Urinary excretion of 3-methyladenine after consumption of fish containing high levels of dimethylamine

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The urinary excretion of the DNA alkylation product, 3-methyladenine (3-MeAde), was measured in human volunteers who were on controlled diets and consumed fresh fish, or frozen-stored fish that contained 50-fold higher levels of dimethylamine (DMA), with or without ingested nitrate. DMA potentially could react with nitrosating agents in the diet or within the body, and produce the potent carcinogen N-nitrosodimethylamine (NDMA), which can then react with DNA to form several adducts including 3-MeAde. Our findings show that there was no increase in urinary levels of 3-MeAde after consumption of fish preserved by frozen storage relative to levels after consumption of fresh fish. Furthermore, consumption of 225 mg sodium nitrate (equal to the nitrate content in a large glass of beet juice) at 1 h prior to consumption of the frozen-stored fish did not increase urinary 3-MeAde levels as would be expected if nitrate enhanced endogenous nitrosation of DNA. In contrast, urinary excretion of 3-MeAde from a volunteer who was a moderate cigarette smoker (11 cigarettes per day) was ~3- to 8-fold higher than dietary 3-MeAde intake. These findings indicate that consumption of high levels of DMA in fish does not result in detectable levels of NDMA formation and genetic damage as measured by the urinary biomarker 3-MeAde.

Introduction

Humans are exposed to N-nitrosodimethylamine (NDMA*), a potent carcinogen, from many sources including endogenous formation (1–4). The secondary amine precursor, dimethylamine occurs naturally in many foods (5,6) including fish where levels increase during freezing (7,8). Although there is indirect evidence that NDMA forms in the stomach (4), the amount formed cannot be readily quantitated in blood or urine because NDMA is rapidly metabolized. NDMA methylates DNA in several positions, including the 3-methyl position of adenine (9,10). The 3-methyladenine (3-MeAde) is rapidly cleaved from DNA by glycosylases, or spontaneously due to weakening of the N-glycosidic bond and excreted in urine (see review in 11). Results from these studies suggest that urinary 3-MeAde can be used as a non-invasive biomarker for estimating exposure to methylating compounds. For example, cancer patients receiving methylnitrosourea, a DNA methylating and chemotherapeutic agent, had a dose-dependent increase in urinary 3-MeAde during the 24 h after treatment from baseline levels (12). Also, administration of two NDMA precursors, aminopyrine and nitrite, to rats resulted in a dose-dependent increase in urinary 3-MeAde (13). Increased levels of 3-MeAde also are detected in urine of cigarette smokers (14–16). Although 3-MeAde represents only ~10% of total urinary adducts resulting from a dose of methylating agent, it is a more sensitive indicator of DNA methylation than other methylated bases, such as 7-methylguanine, because background levels are lower (11,13). However, there are sources of pre-formed 3-MeAde in the diet (13,17), possibly as a result of the use of methylbromide as a fumigant (17,18). Therefore, in order to use this biomarker, it is necessary that subjects consume a controlled diet to minimize background levels of 3-MeAde, which would probably mask the effect of low-level exposure to methylating agents (18–20).

Over the years, concerns have been raised regarding the health risk of consuming high levels of dietary secondary amines, which may result in the endogenous formation of carcinogenic N-nitroso compounds (5,6,21). Licht and Deen (22) developed a mathematical model predicting the rates of nitrosamine and nitrosamide formation in the human stomach and their findings indicate that the endogenous formation of NDMA from a typical Western diet rich in dimethylamine is insignificant with respect to levels of NDMA pre-formed in the diet. However, some studies have reported that endogenous nitrosation of secondary amines can occur and contributes to the exposure to N-nitroso compounds (23–28). In this study, we used urinary 3-MeAde as a biomarker to determine if consumption of fish that had been preserved by freezing and consequently, contained elevated levels of dimethylamine, resulted in higher levels of endogenous NDMA formation and DNA damage than did fresh fish consumption.

Material and methods

The liquid diet Iso shake (Vanilla No. 9175 and Fruits des bois No. 9178) was obtained from Laboratoire Sopharga, Creully, France. Fresh cod was obtained at a local supermarket. Frozen cod was prepared at Nestlé R&D Center, Bjuv, Sweden. Fresh cod fillets were minced, frozen and stored at ~10°C for 14 weeks in order to accelerate DMA formation, so that comparable values of DMA, which are formed following a longer period of storage at ~18°C, could be obtained (C.Steen, unpublished observations). The fillets were then sent on dry-ice to the Nestlé Research Center, Vers-chez-les-Blanc, Switzerland prior to cooking. 3-Methyladenine was obtained from Sigma (Buchs, Switzerland). Sepharose CL-4B resin was purchased from Pharmacia. Deuterium labeled (d3-3-MeAde), tritium labeled 3-MeAde (6000 dpm/ng), and monoclonal antibodies bound to Sepharose CL-4B which recognize 3-MeAde, were prepared as previously described (19,20). All solvents were gradient grade.

Study design

Ten male volunteers, age 22–50, who did not smoke or take medications, participated in the study. A written description of the protocol as well as individual or small group discussions were provided. After giving consent,
volunteers underwent a health-screening, including a medical questionnaire, measurements of blood pressure, pulse, height, and weight, and a blood sample was taken (after a 12 h fast) for routine clinical parameters. The study was approved by the Ethical Committee of the Nestlé Research Center.

Subjects were given a free-choice diet for 2 days (days 1 and 2), and then were given a standardized liquid diet and bottled water for 5 days (days 3–7). On day 5, they were also given 300 g of prepared fresh fish (low dimethylamine content); on day 6, 300 g prepared frozen fish (high dimethylamine content); and on day 7, 325 mg sodium nitrate (equal to the nitrate content of a large glass of beet juice) and 1 h later when peak gastric nitrite levels were present (29), 300 g prepared frozen fish was consumed. Five randomly chosen subjects had the treatments for days 5 and 7 reversed. The fish (300 g each) was placed in plastic bags and cooked in boiling water, 10 min for fresh fish and 30 min for frozen fish. Subjects were asked to consume all of the fish including the juices performed during the cooking. Urine samples were collected throughout the experiment in 2 l bottles containing 1 g sodium azide. Volunteers were instructed to keep urine collections refrigerated whenever possible. Collections were pooled every 24 h from 11:30 a.m., the total volume was recorded and the aliquots were stored at −40°C until analysis. Volunteers kept a journal in which they noted any changes in health and deviations from the protocol. All samples were coded in order to avoid any bias in the data analysis.

The 3-MeAde content in urine from a volunteer who smoked, and who had participated in a previously reported study (14) on the effect of cigarette smoking and urinary 3-alkyladenine excretion, was also measured.

3-Methyladenine quantitation

3-MeAde was quantitated in urine, water, fish and liquid diet samples by high performance liquid chromatography–mass spectrometry (HPLC–MS) after clean-up by immunoaffinity chromatography, as described by Prevost et al. (19,20), with the following modifications. $d_3$-3-MeAde (44.7 ng/3 ml urine) was added as an internal standard prior to clean-up and samples were analyzed in triplicate. Urine was added to 4 vol. of methanol and placed in an ultrasonic bath for 5 min and then centrifuged for 15 min at 3000 g. The supernatant was recovered and the pellet was washed with 2 ml methanol. The combined supernatants were dried to dryness and redissolved in 4 ml PBS. The urine extract was applied to the immuno-affinity column and the column was washed with 3 ml PBS, followed by 10 ml water. Then, 3-MeAde was eluted with 3 ml 1 M acetic acid and evaporated to dryness under vacuum.

The 3-MeAde content in the liquid diet (10 ml) was determined with $d_3$-3-MeAde (15 ng/ml) as internal standard. The liquid diet was added to 30 ml methanol containing 1% acetic acid and placed on ice for 30 min. After centrifugation at 10,000 g for 15 min, the supernatant was rotary evaporated to dryness and suspended in 1 mM HCl (4 ml). The solution was extracted twice with 5 ml ethyl acetate and the aqueous phase was evaporated to dryness. The extract was dissolved in 4 ml PBS and purified by immunoaffinity chromatography as described above.

After cooking, the fish (300 g) was homogenized with 150 ml methanol containing 1% acetic acid. $d_3$-3-MeAde was added as an internal standard (11.9 ng/g fish wt prior to cooking). Aliquots of 10 g of the homogenate were added to 30 ml methanolic:1% acetic acid, and incubated on ice for 30 min. The homogenate was then centrifuged at 10,000 g for 15 min and the supernatant was collected and rotary evaporated to dryness. The extract was dissolved in 1 mM HCl (4 ml), partitioned against ethyl acetate as above for the liquid diet, and the aqueous phase was evaporated to dryness. The residue was extracted with 5 ml methanol, washed twice more with 3 ml methanol and the combined methanol washes were evaporated to dryness. The extract was dissolved in 4 ml PBS and 3-MeAde was purified by immunoaffinity chromatography as described above.

HPLC-MS was performed with a Waters 600-MS pump, a Waters 486-MS UV detector and a Waters 717 auto sampler. Separation was achieved on a Supelco LC-18DB (5 μm, 250×4 mm) at a flow rate of 1 ml/min and injected with 20 μl of the sample. The mobile phase was comprised of solvent A: ammonium acetate, 10 mM, pH 7.6, and solvent B: methanol. The analysis commenced with 100% A over the first 5 min, increased to 100% B over 5 min and remained at 100% B for 5 min. The solvent was split before entering the mass spectrometer using an LC Packings Acurate™ microflow processor (Omnilab, Commugny, Switzerland), which admitted 100 μl into the source. Mass spectrometry was performed with a Finnigan MAT TSQ-700 mass spectrometer (Bremen, Germany), equipped with a Finnigan electrospray interface (ESI-II) that worked at a high voltage of 4.5 kV. The manifold temperature was 70°C and the heated capillary was set at 200°C. Nitrogen was used as sheath gas at a pressure of 4.8 bar. The $[M+H]^+$ ions of 3-MeAde and its internal standard $d_3$-MeAde were monitored at 150 and 153 m/z, respectively.

Determination of secondary and tertiary amines in fish and urinary nitrate

The dimethylamine, trimethylamine and trimethylamine-oxide contents in fish samples were quantitated as previously described (30–33). Urinary nitrate was measured as described by Green et al. (34).

Statistical methods

The cross-over trial was checked for treatment–period interactions and evaluated with the paired Student’s t-tests. The final repeatability standard deviations for 3-MeAde provided estimates of detection and quantification limits.

Results

The DMA and TMA content found in fresh and frozen fish samples are presented in Table I. Consistent with previous reports (3,7,8), the level of DMA increased during frozen storage of fish to values that were as much as 50-fold higher than those found in fresh fish. It should be noted that the amount of TMA detected in fresh fish was highly variable, which indicates that some of the fish designated as ‘fresh cod’ was of low quality and freshness. The levels of pre-formed 3-MeAde in frozen fish were also higher than in fresh fish. Low, detectable levels of 3-MeAde were also found in the liquid diets.

Pre-formed 3-MeAde is present in typical Western diets and contributes substantially to urinary levels of this adduct (13,18–20). In order to decrease this high background level, which would obscure the effects of low-level exposure to NDMA, the volunteers were given controlled liquid diets that had low levels of pre-formed 3-MeAde. An HPLC analysis of immuno-purified 3-MeAde from the urine of a subject on a free-choice diet and a controlled diet with fish consumption is presented in Figure 1. The 3-MeAde adduct was readily detected in the urine of subjects on the controlled diet. The calibration curve of 3-MeAde, with the deuterated internal standard, displayed a linear response over the concentration range from 0.05 to 5 ng/ml, which enabled accurate quantification of 3-MeAde in urine (Figure 2).

The urinary excretion of 3-MeAde as a function of diet for the eight subjects who completed the study is presented in Figure 3, and urinary nitrate levels are presented in Table II. In the first 2 days of the study when subjects consumed a free-choice diet, the levels of 3-MeAde were variable and ranged from 8 to 150 nmol/day. As has been previously reported (20), 3-MeAde was rapidly cleared, and basal levels of 3-MeAde (2–3 nmol/day) were reached on days 3 and 4 when subjects consumed a controlled liquid diet that contained low levels of pre-formed 3-MeAde. A slight increase in the level of 3-MeAde in urine was detected in seven of the eight subjects during the consumption of fresh or frozen fish on days 5 or 7. However, this modest increase is attributed to pre-formed 3-MeAde in fish. Notably, the consumption of 325 mg sodium nitrate with the frozen fish on days 5 or 7, which is readily discerned by the elevated nitrate levels detected in urine (Table II), did not result in increased levels of 3-MeAde as would be expected if nitrate enhanced endogenous nitration of dimethylamine to produce NDMA.

The combined average intake and urinary excretion of 3-MeAde from the eight subjects during the controlled liquid and fish diets of days 3 through to 7 are presented in Figure 4. The urinary excretion of 3-MeAde exceeded the pre-formed 3-MeAde intake by ~1.5- to 2-fold during consumption of the controlled liquid diet (days 3 and 4) and fresh fish (day 5 or 7). The difference in excretion versus intake of 3-MeAde in these volunteers was statistically significant (Student’s t-test;
Table I. Dietary levels of pre-formed 3-methyladenine, dimethylamine, trimethylamine and trimethylamine-oxide

<table>
<thead>
<tr>
<th></th>
<th>3-MeAde nmol/300 g</th>
<th>Dimethylamine µmol/300 g</th>
<th>Trimethylamine µmol/300 g</th>
<th>Trimethylamine-oxide µmol/300 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh fish</td>
<td>0.48 ± 0.06</td>
<td>23–520</td>
<td>76–6310</td>
<td>12 300–13 800</td>
</tr>
<tr>
<td>Frozen fish</td>
<td>2.76 ± 0.36</td>
<td>1860–1890</td>
<td>300</td>
<td>12 500</td>
</tr>
<tr>
<td>Liquid diet</td>
<td>0.16 ± 0.02</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</tbody>
</table>

One can of liquid diet = 375 ml; comparable values for vanilla or fruit drink.

TMA and DMA range values from at least six measurements for fresh fish and two measurements for frozen fish. TMAO range of seven values for fresh fish, a single measurement for frozen fish.

3-MeAde (n = 3 analyses from two samples of 300 g of fish).

NA = not analyzed.

Fig. 1. HPLC–MS analyses of 3-MeAde in urine of a volunteer on either (A) a controlled liquid diet, (B) a free-choice diet, or (C) an internal standard. The [M+H]^+ ions of 3-MeAde and its internal standard d3-MeAde were monitored at 150 and 153 m/z, respectively.

Fig. 2. Calibration curve of 3-MeAde and its trideuterated internal standard (5 ng/ml) over the concentration range of 0–5 ng/ml. The range of 0–0.5 ng/ml has been magnified.

Fig. 3. Urinary excretion of 3-MeAde in subjects as a function of diet (error bars and n = 3 ± SD). Data on frozen fish plus nitrate consumption, day 5 (subjects 9, 10, 11 and 13) and day 7 (subjects 2, 3, 6 and 8) were presented as day 7 to simplify the graphic presentation.

P < 0.0017 for controlled liquid diet; P < 0.016 for fresh fish consumption) and was consistent with previous observations (14,19,20). During the days when the subjects consumed the frozen-stored fish with or without nitrate, the observed differences in excretion versus intake of 3-MeAde were very slight; however, the levels of 3-MeAde excreted in urine were slightly less than the 3-MeAde intake (P < 0.0063). The global repeatability standard deviation (SD) was ~0.5 nmol.
Table II. Urinary excretion of nitrate in subjects on free-choice, controlled diet and controlled diet plus fish

<table>
<thead>
<tr>
<th>Subject</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
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<tr>
<td>2</td>
<td>889</td>
<td>781</td>
<td>616</td>
<td>478</td>
<td>728</td>
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<td>486</td>
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<td>6</td>
<td>1620</td>
<td>1808</td>
<td>896</td>
<td>615</td>
<td>672</td>
<td>580</td>
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<td>769</td>
<td>531</td>
<td>454</td>
<td>2863</td>
<td>673</td>
<td>489</td>
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<tr>
<td>10</td>
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<td>1321</td>
<td>488</td>
<td>698</td>
<td>3581</td>
<td>932</td>
<td>602</td>
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<tr>
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<td>1159</td>
<td>531</td>
<td>522</td>
<td>3301</td>
<td>750</td>
<td>433</td>
</tr>
<tr>
<td>13</td>
<td>647</td>
<td>766</td>
<td>618</td>
<td>521</td>
<td>2890</td>
<td>824</td>
<td>641</td>
</tr>
</tbody>
</table>

Subjects consumed a free-choice diet during days 1 and 2; controlled liquid diet on days 3 and 4; and fish diets during days 5–7. Sodium nitrate (325 mg) was given on either day 5 (subjects 9, 10, 11, 13) or day 7 (subjects 2, 3, 6, 8).

Fig. 4. Combined average intake and urinary excretion of 3-MeAde from all subjects (n = 8) and from one subject who was on a controlled diet ± smoking (11 cigarettes per smoking day, analysis performed in triplicate ± SD) with the mean values of urinary 3-MeAde. Data on frozen fish plus pre-formed 3-MeAde is present in a free-choice diet. There were consistent values seen in eight volunteers of this current study. The difference between 3-MeAde excreted in urine from the 3-MeAde intake during days the subject smoked was 4.3- to 9.7-fold higher than the difference between levels observed during days of non-smoking. These results demonstrate that the HPLC-MS method employed in this study is sufficiently sensitive to detect increased excretion of 3-MeAde in the urine of a moderate smoker (11 cigarettes per day).

Discussion

There is epidemiological data associating consumption of salted, fermented or dried fish containing volatile N-nitrosoamines with an elevated risk of nasopharyngeal, esophageal and stomach carcinoma in humans (35–37). To our knowledge, no data exist which suggests that ingestion of fresh or frozen stored fish is associated with an increased incidence of cancer. Consistent with this premise, the levels of volatile N-nitroso compounds (mainly NDMA) in fresh fish are generally well below 1 p.p.b. (3,38,39). The contamination of NDMA is dependent upon the DMA content in fish, which is low in fresh fish but increases over time during various storage conditions due to chemical, enzymatic or bacterial reactions that convert TMAO into DMA (3,7,8). Consequently, if fish is treated with a nitrosating agent, such as salt containing nitrate or nitrite, NDMA formation may occur. Concerns also have been raised regarding exposure to relatively high levels of dietary secondary amines, such as DMA, which can be nitrosated in the stomach to form potentially carcinogenic N-nitroso compounds (5,6,8,21). Thus, consumption of fish products preserved by frozen storage that contain high levels of DMA could potentially result in the endogenous formation of the carcinogen NDMA. Our data confirm previous observations that the levels of DMA increases in stored frozen-fish by as much as 50-fold (Table I). Therefore, we have used the urinary biomarker 3-MeAde, as a probe to assess the endogenous formation of NDMA or other methylating agents in fish, which can alkylate DNA and cause genetic damage.

Pre-formed 3-MeAde is present in a free-choice diet. Therefore, in order to detect the effects of methylating agents and increased excretion of 3-MeAde in urine following fish consumption, it was necessary for subjects to be placed on a strictly controlled diet that contained low levels of 3-MeAde (20). In addition to the increased DMA content in frozen fish, there is a concomitant increase in pre-formed 3-MeAde content (Table I), which may be attributed to reactive methylating species, methylation by S-adenosylmethionine, or by enzymatic conversion of DNA through methyltransferases during frozen storage (40,41). The presence of 3-MeAde in the marine sponge Topsentia genitrix has been previously reported (42).
urine was actually slightly less than 3-MeAde intake at 24 h after consumption of frozen fish that was high in DMA content. This discrepancy may in part be attributed to a slight overestimation of the 3-MeAde content of frozen fish since two representative fish samples (2×300 g) and not each individual fish per meal per volunteer, were used to determine the 3-MeAde intake. The consumption of 325 mg sodium nitrate at 1 h prior to the frozen-stored fish did not increase urinary levels of 3-MeAde and suggests that endogenous formation of NDMA from DMA is insignificant. The 95% confidence interval of 3-MeAde levels excreted in urine on day 6, when the subjects consumed frozen fish, was 2.78–3.70 nmol 3-MeAde; while the 95% confidence interval for days 5 or 7, when the subjects consumed frozen fish plus nitrate, was 2.32–3.08 nmol. In order to show a significant treatment effect with nitrate, the amount of 3-MeAde excreted in urine during day 5 or 7 when subjects consumed frozen fish plus nitrate would have had to exceed 3.70 nmol 3-MeAde/day; however, during these days, the urinary levels of 3-MeAde did not exceed 3.43 nmol in any of the subjects. In contrast, urinary 3-MeAde levels were increased 3- to 8-fold over dietary intake when a volunteer on a controlled diet resumed moderate smoking (11 cigarettes per day) and supports the notion that N-nitroso compounds or other reactive methylating agents in tobacco react with DNA to form 3-MeAde (45).

The deliberate nitrosation of fish under simulated gastric juice conditions showed that the formation of nitrosamines (mainly NDMA) in the stomach after ingestion of these foods was unlikely (46). In support of these data and theoretical calculations cited above, we did not detect an increase in the urinary excretion of 3-MeAde, a biomarker of NDMA exposure, in subjects who consumed fish conserved by frozen storage with or without ingested nitrate. Therefore, exposure to endogenously formed NDMA from dietary DMA in frozen fish appears negligible compared with exposure from preformed dietary N-nitroso compounds (47,48). These findings should help to allay some of the concerns regarding the health risks of consumption of high levels of DMA in fish products and other daily staples.

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References


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