

# Formation of *N*-(Nitrosomethyl)urea in Stomachs of Experimental Pigs and Human Volunteers Given Fish Sauce in Vivo

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*N*-(Nitrosomethyl)urea (NMU) was characterized in carcinogenic nitrosated fish sauce recently (Deng et al. *J. Agric. Food Chem.* **1998**, 46, 202–205; *Biomed. Environ. Sci.* **1999**, 12, 54–61). To study the possibility of intragastric synthesis of NMU, experimental mini-pigs surgically fitted with a flexible stomach cannula and human volunteers were used. Fish sauce samples (20–30 mL) and nitrite were injected into the gastric lumen through the cannula for pigs or taken orally for human volunteers. Gastric juice samples were taken out 30 min later. Concentration of NMU in condensed extracts of these samples was analyzed with HPLC–photohydrolysis–pyrolysis–thermal energy analyzer. Results showed that there was formation of NMU in the gastric lumen of both models in vivo and that the formation of NMU was nitrite- and pH-dependent. NMU was also detectable in the condensed extract of 100 mL of a mixture of pooled fasting human gastric juice samples and fish sauce sample (9:1, v/v) after treatment with 500  $\mu\text{mol/L}$  of nitrite in vitro. In conclusion, there is intragastric formation of NMU, even at natural amounts of nitrite.

**Keywords:** *Nitrosomethylurea; fish products; nitrosation; pig; human; gastric juice; stomach cancer; nitrosamide; nitroso compounds; nitrite; HPLC*

## INTRODUCTION

*N*-Nitrosamides are considered to be important kinds of candidates of causal factors of human stomach cancer (Mirvish, 1983). We reported previously that the human intragastric exposure level of total *N*-nitrosamides was significantly correlated with a high risk of stomach cancer (Deng et al., 1997; Zhang et al., 1991). However, little is known about the chemical structure, distribution, and metabolism of natural *N*-nitrosamides in human environments.

Fish sauce is a traditional seasoning used daily (~30 mL/capita/day) by the local residents at Changle County, the highest risk area for stomach cancer in China. Its daily intake is significantly correlated to the mortality of stomach cancer (Ye et al., 1994). It is rich in creatinine and other nitrosable amines (Zhang et al., 1993). It was reported that fish sauce samples from this county were carcinogenic after treatment with nitrites under simulated stomach conditions (Deng et al., 1991). *N*-(Nitrosomethyl)urea (NMU) was characterized from the nitrosated fish sauce with HPLC-PHPS-TEA and confirmed with HPLC-ESI-MS and HPLC-UV-DAD (Deng et al., 1998, 1999). Deng et al. (1998) reported that 3–5  $\mu\text{mol/L}$  of NMU was detected in a mixture of fish sauce sample and human gastric juice samples at equal volume after treatment of 5 mmol/L of nitrite (final concentration) at pH 1–3.

Conditions in gastric lumen are more complex than the simulated stomach conditions. For example, normal gastric mucosa secretes ascorbic acid into gastric juice, which could inhibit formation of nitroso compounds. It is unknown whether there is formation of NMU in human gastric lumen after the consumption of fish

sauce and nitrite. This paper reported our further study of the endogenous formation of NMU in stomachs of experimental pigs and human volunteers after administration of fish sauce and nitrite. Pignatelli et al. (1993) reported that the level of nitrite in gastric juice samples of fasting humans in Columbia was up to 472  $\mu\text{mol/L}$ . Therefore, the possibility of the formation of NMU in gastric juice samples treated with 500  $\mu\text{mol/L}$  of nitrite was also studied.

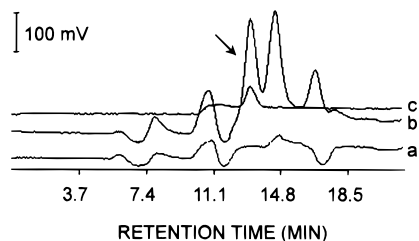
## MATERIALS AND METHODS

**Chemicals and Samples.** Authentic NMU was purchased from Sigma Chemical Co., St. Louis, MO (N-4766). Fish sauce samples were collected from Changle County in Fujian Province, an area with the highest mortality of stomach cancer in China. The main species of fishes were *Sardinella aurita* (Val.) and *Decapterus maruadsi* (T. & S.). Fasting human gastric juice samples were collected from subjects who were examined by gastric fibroendoscope and analyzed for the total amounts of *N*-nitrosamides (Deng et al., 1997). Only samples without *N*-nitrosamides were pooled and stored in liquid nitrogen for study of NMU formation.

**Treatments of Experimental Pigs and Human Volunteers.** Two experimental mini-pigs (male, 3 months old, 15 kg body weight) were purchased from the Breeding Center of Mini Pig, Beijing University of Agriculture. Each pig was surgically fitted with a flexible stomach cannula. Normal feeding and care were resumed after 24 h. One week later, the fistulated pigs were used to study the endogenous formation of NMU in gastric lumen in vivo. Thirty milliliters of fish sauce sample was diluted with 30 mL of distilled water. It was adjusted to pH 1.0 with 6 mol/L of HCl. Solutions of sodium nitrite at doses (micromoles) of 220, 870, and 3480 and 60 mL of the diluted fish sauce sample were injected into the gastric lumen of pigs through the cannula one by one. In the pig model, *N*-nitrosopiperidine was also added to the diluted sample (final concentration = 56  $\mu\text{mol/L}$ ) before injection as the internal marker. Gastric juice samples were taken from the gastric lumen 30 min later.

Twenty milliliters of fish sauce sample was adjusted to pH 2.8 with 1.0 mL of 6 mol/L of HCl, the maximum dose for

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**Figure 1.** Chromatograms of NMU by HPLC-PHPS-TEA from extract of pig gastric juice sample: (a and b) extract of gastric juice sample; (c) 1 mmol/L of authentic NMU; (a) detected without photolysis; (b and c) detected with photolysis. The sample was taken from the animal treated with 60 mL of 50% fish sauce sample and 3480  $\mu\text{mol}$  of sodium nitrite for 30 min, extracted with acetone/dichloromethane, and concentrated. A PRP-1 analytical column and 10 mmol/L trifluoroacetic acid with acetonitrile gradient (0–7% at 1%  $\text{min}^{-1}$ , hold for 5 min, then 7–22% at 1.5%  $\text{min}^{-1}$ , hold for 3 min, and then 22–0% at 22%  $\text{min}^{-1}$ , hold for 26 min) were used in a Waters HPLC system (1.0 mL/min, 20  $\mu\text{L}$ /injection). The chemical marked by the arrow was NMU.

human use first. Fasted healthy human volunteers (two males and two females, 23–38 years old) drank all of the pH-adjusted fish sauce sample and then drank 20 mL of distilled water containing 580  $\mu\text{mol}$  of sodium nitrite immediately. Gastric juice samples were taken by stomach tube 30 min later.

Control experiments were also carried out in the same time in vitro. The same amount of pH-adjusted fish sauce sample and nitrite was mixed and incubated 37 °C for 30 min in vitro. The incubated mixture was extracted and detected as gastric juice sample in the same time.

**Formation of NMU in Pooled Human Gastric Juice Samples at the Natural Level of Nitrite in Vitro.** Ninety milliliters of pooled fasting human gastric juice samples was mixed with 10 mL of fish sauce sample. The mixed sample was adjusted to pH 2.0 with 6 mol/L of HCl and incubated with 50  $\mu\text{mol}$  of sodium nitrite (final concentration = 500  $\mu\text{mol}$ /L) at 37 °C for 30 min.

**Detection of NMU in Gastric Juice Samples.** Purification of samples and detection of NMU were carried out as described in detail previously (Deng et al., 1998). Briefly, nitrosation of gastric juice samples was stopped by sulfamic acid. Nitroso compounds (including NMU) in the samples were extracted and detected with HPLC-PHPS-TEA. The detection limit was 0.3 nmol of NMU. Its  $t_{\text{R}}$  was  $\sim$ 13.0 min. In addition, the total amount of NMU synthesized in gastric lumen of pigs was calculated, depending on the volume of sample, concentration of NMU in the samples, and recovery rate of the internal marker *N*-nitrosopiperidine ( $t_{\text{R}}$  = 25.0 min).

## RESULTS

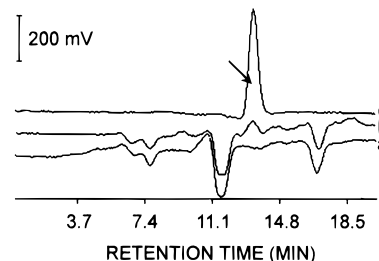
**Incubation Time.** NMU was detectable in the extract of a mixture of fasting pig and human gastric juice and fish sauce samples (1:1, v/v) after treatment with nitrite (final concentration = 50 mmol/L) at pH 1.0 and 37 °C for 30 min in vitro. Concentrations of NMU (micromoles per liter) in the nitrosated mixture of human gastric juice and fish sauce samples after nitrosation for 10, 30, and 60 min were 36.1, 55.6, and 38.8 in one experiment and 61.5, 77.6, and 41.6 in repeat experiment, respectively. The ratios of the average concentration of NMU were 1:1.34:0.82 after nitrosation for 10, 30, and 60 min. Therefore, 30 min was selected as the in vivo incubation/nitrosation time in the following experiments.

**NMU Synthesis in Gastric Lumen of Pigs.** NMU was detected in the gastric juice (pH 3.0) of both pigs at 30 min after injection of 60 mL of the diluted fish sauce (adjusted to pH 1.0) and sodium nitrite (Figure 1). The amount of NMU in gastric juice samples (pH

**Table 1.** Comparison of Formation of NMU at Various Doses of  $\text{NaNO}_2$  in Gastric Lumen of Pig Model in Vivo and Control Experiments in Vitro

amount of $\text{NaNO}_2$ ( $\mu\text{mol}$ )	formation of NMU			
	concn ( $\mu\text{mol}/\text{L}$ )		total amount ( $\mu\text{mol}$ )	
	in vivo	in vitro	in vivo	in vitro
3480	25.40	29.20	4.27	1.75
870	7.97	6.48	1.91	0.38
220	BDL <sup>a</sup>	2.77	BDL	0.17

<sup>a</sup> BDL, below detection limit (184 nmol/L); the value was the average of two independent experiments.



**Figure 2.** Chromatograms of NMU by HPLC-PHPS-TEA from extract of human gastric juice sample: (a and b) extract of gastric juice sample; (c) 3 mmol/L authentic NMU; (a) detected without photolysis; (b and c) detected with photolysis. The sample was taken from a volunteer (male A) treated with 40 mL of 50% fish sauce sample and 500  $\mu\text{mol}$  of sodium nitrite for 30 min. The remaining steps were as in Figure 1. The chemical marked by the arrow was NMU.

6.0) of treated pigs was below the detection limit if the pH of the diluted fish sauce was not adjusted (original pH of 5.5). Concentration and total amount of NMU synthesized in vivo and in vitro were dependent on the dose of nitrite (Table 1).

**NMU Synthesis in Gastric Lumen of Healthy Volunteers.** A total of 4–100 nmol of NMU could be detected in gastric juice from four healthy human volunteers 30 min after the oral administration of 20 mL of fish sauce (adjusted to pH 2.8) and 20 mL of water containing 580  $\mu\text{mol}$  of sodium nitrite (Figure 2 and Table 2). NMU was undetectable in gastric juice samples (pH 3.0, 5.0, 5.0, and 6.0) when the pH of fish sauce sample was unadjusted.

**Formation of NMU at Natural Levels of Nitrite in Vitro.** After nitrosation by 500  $\mu\text{mol}/\text{L}$  of sodium nitrite (final concentration) at pH 1.0 and 37 °C for 30 min, NMU was detectable at a retention time of 12.8 min in the condensed extract of 100 mL of mixture of gastric juice and fish sauce (Figure 3b). A higher peak could be obtained when the extract was spiked by 0.43 mmol/L of authentic NMU (Figure 3c).

## DISCUSSION

Consumption of fish sauce is a high risk factor for stomach cancer among the residents living along the coast of the southeastern Chinese Seas. It is rich in creatinine and other nitrosable amines (Zhang et al., 1993). Fish sauce was mutagenic and carcinogenic after it was nitrosated at simulated stomach conditions (Deng et al., 1991). A strong gastric carcinogen, NMU, was characterized in the nitrosated fish sauce samples (Deng et al., 1998, 1999). Here, we further report that there is endogenous formation of NMU in the gastric lumen of human volunteers after the ingestion of 20 mL of fish sauce and 560  $\mu\text{mol}$  of sodium nitrite. To the best of our knowledge, our study is the first showing the formation of *N*-nitrosourea in human stomach.

**Table 2. Status of Gastric Juice Samples from Four Human Volunteers and Formation of NMU 30 min after the Volunteers Had Taken 20 mL of pH-Adjusted Fish Sauce and 0.5 mmol of Nitrite<sup>a</sup>**

sample origin	age	total vol of sample (mL)	pH of sample	total amount of NMU in gastric lumen (nmol)
male A	38	24	5.0	4
male B	26	110	3.0	85
female A	25	50	2.0	100
female B	28	50	2.0	22

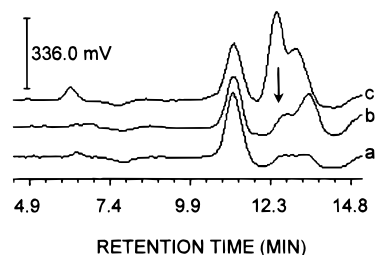
<sup>a</sup> Thirty minutes after administration of 20 mL of pH-unadjusted fish sauce, the volume and pH of gastric juice were 75 mL, pH 6.0, for male A; 44 mL, pH 5.0, for male B; 44 mL, pH 3.5, for female A; and 60 mL, pH 5.0, for female B. No NMU was detected in these samples.

The gastric lumen of pig and human is a more complex environment compared with simulated stomach conditions. For example, normal gastric mucosa secretes ascorbic acid into the gastric lumen, which might inhibit the endogenous synthesis of *N*-nitroso compounds in the stomach. Gastric mucosa contains hydrosulfide compounds, which might destroy the formed *N*-nitroso compounds. In our study, the amount of NMU formed in pig stomach was not less than that of NMU synthesized under simulated stomach conditions (Table 1). Formation of NMU in pig stomach was nitrite- and pH-dependent, which was similar to the results observed for the simulated stomach conditions (control experiment) and also similar to the work of Maragos et al. (1990). Administration of pH-unadjusted fish sauce resulted in a decrease of the acidity of the stomach lumen of experimental pigs and human volunteers. This might account for the absence of NMU in the gastric juice of both models after the inclusion of the original fish sauce sample. Acidic fish sauce would minimize the effect of a given sample on stomach acidity. This might contribute to the easy intragastric formation of NMU. Fish sauce is used as a delicious seasoner by residents in high-risk areas for stomach cancer in China. These people consume mainly other kinds of foods at the same time. Although the exact effect of the consumption of fish sauce on the acidity of their stomach lumen is unknown, we do not think that the decrease of stomach acidity, as observed in the experimental pigs and volunteers, would happen in the general population. Therefore, the intragastric environment of the resident may be prone to the chemical formation of NMU when fish saue is taken with other foods.

Pignatelli et al. (1993) reported that the level of nitrite in fasting gastric juice in Columbia was up to 472  $\mu\text{mol/L}$ . Zhang et al. (1984) reported that the concentration of nitrite in fasting gastric juice samples in China was up to 100  $\mu\text{mol/L}$ . We observed that treatment of 500  $\mu\text{mol/L}$  of nitrite resulted in the formation of NMU in pooled samples of fasting human gastric juice containing 10% fish sauce. This indicates that low amounts of *N*-nitrosoureas can be formed in the normal stomach when nitrite is consumed in amounts to which humans are commonly exposed. Because of high exposure levels of nitrite and nitrate, NMU could form endogenously in the stomachs of residents in high-risk areas and might contribute to the causes of the high incidence of stomach cancer.

#### ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; NMU, *N*-nitrosomethylurea; PHPS, photohydrolysis-



**Figure 3.** Chromatograms of NMU by HPLC-PHPS-TEA from extract of pooled human gastric juice sample taken from subjects with high risk for stomach cancer: (a and b) extract of gastric juice sample; (c) 0.43 mmol/L authentic NMU-spiked extract of gastric juice sample; (a) detected without photolysis; (b and c) detected with photolysis. Ninety milliliters of gastric juice was mixed with 10 mL of fish sauce first and adjusted to pH 2.0 and then treated with 50  $\mu\text{mol}$  of sodium nitrite for 30 min. The remaining steps were as in Figure 1. The chemical marked by the arrow was NMU.

pyrolysis; TEA, thermal energy analyzer; ESI, electronic spary ionization; MS, mass spectrometry; UV, ultraviolet; DAD, diode array detection;  $t_R$ , retention time.

#### ACKNOWLEDGMENT

We thank Ruming Wang and Liankuan Gu for their skilled technical assistance.

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Received for review August 27, 1999. Revised manuscript received March 13, 2000. Accepted March 28, 2000. This study was supported by grants from the Beijing Municipal Commis-

sion for Science and Technology and from the Ministry of Health. A preliminary report of these results was presented at the Third International Gastric Cancer Congress, Seoul, Korea, April 27–30, 1999.

JF990967Q