Nitrate Contamination of Drinking Water: Evaluation of Genotoxic Risk in Human Populations

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Nitrate contamination of drinking water implies a genotoxic risk to man due to the endogenous formation of carcinogenic N-nitroso compounds from nitrate-derived nitrite. Thus far, epidemiological studies have presented conflicting results on the relation of drinking water nitrate levels with gastric cancer incidence. This uncertainty becomes of relevance in view of the steadily increasing nitrate levels in regular drinking water supplies. In an attempt to apply genetic biomarker analysis to improve the basis for risk assessment with respect to drinking water nitrate contamination, this study evaluates peripheral lymphocyte chromosomal damage in human populations exposed to low, medium, and high drinking water nitrate levels, the latter being present in private water wells. It is shown that nitrate contamination of drinking water causes dose-dependent increases in nitrate body load as monitored by 24-hr urinary nitrate excretion in female volunteers, but this appears not to be associated with peripheral lymphocyte sister chromatid exchange frequencies.

Introduction

Chemical compounds of toxic potential entering a public water supply may impose an immediate risk on human health. In most European countries, nitrate levels in rivers and groundwaters have increased gradually over the last decade mainly as a consequence of large-scale agricultural application of manure and fertilizers, thereby threatening drinking water quality (1). World Health Organization (WHO) and European Economic Community (EEC) guidelines on the quality of drinking water indicate a maximally admissible nitrate concentration of 44.3 and 500 mg nitrate/L, respectively, and international future scenarios predict that these standards will be exceeded at high incidence in the year 2000 (1,2). The WHO Joint Expert Committee on Food Additives has set the acceptable daily intake for nitrate at 5 mg/kg body weight based on no-effect levels observed in numerous experimental animal as well as in human studies on the induction of methemoglobinemia by nitrate-derived nitrite; bottle-fed infants of 3–5 kg body weight are considered to represent the population subunit at high risk for the resulting cyanosis (1,3–5).

Endogenous nitrosation of nitrate-derived nitrite resulting in the formation of carcinogenic N-nitroso compounds may induce a second health risk possibly associated with increased intake of nitrate (5). After gastrointestinal resorption and recirculation of foodborne nitrate, reduction to nitrite occurs in the oral cavity by bacterial activity, and carcinogenic N-nitroso compounds may subsequently be synthesized in the stomach from reingested nitrite and secondary amines also present in food. However, epidemiological studies have presented conflicting evidence to support this hypothesis: both inverse or absent associations (6–10) and positive correlations (9,11–14) have been reported, the latter indicating an increased stomach cancer risk at nitrate drinking water levels of 20 to 30 mg/L (12,13). The WHO concluded (5) that no firm epidemiological evidence has been provided to link increased incidence of gastric cancer to high nitrate levels in drinking water. However, it was stated that the available data are inadequate. The controversy on the relation between environmental nitrate burden and carcinogenic risk again may become of relevance in view of the steadily increasing levels of nitrate contamination of drinking water supply, in particular since it has been calculated that at nitrate concentrations of 50 mg/L, consumption of drinking-waterborne nitrate considerably contributes to overall nitrate intake (15).

In The Netherlands, in the Province of Limburg, drinking water quality is threatened as a consequence of nitrate pollution of groundwater due to soil fertilization with manure mainly from animal origin. Dilution with nitrate-free water is already required to prevent exceeding the EEC nitrate standard of 50 mg/L. In a pilot study, we correlated gastric cancer mortality rate in 11
towns in the Limburg area to the respective nitrate levels of drinking water thereby also using data on esophageal and bladder cancer incidence since carcinogenic N-nitroso compounds show distinctly organotropic actions involving various other targets than only the stomach (16). No significant association was found, partly because of the unavoidably insufficient statistical power. In view of recommendations to apply validated biomarkers to environmental health field studies to cope with these methodological limitations of cancer epidemiology (17), we subsequently considered evaluation of biological effect parameters with respect to the possibility of carcinogenic risk of nitrate ingestion via drinking water. The aim of this study, therefore, was to evaluate the relation between consumption of nitrate and genotoxicological events indicative for cancer risk in humans living in areas with different levels of drinking water nitrate contamination.

Materials and Methods

Three test populations increasingly at risk for nitrate intake via drinking water were identified. Housewives from two municipalities with regular drinking water supply represented the “low” risk group exposed to 0.13 mg nitrate/L of drinking water and the “medium” risk group exposed to a nitrate level of 32.0 mg/L and volunteered to participate by informed consent. The participants had no diseases, no occupational exposures, no use of medicine, were not pregnant, and were standardized for socioeconomic status by selecting families living in the same type of governmentally subsidized houses. Furthermore, they were individually matched on age (mean age 33 ± 5 years for the low risk group and 33 ± 4 years for the subgroup at medium risk), smoking behavior (mean smoking years 13.2 ± 4.7 versus 14.9 ± 3.4 years), as well as period of inhabitation (14.7 ± 12.1 versus 18.1 ± 12.1 years). Subjects agreed to answer to a questionnaire on food consumption and lifestyle habit, as well as to donate samples of saliva, 24-hr urine, and venous blood. As subgroup at high risk for nitrate intake via drinking water, female subjects were approached who make use of private wells for drinking water supply; these waters were verified by our laboratory to contain nitrate levels ranging from 560 to 3110 mg/L (mean 133.5 ± 68.5 mg/L), at least over the last 2 years. Both test groups at low and medium risk consisted of 30 individuals. Due to the limited occurrence of highly contaminated private drinking water wells, the population at high risk was restricted to 18 females, who furthermore tended to be older (average 40 ± 8 years) and therefore could not be individually matched to persons from the low exposure group. Sampling occurred during the months of May, June, and August of 1989, between 9:00 and 12:00 A.M. Subjects were asked to refrain from food until sample collection. Acutely produced, 5-mL saliva samples and 24-hr urine excretions were handled as recommended (18) and stored on ice. Five-milliliter venous blood samples were collected in heparinized tubes. All samples were transported to the laboratory as soon as possible (in general within 1 to 2 hr).

The questionnaire inquired about lifestyle factors that are known to confound sister chromatid exchange (SCE) frequency, and frequency and amount of consumption of foods as subdivided into three categories: drinking of alcohol as well as nonalcoholic beverages with special attention for consumption rate of coffee, tea, beer, milk, and tap water; intake of potatoes, fruits, and vegetables that are generally known to contain rather high concentrations of nitrite and nitrate; and consumption of bread, sandwiches, cheese, and sandwich meats also known for their high nitrite and nitrate levels. Oral intake of nitrate was calculated from these food inquiry data in combination with nitrate concentrations as determined in municipal drinking water supplies or private water wells, respectively, and nitrate food contents as analyzed in Dutch foods by various national research institutes (National Institute for Public Health and Environmental Protection, Bilthoven; TNO-CIVO Toxicology and Nutrition Institute, Zeist; etc.). For analysis of nitrite in saliva, 5-mL saliva samples were collected in tubes containing 0.5 mL of 1M NaOH dried under N2 as a preservative and upon transportation to the laboratory, stored at 4°C. Before analysis, 0.1 mL of 1 M ZnSO4 was added to 2 mL of saliva, which was placed on ice for 15 min. The mixture was subsequently centrifuged for 10 min at 3000g. The nitrite content was determined by a standardized colorimetric method (ISO method no. 2918) (18).

Before analysis of nitrate content of saliva, 1 to 2 mL of 0.15 M ZnSO4 was added to 2 mL of saliva, and the mixture was placed on ice for 15 min. After centrifugation for 5 min at 12,000g, supernatants were filtered through 22-μm Millex GM filters (Millipore). The nitrate concentrations were determined by HPLC (19), using a Kratos Spectroflow 400 pump, a Kratos Spectroflow 980 programmable UV detector set at 214 nm, a Chrompack Microsphere C18 column (100 x 3.0 mm ID), and a precolumn packed with Microsphere C18. The mobile phase consisted of Pic A reagent (0.005 M aqueous tetramethylammoniumphosphate). Flow rate was 1.0 mL/min.

For the determination of urinary concentrations of nitrate, 7.5 g NaOH was added to the 24-hr urine samples that were subsequently stored at 4°C. Immediately before analysis, ZnSO4 (2 M) was added to 10 mL or urine, and the mixture was allowed to stand on ice for 15 min and centrifuged for 10 min at 3000g. A 50-μL sample was taken for the analysis of nitrate using the spectrophotometric method by Boehringer Applying nitrate reductase (20). It appeared to be impossible to determine urinary nitrate levels by HPLC or nitrate concentrations in saliva by spectrophotography due to interference by other compounds. Twenty-four-hour urinary nitrate excretion was calculated by multiplying urinary nitrate concentrations with the amount of urine excreted daily. Evaluation of genetic risk in the three test populations was performed by determination of chromosomal damage in peripheral lymphocytes sampled by venous puncture, by application of the SCE assay, which has proven to be a reliable tool for genotoxicological testing in man (21). The method for SCE frequency assessment has been described previously (22). In essence, 0.4 mL of full blood was cultured for 72 hr at 37°C with 5 mL of RPMI 1640 medium enriched with 10% fetal calf serum and containing 125 U/mL penicillin, 125 μg/mL streptomycin, 5 mM l-glutamine, and 50 μ/mL heparin, with phytohemagglutinin (PHA) added in order to stimulate cell growth. 5-Bromo-deoxy-uridine (BrDU) was added to the lymphocyte cultures in a final concentration of 58 μM after a 24 hr of PHAincubation. After 72 hr of culturing, metaphases were harvested by treatment with colcemid (0.2 μg/mL) for 2 hr. After induction of cell lysis by hypotonic KCl treatment, lymphocyte SCE slides were prepared and stained by means of the 33258 Hoescht-plus-Giemsa technique. Per individual, two lymphocyte cul-
ures were initiated, and per cell culture, at least 10 metaphases (20 metaphases per individual) were analyzed, with a minimum of 40 chromosomes. Numbers of SCE were normalized over 46 chromosomes and averaged as number of SCE per cell per individual. Slides were encoded and analyzed blindly by two independent observers.

Statistical evaluation of differences between several groups with respect to nitrate intake via drinking water and food, as well as urinary nitrate excretion, was performed by means of one-way analysis of variance. Statistical significance of differences between two respective subgroups was analyzed by means of Student's t-test for unpaired values. Linear and multiple regression analysis was applied to evaluate the reliability of associations between various effect parameters. Odds-ratio analysis was used to study the elevated risk for SCE incidence in specific population subunits in comparison with the overall population. Relationship of SCE frequencies with various levels of nitrate exposure and with smoking behavior has been evaluated by means of two-way analysis of variance (unbalanced design).

Results

Figure 1 shows overall nitrate body burden in exposed populations calculated as nitrate doses from intake of drinking water (Fig. 1A) and of food (Fig. 1B), in relation to 24-hr urinary nitrate excretion (Fig. 1C). One-way analysis of variance indicated that calculated nitrate doses from drinking water intake differ significantly between groups at various nitrate exposure levels ($p = 0.0001$). Calculated nitrate doses from food consumption did not differ between the three populations ($p = 0.394$). Mean saliva concentrations of nitrate appeared to be $6.01 \pm 2.41 \mu g/mL$, $5.41 \pm 1.14 \mu g/mL$, and $23.39 \pm 8.18 \mu g/mL$ in the respective populations consuming drinking water with low, medium, and high nitrate concentrations. Twenty-four-hour urinary excretion of nitrate increased correspondingly with the risk for increased intake of nitrate (ANOVA level of significance at $p = 0.009$), and therefore appears to reflect daily nitrate consumption via drinking water reliably. This was confirmed by means of regression analysis that was applied to the total number of subjects ($n = 78$) without discrimination between smokers and nonsmokers, since it was demonstrated that individual 24-hr urinary nitrate excretions (Fig. 2) as well as individual saliva nitrate concentrations (data not shown) significantly (urinary, $r = 0.23, p < 0.05$; saliva, $r = 0.25, p < 0.05$) correlated with individual nitrate doses calculated from reported intake of drinking water.

Average saliva levels of nitrite in this study were $7.65 \pm 7.70 \mu g/mL$, $5.49 \pm 5.10 \mu g/mL$, and $11.65 \pm 7.67 \mu g/mL$ in humans exposed to low, medium, and high nitrate drinking water concentrations, respectively. Nitrite concentrations of saliva did not, however, reliably monitor oral nitrate intake since multiple regression analysis showed saliva nitrite content to be related to both nitrate and nitrite consumption at relatively large interindividual variation.

It is therefore concluded that determination of 24-hr urinary excretion of nitrate can be used as a marker to monitor whole-body nitrate load in cases of increased nitrate intake. It is furthermore concluded that nitrate contamination of drinking water results in dose-dependent increases in body nitrate burden as measured by both saliva nitrate concentration and 24-hr urinary nitrate excretion.
Table 1 shows the average SCE frequency in peripheral lymphocytes derived from subjects exposed to low, medium, and high nitrate levels in their drinking water. It is indicated that increased body nitrate burden does not affect lymphocyte SCE frequency in the total population. A statistically significant interaction with respect to peripheral lymphocyte SCE frequency occurred between nitrate exposure and smoking behavior. Odds-ratio analyses also showed no increased risk for periperal lymphocyte SCE incidence in population subunits exposed to relatively high nitrate levels in their drinking water. Table 1 furthermore indicates that cigarette smoking induces peripheral lymphocyte chromosomal damage: SCE frequencies in smoking individuals were significantly elevated in comparison with nonsmokers in the studied populations at medium and high risk for increased nitrate intake. Furthermore, Student's t-test analysis showed that nonsmokers with an historical habit of cigarette smoking had no higher lymphocyte SCE frequencies than subjects who have no smoking history: averaged SCE frequency in nonhistory nonsmokers was 7.3 SCE/cell (n = 33) versus 7.2 SCE/cell in nonsmoking subjects with a smoking history (n = 17). This was confirmed by means of odds-ratio analysis.

Table 1. Average frequency of peripheral lymphocyte sister chromatid exchanges (mean ± SD) in populations at different levels of drinking water nitrate exposure.*

| Group     | Low nitrate (0.13 mg/L) | Medium nitrate (32.0 mg/L) | High nitrate (133.5 ± 68.5 mg/L) |
|-----------|-------------------------|-----------------------------|-----------------------------------|
| Nonsmokers| 7.7 ± 1.6               | 6.9 ± 1.0*                  | 7.2 ± 0.9*                        |
|           | (18)                    | (19)                        | (13)                              |
| Smokers   | 8.0 ± 1.4               | 9.3 ± 1.9*                  | 8.6 ± 1.0*                        |
|           | (10)                    | (11)                        | (4)                               |
| Total     | 7.8 ± 1.5               | 7.8 ± 1.8                   | 7.5 ± 1.1                         |
|           | (28)                    | (30)                        | (17)                              |

*Relationship of SCE frequencies with nitrate exposure level and smoking was evaluated by means of two-way analysis of variance (unbalanced design): effect of nitrate exposure was not statistically significant (p = 0.774); effect of smoking was highly significant (p = 0.0003); significant interaction of nitrate exposure and smoking occurred at p = 0.022.

*Numbers in parentheses are numbers of subjects.

*Significant difference between smoking and nonsmoking subgroups at p < 0.05, Student's t-test for unpaired values.

Figure 3 demonstrates individual SCE frequency in relation to 24-hr urinary nitrate excretion and shows a statistically nonsignificant tendency toward a negative correlation. Multiple regression analysis evaluating the possible contribution to peripheral lymphocyte SCE frequency of urinary nitrate excretion, smoking (number of cigarettes smoked per day, number of cigarette-years), age, and use of oral contraceptives indicated that only the number of cigarettes smoked daily was significantly (beta = 0.14) associated with lymphocyte frequency. It is therefore concluded that increased body nitrate burden caused by nitrate contamination of drinking water supply does not present a genotoxic risk as monitored by peripheral lymphocyte SCE frequency.

Discussion

Data on body nitrate load as reported in the literature tend to vary rather extremely. Reported saliva concentrations range from 7 to 62 μg/mL (7,23,24), while levels of 24-hr urinary nitrate excretion vary from approximately 50 to 90 mg/day (25,26). Furthermore, evaluation of the relation between body nitrate load and exposure to nitrate in the general environment has yielded conflicting results (7,26). The present study reports saliva concentrations and 24-hr urinary excretions of nitrate which fit well within this wide range, but furthermore shows distinct relations between body nitrate levels and oral doses as calculated from drinking water intake, probably demonstrating an endogenous steady state in nitrate kinetics.

At these levels of nitrate intake, the formation of carcinogenic N-nitroso compounds from nitrate-derived nitrite should be expected. In man, endogenous nitrosation has been observed to increase steeply at rather high oral nitrate doses (> 260 mg/day) but has also been shown to occur at normal dietary nitrate intake (27). Data from food inquiries in the present study indicate that in the investigated populations at low, medium, and high risk for increased nitrate consumption, 30, 33, and 61%, respectively, exceed this nitrate threshold dose of 260 mg/day. However, in view of the fact that subjects at low nitrate exposure risk reported an average daily nitrate consumption of 209.7 ± 89.5 mg/day, which is considerably higher than previously published data on total dietary nitrate intake (1,2,7,15), overreporting of nitrate-containing food consumption may be possible. Despite this possible bias, it may still be assumed that endogenous nitrosation in these subjects does occur since consumption of drinking water with nitrate levels of approximately 50 mg/L and higher has recently been demonstrated to be correlated to increased exposure to endogenously produced N-nitroso compounds (28). In this respect, it should be noted that evaluation of genotoxic risk in general cannot solely be based on peripheral lymphocyte SCE frequency determination but, as recommended (21), should implement analyses of other cytogenetic end point parameters, for instance HGPRT locus point mutations or formation of micronuclei as well.

The negative although statistically nonsignificant association between 24-hr urinary nitrate excretion and peripheral lymphocyte SCE frequency observed in this study is of interest in view of previous epidemiological results that show negative correlations between estimated nitrate consumption and gastric cancer mortality (7-9). Our pilot epidemiological study among 11 municipalities in the Limburg area based on standardized
mortality rate analysis also indicated an inverse, statistically non-significant relation between regular drinking water nitrate concentrations and stomach carcinogenesis. The negative relation between endogenous nitrate load and lymphocyte chromosomal damage should not, however, be interpreted as an indication for a protective mechanism induced by nitrate consumption, since there are no data on antimutagenic or anticarcinogenic effects of nitrate available (8).

Recent investigations have shown nitrite to possess genotoxic potential in laboratory rodents (29), although no indication for carcinogenicity was found in rats (30). Forman et al. (7) concluded that at saliva nitrite concentrations of 1.8 to 2.85 μg/mL, no association with the incidence of gastric cancer can be demonstrated. Individual saliva nitrite levels as observed in this study, however, were not positively correlated with peripheral lymphocyte SCE frequency, indicating no genotoxic potential of nitrite in man at these endogenous levels.

In conclusion, nitrate contamination of drinking water causes dose-dependent increases in body nitrate burden in exposed humans. This is probably not associated with genotoxic risk at drinking water nitrate levels that do not exceed the WHO guideline (5), as is demonstrated by the absence of increased chromosome breakage tendency of peripheral lymphocytes. Future studies should use analysis of peripheral lymphocyte DNA point mutation indices in relation with determination of endogenous nitrosation to improve genotoxic risk assessment in human populations exposed to increased nitrate levels in their environment.

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