N-Nitrosoproline Excretion by Rural Nebraskans Drinking Water of Varied Nitrate Content

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Abstract

The N-nitrosoproline (NPRO) test for in vivo nitrosation was applied in a study of 44 rural Nebraska men drinking high- or low-nitrate water from private wells. The subjects followed diets low in NPRO and nitrate for 5 days. On days 4 and 5 they avoided ascorbate-rich foods. Urine was collected for 24 h on day 4 while the subjects followed normal activities and on day 5 after an overnight fast and taking 500 mg l-proline. We determined NPRO, nitrate, creatinine, and specific gravity in the urines, and nitrite and nitrate in single saliva specimens collected on days 4 and 5. Results for all variables were separated into those above and below the median values and were analyzed by univariate and multivariate consideration of the contingency tables. Nitrate concentration in drinking water (≥ or <10 ppm nitrate-nitrogen) was significantly associated with both day 4 and day 5 NPRO (≥ or <1.5 μg/day; P < 0.04); and with urine nitrate (≥ or <1.5 mmol/day), saliva nitrite (≥ or <5 mg/liter), and saliva nitrate (≥ or <25 mg/liter) (P = 0.002). Urine nitrate was significantly associated with both day 4 and day 5 NPRO, with odds ratios of 4.2 and 5.4, respectively. Creatinine was positively associated with NPRO on day 4 (P = 0.04). These findings, like those of a recent study in Denmark, showed an association between nitrate intake in water and NPRO formation. Their significance for people drinking high-nitrate water remains to be determined.

Introduction

In 1981 Ohshima and Bartsch (4) demonstrated in vivo nitrosation in humans using a test in which 24-h urine samples were analyzed for NPRO.1 When a fasting man ingested 325 mg nitrate and, 30 min later, 500 mg of the natural amino acid l-proline, he excreted 23 μg NPRO in his urine. NPRO excretion was proportional to proline dose and to nitrate dose squared and was inhibited 80% by also taking 1.0 g ascorbate. The test is considered safe because NPRO is noncarcinogenic in rats (5). NPRO formation was low in cases of achlorhydria, indicating that most nitrosation of proline is an acid-catalyzed gastric reaction (6). Proline is nitrosated with moderate facility compared to other amines and hence should be a useful marker of amine nitrosation in the stomach, even though the kinetics of its nitrosation, which proceeds maximally at pH ≤ 2, differs somewhat from those for most amines, which show maximum rates at pH 3.0–3.4 (7). NPRO formation increased in smokers given proline and nitrate (6, 8, 9). In tests conducted by feeding proline but not nitrate, NPRO was elevated in areas of China and Japan with high incidences of esophageal and stomach cancer (10, 11).

Nitrate levels are rising in the ground and surface water of certain rural areas of the United States (12). In many areas of Nebraska susceptible to leaching from the surface, nitrate in ground water (the source of well water) is associated with excess fertilization (13). This might contribute to the etiology of certain cancers if high levels of nitrate in ingested water produce significant amounts of carcinogenic N-nitroso compounds in the stomach. The cancers induced by these compounds could include some of those which show raised incidences in the Midwest. In this connection, rural residents or farmers of Nebraska and/or Iowa show excess incidences of various types of leukemia and lymphoma (14–16) and of stomach and prostate cancer (14). Non-Hodgkins lymphoma was associated with exposure to certain herbicides, but this factor may account for only 10% of the disease (17).

The average U.S. intake of nitrate is 75 mg/person/day, 90% of which comes from vegetables (18). Total nitrate intake is doubled if drinking water contains 50 ppm nitrate (11.3 ppm nitrate-N) and 1.5 liter/day is drunk. Above this level, water becomes the main source of nitrate. Nitrate in water might be more hazardous than nitrate in vegetables because the latter is usually counterbalanced by ascorbate and polyphenols that inhibit gastric nitrosation (18, 19).

The EPA sets 11.3 ppm nitrate-N as the limit for safety, based on the dose that can produce methemoglobinemia in infants (20). Of 3456 well water samples

1 The abbreviations used are: NPRO, N-nitrosoproline; EPA, Environmental Protection Agency; GC, gas chromatography; NPIPC, N-nitrosopipelic acid; nitrate-N, nitrate-nitrogen. [Nitrate in water is expressed as nitrate-N (23% of NO₃⁻), as is customary in U.S. agriculture.]

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2 To whom requests for reprints should be addressed, at Eppley Institute for Research in Cancer, University of Nebraska Medical Center, 600 South 42nd Street, Omaha, NE 68198-6805.
submitted in 1988 for analysis by the Nebraska Health Department. 6% contained 30-120 ppm; 4%, 20-30 ppm; 12%, 10-20 ppm; and 78%, 0-10 ppm nitrate-N. Hence, nitrate in water is clearly of concern in this state, even though these were not random samples.

The current project was designed to test whether men drinking high-nitrate water might form excessive amounts of carcinogenic nitrosamines in vivo, as indicated by the amount of NPRO in 24-h urine specimens when doses of proline (but not of nitrate) are given. The NPRO test was taken by rural Nebraskans with high or low nitrate levels in their water. Only men were selected because they have higher incidences of gastric and esophageal cancer than women (19, 21) and because it is somewhat easier for men to collect urine. In addition to NPRO, we determined nitrate, creatinine, and specific gravity in the 24-h urines and nitrate and nitrite in single samples of saliva. Urinary nitrate is a useful measure of total nitrate load because about 55% of ingested nitrate is excreted in urine and urinary nitrate includes a contribution from endogenous nitrate (22). Urinary creatinine and specific gravity were determined to indicate the completeness of the urine collections. Salivary nitrite and nitrate were determined because saliva nitrate is reduced by oral bacteria to saliva nitrite, which normally supplies 80% of the gastric nitrite (2, 18, 19, 23, 24).

Materials and Methods

Conduct of the Test. Inquiry letters were sent to all male rural Nebraskans whose well water had >20 ppm nitrate-N in analyses performed 1-2 years previously by the Nebraska Health Department. After we received preliminary information, 1 man with <5 ppm nitrate-N water (determined in similar analyses) was recruited for each high-nitrate man by writing to three such men. Final interviews were done by telephone. Men with high- and low-nitrate water were matched by county or adjacent county, age ±5 years, and smoking habits. The men were 19-60 years old and were excluded if they took medications or had diseases that affect gastric pH.

The volunteers came to meetings at three locations, where we collected the questionnaires and informed consent forms and handed out detailed instruction lists of proscribed foods (see below), food diaries, cans of apple juice, gelatin capsules containing 500 mg L-proline, bottles for collecting urine, tubes and plastic funnels for collecting saliva, and forms for arranging modest payments, including mileage charges. We explained the instructions and the project’s significance and reinforced the volunteers’ motivation to perform the test properly. The men carried out the test on their own in August-September 1988, while they pursued their normal activities and consumed their usual diets, except that during the 5-day test they did not eat (a) foods that contain NPRO, including nitrite-preserved or smoked meat and fish products, beer, wine, and yellow cheese (25, 26); (b) gelatin, because of its high proline contents; and (c) beets, celery, radishes, spinach, and lettuce, because of their high nitrate content (18). To increase the likelihood of obtaining positive results, on days 4 and 5 the men (a) avoided foods high in ascorbate, including citrus fruit, melons, strawberries, pineapples, tomato products, cabbage family, spinach, potatoes, vitamin-fortified foods and vitamin supplements and (b) did not chew gum or tobacco, because this stimulates the flow of saliva. Food and beverage intakes were recorded, including approximate amounts and times of consumption.

Urine was collected for 24 h on each of days 4 and 5 from 6 to 7 a.m. in brown 3-liter polypropylene bottles that were kept refrigerated whenever possible and contained 50 ml sulfamate-H2SO4 “stopping solution” (15). No other treatment was given on day 4. The men fasted from 8 p.m. of day 4 until 1 p.m. on day 5 but drank freely from their well water, including 2 glasses (450 ml) when rising on day 5. One h later on that day, they took 500 mg L-proline (emptied from the capsule) in 6 ounces of canned apple juice and started to collect the day 5 urine. Saliva samples (5 ml) were collected at 7-8 a.m. on days 4 and 5 in screw-capped test tubes containing 1 ml of 0.5 N Na2CO3 in water. The men collected one 100-ml sample of drinking water in bottles containing 5 ml of 1 N H2SO4 during the test and another similar sample 3 months later. They handed in the samples and food diaries at the original three meeting locations.

Chemical Analyses. After transport to Omaha, the urines were well mixed and their volumes measured. Samples were analyzed at a clinical laboratory for specific gravity (derived from refractive index) and for creatinine by the Jaffe reaction. All urines had >1 g creatinine. Other urine samples (500 ml) were stored at −15°C in polypropylene bottles for the NPRO and nitrate analyses. Storage for 1 year did not affect the results. All analyses were performed in full duplicate. For all 88 duplicate analyses for NPRO, the mean value for the percentage variation [difference between individual and mean results) × 100/mean result] was 13.4%. The same parameter for 15 consecutive duplicate analyses was 9% for urine nitrate, 1.7% for saliva nitrate, and 4% for saliva nitrate. In the NPRO analyses, the percentage variation was 10.1% for 60 analyses with mean values of >1.0 µg NPRO/day and 20.4% for 28 results with ≤1.0 µg/day. The larger variation in the lower values was expected (because they were close to the detection limit of 0.2 µg/day) and would not affect the analysis of our data, which were separated into those with ≤ and <1.5 µg NPRO/day.

NPRO. This was determined by a modification of the method of Stillwell et al. (27). Urine samples (20 ml) were mixed with NaCl, stopping solution, and 400 ng NPIC and absorbed on columns of 125 ml unsieved Celite 560 (Cron Chemical Corp., Houston, TX). The columns were left for 10 min and eluted with 20 ml CH3Cl and 280 ml CH3Cl,MeOH (92:8). The first 20 ml of eluate were discarded. The remaining eluate was dried over Na2SO4 and evaporated. The residues were treated with 14% BF3 in methanol to produce the methyl esters. CH3Cl extracts of the product were concentrated to 200 µl and three 5-µl samples were analyzed by GC-thermal energy analysis. GC was performed at 185°C on a 2 mm × 6 ft glass column of 10% Carbowax 20M on 80/100 Chromosorb WAW (20 ml H2/min; detector, Thermal Energy Analysis; Thermedics, Inc., Woburn, MA). Retention times were 6-8 min for NPIC and 7-9 min for NPRO methyl esters. NPRO was calculated using NPIC as the internal standard. The results of the GC analyses/sample generally differed by <20%. If the full duplicate analyses did not agree within 30 or 50% (for > and <1

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*J. Sahs, personal communication.*
μg NPRO/day, respectively), additional samples were analyzed. NPIC showed 81 ± 3% recovery (mean ± SE, based on nitrosomorpholine standards from the same GC run) for 26 consecutive urines.

**Nitrate.** We followed a method involving the conversion of nitrate to nitrobenzene (28). Urine or saliva samples (5 ml) were mixed with 50 mg Ag₂SO₄ and 0.1 ml saturated ZnSO₄ and filtered. One ml H₂SO₄ was added to 0.5 ml filtrate, which was reacted with benzene at 70°C and made basic with NaOH. Nitrobenzene was extracted with ethyl acetate. The extract was concentrated to 1.5 ml and made basic with NaOH. Nitrobenzene was extracted with benzene at 70°C (5 ml) were mixed with 50 mg Ag₂SO₄ and 0.1 ml saturated ZnSO₄ and filtered. One ml H₂SO₄ was added to 0.5 ml filtrate, which was reacted with benzene at 70°C and made basic with NaOH. Nitrobenzene was extracted with ethyl acetate. The extract was concentrated to 1.5 ml and made basic with NaOH. Nitrobenzene was extracted with benzene at 70°C (5 ml) were mixed with 50 mg Ag₂SO₄ and 0.1 ml saturated ZnSO₄ and filtered. One ml H₂SO₄ was added to 0.5 ml filtrate, which was reacted with benzene at 70°C and made basic with NaOH. Nitrobenzene was extracted with ethyl acetate. The extract was concentrated to 1.5 ml and made basic with NaOH. Nitrobenzene was extracted with benzene at 70°C (5 ml) were mixed with 50 mg Ag₂SO₄ and 0.1 ml saturated ZnSO₄ and filtered. One ml H₂SO₄ was added to 0.5 ml filtrate, which was reacted with benzene at 70°C and made basic with NaOH. Nitrobenzene was extracted with ethyl acetate. The extract was concentrated to 1.5 ml and made basic with NaOH. Nitrobenzene was extracted with benzene at 70°C.

Results

The first 3 days of the 5-day test were included because dietary-bound NPRO (NH₃-terminal NPRO in proteins) takes 3 days to be eliminated from the body (25, 26, 30). Urine was collected on day 4 to give the “background” urine NPRO and on day 5 after an overnight fast and taking 500 mg proline to give the “test” NPRO. Fasting was introduced on day 5 to increase the likelihood of positive results, based on our finding (2, 3; unpublished data) that 3 times more NPRO was produced when proline and nitrate were taken while fasting than when they were taken with a standard meal. L-Proline was administered in 1 cup (6 ounces, 178 ml) of apple juice. In retrospect, the proline vehicle should have been water or a soft drink, because 6 ounces of canned apple juice contains 2.3 mg ascorbate according to food tables (31) and unknown amounts of polyphenols, both of which react with nitrite and could have reduced the NPRO yield. As little as 9 mg (0.05 mmol) of ascorbic acid lowered the NPRO yield in volunteers given proline and nitrate, but 2 mg ascorbic acid had no effect (32). In the study by Leaf et al. (32), all three compounds were taken 2–3 h after a meal, whereas proline and ascorbic acid were taken with a meal in our tests, to simulate the normal situation where most nitrosatable compounds are food components. This would probably lessen the effect of small doses of ascorbic in our study.

Forty-four men performed the test. All used drinking water from private wells, 23 had <10 and 21 had >10 ppm nitrate-N in their water, and 22 were farmers. Seven men (four from those with ≥10 ppm nitrate-N) were cigarette smokers, with no apparent effect on NPRO. Matching for nitrate (1 low-nitrate man/high-nitrate man) was imperfect because nitrate levels for water collected for the test sometimes differed from those for the samples used to select the volunteers. The mean age was 39 and 41 years in the high- and low-nitrate groups, respectively. From the diaries and food composition tables, nitrate intake from food was estimated to be 20 ± 4 mg on day 4 and 29 ± 9 mg on day 5 for all 44 subjects (mean ± SE). These values are much less than the 75 mg/day standard in the United States (18), presumably in part because high-nitrate foods were restricted.

The detection limit for NPRO was 0.3 μg/24 h urine (μg/day). Mean basal NPRO for no nitrate in the water was about 1.0 μg/day. Table 1 shows the results on days 4 and 5 expressed as mean ± SE for all 44 subjects and for the high- and low-nitrate groups. These results suggested that on both days the high-nitrate group showed higher values than the low-nitrate group for urine NPRO, but the individual results for NPRO on days 4 and 5 plotted against water nitrate. Of the 88 NPRO results for both days, 45 were <1.5, 28 were 1.5–3.0, and 15 were 3.0–

### Table 1

Mean values for all measured parameters of urine, saliva, and water samples for all subjects and for those with ≥ and <10 ppm nitrate-N in their drinking water

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>All 44 subjects (mean ± SE)</th>
<th>21 subjects with ≥10 ppm nitrate-N (mean ± SE)</th>
<th>23 subjects with &lt;10 ppm nitrate-N (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 4</td>
<td>Day 5</td>
<td>Day 4</td>
</tr>
<tr>
<td>Nitrate</td>
<td>mg nitrate-N/liter</td>
<td>19.5 ± 3.2</td>
<td>38.1 ± 3.6</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>Nitrate</td>
<td>μg/day</td>
<td>1.98 ± 0.20</td>
<td>1.64 ± 0.17</td>
<td>1.54 ± 0.20</td>
</tr>
<tr>
<td>Nitrite</td>
<td>mmol/day</td>
<td>2.6 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Creactinine</td>
<td>g/day</td>
<td>2.7 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Volume</td>
<td>ml</td>
<td>1.70 ± 0.10</td>
<td>1.50 ± 0.08</td>
<td>1.63 ± 0.14</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>g/ml</td>
<td>1.023 ± 0.001</td>
<td>1.021 ± 0.001</td>
<td>1.024 ± 0.001</td>
</tr>
<tr>
<td>Saliva</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>mg/liter</td>
<td>10.1 ± 2.6</td>
<td>14.0 ± 2.5</td>
<td>16.1 ± 5.1</td>
</tr>
<tr>
<td>Nitrate</td>
<td>mg/liter</td>
<td>35 ± 5</td>
<td>54 ± 10</td>
<td>45 ± 10</td>
</tr>
</tbody>
</table>

* Collected once only.
Nitrosoproline Yield with High-Nitrate Water

Fig. 1. Values of day 4 NPRO (A) and day 5 NPRO (B) plotted against nitrate-N in the drinking water.

6.1 μg/day. Because of this skewed distribution, using the t test and linear regressions were not ideal approaches. Accordingly, all results were separated into those above and those below their median values and were analyzed by straightforward 2 x 2 tables using the Mantel-Haenszel χ² test (33). In this approach, the prevalence (proportion) of values of dependent parameters which exceeded the median value was compared for high and low categories of the independent variable.

Table 2 uses this approach to show the effect of nitrate in drinking water on day 4 and day 5 NPRO, on urine nitrate, and on saliva nitrite and nitrate. All of these variables were significantly associated with water nitrate, as shown by the P values. To illustrate the meaning of odds ratios, the ratio of 8.0 in the first group of Table 2 means that the likelihood of a high NPRO (NPRO above the median value) was 8 times greater in the high than in the low water nitrate group. The values entered in Table 2 for urine nitrate, saliva nitrite, and saliva nitrate were the means for each individual of the results for days 4 and 5. Use of these mean values was reasonable because these parameters did not differ significantly between the two days (Table 1), and the means of results on the two days were thought to give more accurate estimates than the individual results. When we calculated the effect of nitrate in drinking water on the separate day 4 and day 5 nitrate/nitrite parameters, similar associations were observed, all of which showed P < 0.03, but χ² values were lower and P values were higher than those in Table 2.

Table 3 shows the association of urine nitrate and creatinine with day 4 and day 5 NPRO and of day 4 NPRO with day 5 NPRO. NPRO excretion is tabulated against nitrate and creatinine excretion on the same day, i.e., this table mostly examines the effects of all three components of the same urine sample on each other. The associations of urine nitrate with NPRO were positive. The crude prevalence odds ratio for the dependence of day 4 NPRO on day 4 urine nitrate was 4.2, with P = 0.03 and a 95% confidence interval of 1.2-15.1 between the high and low categories of urine nitrate. Similarly, day 5 NPRO was associated with urine nitrate on day 5, with a crude odds ratio of 5.4 (P = 0.01). Day 5 NPRO correlated somewhat better with urine nitrate than with water nitrate, whereas the reverse was true for day 4 NPRO. On day 4, NPRO was also positively associated with creatinine, with a crude odds ratio of 3.9 and P = 0.03. No such association was observed on day 5. Surprisingly, there was little association between day 4 and day 5 NPRO, probably because of the different experimental conditions on the two days. Urine volume (median value, 1.5 liters) and specific gravity (median value, 1.02) did not affect NPRO excretion.

Multivariate analysis (34) was used to derive the adjusted odds ratios in Table 3. On day 4 the effect of urine nitrate on NPRO was somewhat reduced by correcting this effect for the influence of creatinine on NPRO, with an adjusted odds ratio of 3.4 (P = 0.08). On day 5, a combined consideration of day 5 creatinine and day 4 NPRO had little influence on the effect of urine nitrate on NPRO, i.e., the adjusted odds ratio for the effect of urine nitrate on NPRO was similar to the univariate estimate.

Discussion

The most important results were the significant associations between water nitrate and urine NPRO and between urine nitrate and urine NPRO. The median level of 10 ppm nitrate-N in the water is the EPA-recommended limit for safe drinking water, and the prevalence of high NPRO values was significantly greater above this value than below it. This supports the current EPA limit, if formation of the noncarcinogenic NPRO indeed reflects that of carcinogenic nitrosamines or other nitrite-derived carcinogens. A correlation between NPRO and water nitrate was also observed in rural Denmark by Møller et al. (35), who found a nearly significant (P = 0.08) association of urinary NPRO with water nitrate using statistical methods similar to those used here. All
Table 2  Effect of nitrate concentration in drinking water on NPRO, urinary nitrate, and salivary nitrite and nitrate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nitrate in water (ppm nitrate-N)</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Day 4 NPRO (µg/day)</td>
<td>17 (81%)</td>
<td>8 (35%)</td>
</tr>
<tr>
<td>≥1.5</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>&lt;1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 5 NPRO (µg/day)</td>
<td>12 (57%)</td>
<td>6 (26%)</td>
</tr>
<tr>
<td>≥1.5</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td>&lt;1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary nitrate (mmol/day)</td>
<td>16 (76%)</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>≥1.5</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td>&lt;1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salivary nitrite (mg/liter)</td>
<td>19 (90%)</td>
<td>9 (39%)</td>
</tr>
<tr>
<td>≥5</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>&lt;5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salivary nitrate (mg/liter)</td>
<td>17 (81%)</td>
<td>8 (35%)</td>
</tr>
<tr>
<td>≥25</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>&lt;25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Univariate analysis of differences between results above and below the median values, using 2 x 2 tables.

Numbers in parentheses, 95% confidence limits of the odds ratios.

Table 3  Univariate and multivariate analysis of the influence of urinary nitrate and creatinine on NPRO in urine, and of the influence of day 4 NPRO on day 5 NPRO

<table>
<thead>
<tr>
<th>Day 4</th>
<th>Urinary nitrate (mmol/day)</th>
<th>Crude odds ratio</th>
<th>Adjusted odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥1.5</td>
<td>&lt;1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 (75%)</td>
<td>5</td>
<td>4.2 (1.2-15.1)</td>
</tr>
<tr>
<td></td>
<td>10 (42%)</td>
<td>14</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>x² = 4.94, P = 0.03</td>
<td></td>
<td>x² = 3.11, P = 0.08</td>
</tr>
<tr>
<td></td>
<td>16 (73%)</td>
<td>6</td>
<td>3.9 (1.1-13.5)</td>
</tr>
<tr>
<td></td>
<td>9 (41%)</td>
<td>13</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>x² = 4.54, P = 0.03</td>
<td></td>
<td>x² = 2.69, P = 0.10</td>
</tr>
<tr>
<td></td>
<td>12 (63%)</td>
<td>7</td>
<td>5.4 (1.5-20.1)</td>
</tr>
<tr>
<td></td>
<td>6 (24%)</td>
<td>19</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>x² = 6.85, P = 0.01</td>
<td></td>
<td>x² = 6.26, P = 0.01</td>
</tr>
<tr>
<td></td>
<td>11 (48%)</td>
<td>12</td>
<td>1.8 (0.5-6.3)</td>
</tr>
<tr>
<td></td>
<td>7 (33%)</td>
<td>14</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>x² = 0.95, P = 0.37</td>
<td></td>
<td>x² = 0.79, P = 0.43</td>
</tr>
<tr>
<td></td>
<td>11 (44%)</td>
<td>14</td>
<td>1.4 (0.4-4.6)</td>
</tr>
<tr>
<td></td>
<td>7 (37%)</td>
<td>12</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>x² = 0.23, P = 0.63</td>
<td></td>
<td>x² = 0.19, P = 0.67</td>
</tr>
</tbody>
</table>

Analysis of differences between results above and below the median values. Values from the Day 4 section are all from day 4. Values from the Day 5 section are from day 5 (except that they include day 4 NPRO), i.e., values from each day were used and not the means for the two days.

These ratios are mutually adjusted within the day 4 group or within the day 5 group. On day 4, the influence of urine nitrate on NPRO is adjusted for the influence of creatinine. On day 5, the influence of urine nitrate on NPRO is adjusted for the influence of day 5 creatinine and day 4 NPRO, and similarly for the other influences on NPRO.

Numbers in parentheses, 95% confidence limits of the odds ratios.
the low water nitrate group (Table 1). These results are in the same range as the mean NPRO values of 1.9–3.8 μg/day in the Danish project (36). A study of areas with varied stomach cancer incidences in Poland showed median values of 1.8–2.8 μg NPRO in groups that were untreated or that took 100 mg proline 1 h after each of the three meals (35). A survey in an area of Colombia with a high incidence of stomach cancer found a mean of 0.93 μg NPRO/day for subjects not dosed with proline (27).

In the current project, the association of NPRO with water nitrate was somewhat stronger on day 4 than on day 5, when proline was taken and the subjects fasted overnight (Table 2). Also, mean NPRO yield was similar on days 4 and 5 (Table 1). These results were surprising because others have reported an increase in NPRO formation when proline was given, e.g., in the original report by Ohshima and Bartsch (4), in which a fasted man produced 1.7 μg NPRO after taking 375 mg (6 mmol) nitrate without proline and 14 μg NPRO after taking the same nitrate dose and 250 mg proline. In our current studies on men taking 400 mg (6.5 mmol) nitrate with a standard meal (3), mean NPRO yield was 2.4 μg when 500 mg proline were given with the meal and 1.1 μg when the proline was omitted. The effect of the proline dose was less clear when proline was taken by subjects eating their normal diets. The ingestion of 100 mg proline 1 h after each of the 3 daily meals increased the median NPRO yield 1.5- and 2.6-fold in studies on healthy subjects in areas of China with high and low rates of esophageal cancer, respectively (10); 3.3- and 1.2-fold in areas of Japan with high and low rates of gastric cancer, respectively (11); and 1.6- and 1.2-fold in areas of Poland with high and low rates of gastric cancer, respectively (36). The similarity in NPRO yield on days 4 and 5 despite the ingestion of proline only on day 5 (Table 1) may be explained by two factors: (a) dietary proline consumption in meat and other protein-rich foods may have been less on day 5 than on day 4 because of the partial fast on day 5, so that total proline consumption (including the proline dose) may have been similar on the two days; (b) total nitrate consumption was probably similar on the two days, as indicated by the similar nitrate excretions on these days (Table 1).

The association of NPRO with urinary nitrate on both days 4 and 5, with P < 0.03 (Table 3), has been observed before, e.g., in surveys in Colombia (27) and Poland (36). These studies suggest that urine nitrate is a useful measure of in vivo exposure to nitrate.

The association between creatinine and NPRO on day 4 (with P = 0.03), but not on day 5 (Table 3), was unexpected, although a similar association was observed in the Danish study (34; unpublished data). This association was less significant (P = 0.10) when the effect of creatinine on NPRO took into account the effect of urinary nitrate on NPRO, i.e., when the adjusted odds ratio was used (Table 3). If real, this relationship is attributed (a) to an increased intake of proline or NPRO in protein or more endogenous nitrate formation when more meat was consumed, because urinary creatinine arises partly from creatine in meat, and (b) to a parallel clearance of creatinine and NPRO from the blood by the kidneys (37). The lack of association between creatinine and NPRO on day 5 may reflect the origin of much of the proline on that day from the administered dose, rather than from meat.

The highly significant associations of water nitrate with saliva nitrate and nitrite on both days 4 and 5 (Table 2) occurred even though only single saliva samples were collected on each day. Saliva nitrate and nitrite levels are well known to rise after nitrate is ingested (23, 24, 38), but this appears to be the first report of such an association for people drinking high- and low-nitrate water under near-normal living conditions.

In conclusion, the prevalence of high NPRO excretion in Nebraskans drinking water with ≥10 ppm nitrate-N was significantly greater than that for men drinking water with <10 ppm nitrate-N, similar to findings in Denmark (35). However, our study was done under “worst case” conditions, with a nitrate intake from the diet of only 20–30 mg compared to the normal U.S. intake of 75 mg (18) and a restricted intake of ascorbate, and the association of NPRO with water nitrate may be less evident with a more normal diet. The significance of these findings for people drinking high-nitrate water remains to be determined.

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