.0002</td>
<td>38</td>
</tr>
<tr>
<td>Group 4 + 5 (-1 outlier)</td>
<td>0.64</td>
<td>0.60</td>
<td>0.0001</td>
<td>37</td>
</tr>
</tbody>
</table>

Fig. 1. Plot of the linear regression line of the amount of urinary nitrate versus that of N-nitrosoproline (N-proline) excreted per individual in 24 h by subjects in group 2 [superficial gastritis (SG)].

Fig. 2. Plot of the linear regression line of the amount of urinary nitrate versus that of N-nitrosoproline (N-proline) excreted per individual in 24 h by subjects in group 3 [chronic atrophic gastritis (CAG)].

Fig. 3. Plot of the linear regression line of the amount of urinary nitrate versus that of N-nitrosoproline (N-proline) excreted per individual in 24 h by subjects in group 4 + 5 [intestinal metaplasia and dysplasia (IM + D)], with one outlier deleted.

DISCUSSION

Previous studies carried out in the Nariño population identified cancer precursors, biochemical markers, and dietary factors that led to an etiological hypothesis of gastric carcinogenesis (1, 5, 12). Part of this hypothesis is the postulate that high levels of nitrite in the gastric cavity may lead to in vivo synthesis of N-nitroso carcinogens. This is supported by the identification of a potent mutagen formed by nitrosation of 4-chloro-6-methyl...
oxyindole, present in fava beans, which are ingested abundantly by the population under study (13). One major objection to the hypothesis has been the well-known optimal nitrosation of proline and other amines at low pH values, which are not found in the presence of intestinal metaplasia. Our findings demonstrate that, when nitrate excretion is taken into account, nitrosation of proline is associated with nitrate excretion in patients with intestinal metaplasia and dysplasia, in whom the gastric pH is elevated.

A significant relationship was observed between NPRO and nitrate excretion in the Colombian group under study. A strong correlation was found for these two indices in subjects histologically characterized with gastric intestinal metaplasia and dysplasia.

Recently, several groups have reported that urinary NPRO excretion in patients with gastric lesions, including chronic atrophic gastritis and gastric carcinoma, showed a lack of relationship between gastric disease and NPRO excretion (14, 15). Studies by Bartsch et al. showed that urinary NPRO levels in patients with chronic atrophic gastritis were dependent on gastric pH, showing maximal yields at about pH 2, with large interindividual variations of excreted nitrosated amino acids (16). Patients, as compared to healthy controls, excreted no apparent excess NPRO. Our results lend support to these studies and show that, in cases of severe gastric abnormality, NPRO excretion was not elevated. Analysis of 117 subjects in the three groups revealed no significant differences in average levels of urinary NPRO excretion. Shuker et al. proposed that 3-methyladenine levels in urine might be used as an index of nucleic acid methylation occurring in vivo (11). 3-Methyladenine is not found as a normal constituent of RNA or DNA, but most of the 3-methyladenine excreted in urine can be accounted for by the diet. The use of 3-methyladenine excretion levels as an index of exposure to methylation reactions may thus be possible if the diet is controlled. In the present study we did not find differences in the excretion levels of 3-methyladenine in subjects consuming a normal diet when compared to the extent of pathologic change present in the group. However, an increase in the mean and median levels of 3-methyladenine excretion was found in the smokers in this study when compared to the nonsmokers.

Urinary levels of 7-methylguanine were also measured in the study population. This compound is normally present in urine and is excreted in relatively high amounts in humans. However, it is also known to be a major methylation product of certain methylating agents in animals and its excretion may thus reflect to some extent in vivo methylation reactions. On the average, smokers in this study excreted higher amounts of 7-methylguanine when compared to nonsmokers. Tobacco smoke contains volatile N-nitrosamines, including N-nitrosodimethylamine, as well as tobacco-specific N-nitrosamines capable of forming active methylating species (Ref. 17 and references cited therein), which may contribute to the somewhat higher levels of urinary methylated purines found in the smokers. We found a slight correlation between nitrate and 7-methylguanine excretion in the Nariño population.

In earlier work the possible role of nitrite with respect to the etiology of gastric cancer was discussed (12, 18, 19). In particular, the intragastric formation of N-nitroso compounds was implicated as an initiating factor in the development of gastric carcinoma. The formation of these substances in the stomach may be influenced by promoters and inhibitors present in the gastric contents. In the present population group from the high-risk gastric cancer region of Nariño, Colombia, we found a strong correlation between excretion of urinary nitrate and excretion of NPRO in the individuals with increasingly severe gastric lesions, which are considered gastric cancer precursors. Gastric pH in this diagnostic group is elevated; thus, hypochlorhydria, by depleting gastric juice of its natural protective factors, may lead to an elevated risk of gastric cancer. The relationship between ascorbic acid intake and NPRO excretion in humans on a controlled diet has been established, and further evidence indicates that nitrosation of proline in the stomach is inhibited when ascorbic acid is administered concurrently with proline (20). Studies by Kamiyama et al. showed that subjects from a high-incidence area for stomach cancer in Japan have an enhanced nitrosation potential of proline when compared to individuals from a low-incidence area (21). They also observed that administration of ascorbic acid led to a reduction of endogenous nitrosation of proline. In a related study Lu et al. demonstrated that inhabitants of a high-risk area for esophageal cancer in China excrete higher levels of N-nitrosamines and nitrate in their urine than those living in a low-risk area (22). They also found that ingestion of ascorbic acid with proline by the high-risk subjects reduced the urinary levels of NPRO and other N-nitrosamines to levels found in undosed subjects of the low-risk area. Significantly, Sobala et al. (23) recently reported that gastric ascorbic acid concentrations are particularly low in individuals with chronic gastritis. It was proposed that oxidation of gastric ascorbic acid to dehydroascorbic acid is increased in the hypochlorhydric stomach. Available data indicate that a high gastric concentration of nitrite, which is also associated with hypochlorhydria, may in turn undergo reaction with ascorbic acid in gastric juice of high pH to form dehydroascorbic acid and nitrous oxide. The presence of low gastric ascorbic acid concentrations in hypochlorhydria, thus, may be a result of oxidation of ascorbic acid by bacterial nitrite. In view of the fact that we found a high correlation between urinary nitrate and NPRO excretion in the subjects who would be expected to have elevated gastric juice pH, as well as low ascorbic acid concentrations, it may be assumed that these subjects are at high risk for gastric nitrosation. The role of ascorbic acid as a protective agent blocking synthesis of N-nitroso compounds in the stomach may be significant and important in the context of the model of gastric carcinogenesis proposed in previous studies (5, 12).

In summary, our results are consistent with those of previous investigations and lend support to the hypothesis that urinary N-nitrosopropionyl excretion is indicative of the involvement of nitrate and N-nitroso compounds as factors in the etiology of gastric cancer. Further work will be carried out to substantiate these results in a larger population group. Finally, the association of lowered levels of gastric ascorbic acid with the severity of gastritis provides a basis for further studies in the area of identifying the dietary components that may prevent the development of the disease.

REFERENCES

EXCRETION OF NITRATE, NPRO, 3-METHYLADENINE, AND 7-METHYLGUANINE


Urinary Excretion of Nitrate, N-Nitrosoproline, 3-Methyladenine, and 7-Methylguanidine in a Colombian Population at High Risk for Stomach Cancer


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/51/1/190

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.