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*Editorials*

# progress of gastric cancer etiology: N-nitrosamides 1999s

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## INTRODUCTION

Stomach carcinoma is still the leading cause of cancer death in China and the second one in the world. Its possible causes include: A) chemical factors such as intragastric formation of *N*-nitroso compounds (NOC) and high salt intake; B ) biological factors such as infection of *Helicobacter pylori* and biotoxins intake; and C) nutritional factors such as deficiency of vitamin C, selenium, a nd other antioxidants. Nitrogenous precursors of NOC, e.g. alkylamines, alkylur eas, alkylguanidines, and alkylamides, occur widely in nature and potential nitrosating agents, e.g., nitrite ($NO_2$-) and $NO_x$ (the gaseou s oxides of nitrogen) are similarly widespread. Relationship between exposure to NOC and causes of human cancer was investigated extensively ten years ago. Resu lts indicated that the exposures of NOC might contribute to the occurrences of m alignancy in the upper digestive tracts including stomachs. It was also observed that both high salt intake and deficiency of some micronutrients enhanced NOC- induced carcinogenicity. Recent studies show that infection of *H. pylori* can lead to atrophic gastritis and achlorhydria, and promote endogenous formation of NOC indirectly[1]. Much attention has been paid to stomach cancer and NOC regarding the characterization of natural *N*-nitrosamides in human envir onment in the 1990s.

*N*-nitrosamides, one kind of direct-acting NOC, can be synthesized endogenously in stomach lumens and damaged DNA of gastric mucosal epithelium *in situ*. Most of epidemiological investigations showed that the occurrence of stomach cancers was correlated positively with exposure levels of nitrosating agents and ni trogenous precursors of NOC[2]. Laboratory synthesized *N*-nitrosamides are strong animal stomach carcinogens. Intragastric *N*-nitrosamide formation may play an important role in the etiology of gastric carcinomas[3]. However, most *N*-nitrosamides are chemically reactive, thermal, photo, and alkali-labile compounds. It is very difficult to detect *N*-nitrosamid es in human environments chemically. Little was known about the existence of n atu ral *N*-nitrosamides before the 1980s because of lack of a convenient sensi tive method to detect them precisely [4]. Several progresses have been ma de on the study of *N*-nitrosamides in the past ten years, including setup of detection meth ods for trace *N*-nitrosamides in the early 1990s[5,6] resulting in recent discovery of natural *N*-nitrosoureas in human environments[7-9].

This article will present and discuss results of studies on stomach cancer and *N*-nitrosamides in the past decade.

## DEVELOPMENT OF SENSITIVE AND SELECTIVE METHODS TO DETECT *N*-NITROSAMIDES CHE MICALLY

Many *N*-nitrosamines can be analyzed sensitively (detection limit, less than 1 ng/injection) by a standard commercial Thermal Energy Analyzer Detector (TEA), relying on their thermal cleavage of the *N-N* bond to produce a nitrog en oxide (NO) radical. However, *N*-nitrosamides and related compounds, unlike *N*-nitrosamines, typically rearrange on pyrolysis to yield molecular nitro gen ($N_2$) instead of nitrogen oxide. Because of the possible etiological role o f *N*-nitrosamides in human gastric carcinogenesis, it is necessary to setup a sensitive and selective method to detect *N*-nitrosamides in human environm ents.

A liquid chromatography (HPLC) with postcolumn photolysis device was assembled first at Shuker and Tannembaum's laboratory, Massachusetts Institute of Technol ogy in 1983 (Figure 1A)[10]. In the device *N*-nitrosamides were cle aved photolytically by ultraviolet (UV) irradiation to produce nitrite ion in aqueous solution, which was determined colorimetrically with Griess reagent in a postcolumn reactor. However this method is not *N*-

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nitrosamide-specific. It is of low sensitivity (detection limit, ng/injection): 20 for *N*-me thyl-*N*-nitrosourea (NMU) and 8 for *N*-methyl-N'-nitro-*N*- nit rosoguanidine (MNNG). The polarity of *N*-nitrosamides ranges widely from str ongly polar to non-polar. Non-polar *N*-nitrosamides do not unde rgo photohydrolysis because of their nondissolubility in aqueous so lution. Though the device could not be used to detect trace amounts of *N*-ni trosamides in nature, it has been often used to further develop sensitive met hods (Figure 1B and 1C)[5,11]. Fine *et al*[12] at New Englan d Institute for Life Sciences have modified the pyrolysis chamber in a standard TEA such that *N*-nitrosamides release nitric oxide on pyrolysis (sensitivities for standards, less than 1 ng/injection) in 1987. However, details o f the instrument were not provided and further development was needed.

An HPLC-photolytic interface-TEA method was reported to precisely detect *N*-nitrosamides and other non-volatile NOC at Hotchkiss's laboratory, Institute of Food Science, Cornell University in 1988 (Figure 1B)[5]. A chromat ographic effluent containing separated NOC is introduced into a glass coil with a purge stream of He and irradiated with UV in a photolysis device. Nitric oxide, cleaved by photolysis, is separated rapidly from the solvent through a series of cold traps and carried by the He into the reaction chamber in a standard TEA. The maximum sensitivity of the approach was approximately 8 ng for NMU and 16 ng for MNNG, and less than 1 ng for *N*-nitrosoamino acids and *N*-nitrosam ines. The selectivity for *N*-nitrosamides was not mentioned.

A selective and sensitive HPLC-Photolysis/ Pyrolysis-TEA method (Figure 1C) was set up for *N*-nitrosamides at Beijing Institute for Cancer Research, Beijing Medical University School of Oncology (Chen *et al*; Li and Deng)[6,11]. In the photolysis device, *N*-nitrosamides are first cleaved photolytic ally by UV irradiation to produce nitriteion in aqueous chromatographic effluen t. Then nitrite ion in the effluent is introduced into a pyrolysis tube (made of $Al_2O_3$) at 550℃ with carrying gas stream of He or $N_2$, and releases nitric oxide on pyrolysis. Nitric oxide is separated rapidly from the solvent-spray t hrough a series of cold traps, and is lead into the reaction chamber in TEA by t he carrying gas. Detection limits (ng/injection) are: 5.2 for NMU, 8.5 for MNNG, about 1 for *N*-nitrosamines, and 16 for *N*-nitrosoproline. In addition, when UV lamp of the photolysis device is turned off, the responses decreased up to 90%-100% for *N*-nitrosamides, but only 0%-45% for other kinds of NOC. I t indicates that the method is a selective one, which can be used to differentiate *N*-nitrosamides preliminarily from other kinds of NOC by comparing the difference in response UV when the lamp is on or off. This special feature can be useful for chemists to select the right chromatogra phic components for further identification of trace *N*-nitrosamides in human environments.

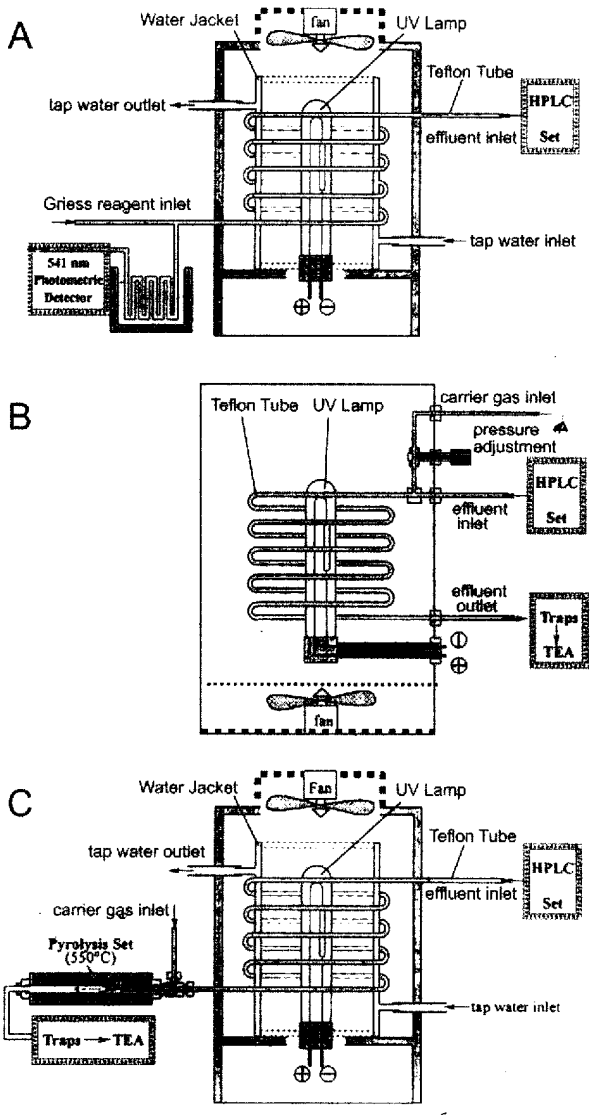


**Figure 1** Diagrams of HPLC-Photolysis Device-UV Detector (A), HPLC-Photolytic Interface-TEA (B), and HPLC-Photolysis/ Pyrolysis-TEA (C).

Zhang *et al*[13] developed a method to detect the total amount of *N*-nitrosamides in biological samples with the Photolysis/Pyrolysis-TEA detector. It can be used only to roughly evaluate exposure levels of total *N*-nitr osamides, because calculation of the concentration of total *N*-nitrosamides is based on the difference inresponse when the UV lamp is on or off. Sampl e purification by extraction with organic reagents is required in the method. Th erefore, *N*-nitrosamides with strong polarity in the samples will be lost du ring the process of extraction. Only medially polar ones can be detected by the method.

## IDENTIFICATION OF *N*-NITROSAMIDES IN NATURE

There is an indispensable evidence to show existence of *N*-nitrosamides in human environments in order to prove etiological role of *N*-nitrosamides in gastric carcinogenesis. However, little is known about the detailed chemical structures of the natural *N*-nitrosamides except stre-

ptozotocin and therape utic *N*-nitrosoureas[14]. Establishment of above the sensitive methods to determine *N*-nitrosamides make it possible to detect the trace amounts of *N*-nitrosamides in nature.

Caffeine is a normal component of coffee. Kumar *et al*[15] rep orted fo rmation of caffeidine and caffeidine acid from pure caffeine treated under condi tions similar to preparation of salted tea practised in Kashmir, which could result in two *N*-nitrosamides, dinitrosocaffeidine and N, N'dimethyl-*N*-nitrosourea by nitrosation. However, there is no report to show that th ese *N*-nitrosamides are detectable in the nitrosated-salted tea. Mende *et al*[16] characterized the nitrosamide precursor pyrrolidin(2)one in food and tobacco. A volatile *N*-nitrosamide, *N*-nitroso pyrrolidin (2 ) one, was detectable in the nitrosated precursor by gas chromatography-TEA me thod. They also mentioned the existence of trace amounts of the *N*-nitrosami de in natural Indian nasal snuff. But no detailed supporting materials were p rovided.

Fish sauce is a liquid product of small marine fish and sodium chloride (7:3). T he main species of fishes used are Sardinella aurita (Val.) and Decapt erus maruadsi (T & S). The fishes are completely liquidized after being fermented for 1-2 years. The product is consumed daily (about 30 mL/capita) by r esidents as a traditional seasoning in the Chinese southeast coast, the highest risk area for stomach cancer in China (the male standard mortality of stomach ca ncer in Changle County, 134.44/$10^5$ in 1986-1988). Epidemiological studies sho wed that the intake of fish sauce is a high risk factor for gastric carcinogenes is for the local residents[17]. It was reported that extract of fish sauce samples is markedly and directly mutagenic toward S. typhimurium TA100 induced high sister chromatid exchanges and micronucleus in Chinese hamster V79 cells after nitrosation with sodium nitrite under the simulated gastric conditions. But the extract of non-nitrosated samples had no such effect. The nitrosated fish sauce also induced SOS in *E. coli* PQ37 and alkylation of calf thymus D NA. The potency of nitrosated fish sauce to induce unscheduled DNA synthesis in human normal gastric mucosal cells was increased about fivefold compared with fish sauce. When the extract of nitrosated fish sauce was given to newborn rats by gavage, dysplasia and adenocarcinoma were induced in the glandular stomach in t he 4th and 16th experimental week, respectively[13,18,19]. Because of the high exposure level of nitrosating agent nitrite for the local residents [20], dietary fish sauce may contribute to the causes of the high mortality due to stomach cancer in the areas. It is necessary to identify che mical carcinogens in the nitrosated fish sauce.

Chen *et al*[21]reported that 100% ($n$ = 21) of fish sauce samples contained some kinds of volatile *N*-nitrosamines by HPLC -TEA and gas chromat ography-TEA. Concentrations of total NOC in 49 fish sauce samples ranged from 0 .2 to 16 μmol/L and rose by up to 4800- and 100-fold after being nitrosated at pH 2 and pH 7, respectively[19]. Deng *et al*[7]characterized a chromatographic component of thermal-unstable *N*-nitroso compounds in nitrosated fish sauce by above HPLC-Photolysis/Pyrolysis-TEA method. A strong chromatographic peak with the same retention times as that for authentic NMU, was obtained under two different liquid chromatographic conditions after the sample was nitrosated by 5 mmol/L of sodium nitrite (final concentration) at 37℃ and pH 2.0 for 1 h. Like NMU, the chemical could not be detected by the method when UV lamp in the photolysis device was turned off. In a confirmation study, the chemical structure of the component was compared with authentic NMU by HPLC-elec tric spray ionization-mass spectrometer and HPLC-UV diode array detector. The chemical showed the same mass spectrum (m/zvalues 64, 102, 145) and spectrum of ultraviolet-absorbency ( $\lambda_{max}$ = 230 nm) as those of NMU[8]. These results indicated that the component was NMU. This was the first study reporting that there is *N*-nitrosamide, NMU, in nitrosated food. In addition, NMU could also be detected in the nitrosated human gastric juice sample spiked with fish sauce[7]. The formation of NMU in the sample was pH- and nitrite-dependent (Table 1). These results provide direct evidences that NMU formation could occur in human gastric juice samples spiked by fish sauce during nitrosation under simulated gastric conditions *in vitro*. Another *N*-nitrosamide, one of *N*-nitrosodipeptides, was separated recently and confirmed in our laboratory.

**Table 1 Comparison of formation of NMU at various doses of $NaNO_2$ in gastric lumen of pig model *in vivo* and control experiments *in vitro*[9,22]**

| Amount of $NaNO_2$ (μmol) | Formation of NMU | | | |
|---|---|---|---|---|
| | Concentration (μmol/L) | | Total amount (μmole) | |
| | *in vivo* | *in vivo* | *in vivo* | *in vivo* |
| 3480 | 25.40 | 29.20 | 4.27 | 1.75 |
| 870 | 7.97 | 6.48 | 1.91 | 0.38 |
| 220 | ND | 2.77 | ND | 0.17 |

ND, below detection limit; the value was the average of two independent experime nts.

Furthermore, experimental mini-pig model and human volunteers were used to study the possibility of intragastric formation of NMU *in vivo*[9,22]. Fish sauce sample (20-30 mL) and nitrite were administrated into gastric lumen of experimental pigs by perfusion via pig stomach cannula, or taken orally by hum an volunteers. Gastric juice samples were taken out 30 min later. Concentration of NMU in condensed extracts of these samples was analyzed with HPLC-Photolysis / Pyrolysis-TEA. Results showed that NMU was formed in gastric lumens of both models *in vivo* and also that the formation of NMU was nitrite and pH-dependent (Tables 1 and 2).

Zhang *et al*[20] reported that concentration of nitrite in

fastin g g astric juice samples in China was up to 100 μmol/L. Pignatelli *et al*[23] reported that the level of nitrite in fasting gastric juice in Columbia w as up to 472 μmol/L. It was reported that NMU was still detectable in the conde nsed extract of 100 mL of mixture of pooled fasting human gastric juice samples and fish sauce sample (9 v:1 v) after treatment of 500 μmo l/L of nitrite *in vitro*, which is within the range reported in human gastric contents as veported by Deng *et al*[9]. These results suggest that low micromolar amo unts of *N*-nitrosoureas can be formed in the normal stomach when nitrite is consumed in amounts to which humans are commonly exposed.

**Table 2 Status of gastric juice samples from four human volunteers and formatio n of NMU 30 min after taking 40 mL of diluted fish sauce and 500 μmol of nitri te[9]**

| Sample's origin | Total volume of sample (mL) | pH of sample | Total amount of NMU detected in gastric lumen (nmol) |
|---|---|---|---|
| Male A | 24 | 5.0 | 4 |
| Male B | 110 | 3.0 | 85 |
| Female A | 50 | 2.0 | 100 |
| Female B | 50 | 2.0 | 22 |

Fish sauce is rich in nitrosable amines, i.e. dipeptides, free amino acids, crea tine, creatinine, and putrescine[24]. It was reported that 16-31 mg/kg of methylurea could be detected in dried, salted bonito fish after nitrosation and denitrosation, though no methylurea could be detected in the fish directly[25]. Further studies showed that methylurea was synthesized through 5-oxocreatinine 5-oxime and 1-methyl-5-oxohydrantoin 5-oxime duri ng nitrosation of creatinine[26,27]. NMU in the nitrosated fish sauce might be synthesized from creatinine.

## POPULATION STUDY ON TOTAL *N*-NITROSAMIDES IN STOMACH

Previous knowledge on status of human exposure to *N*-nitrosamides is deduced indirectly from data obtained in studies to exposure of NOC precursors. The situation has been changed since the setup of chemical methods to detect total *N*-nitrosamides in biological specimens[13]. To elucidate the correlat ion between exposure level of *N*-nitrosamides and causes of gastric carcinom as, a pilot cases-control study and a population study were reported by Zhang *et al* in 1991 and Deng *et al* in 1997[13,28]. Total amount of natural *N*-nitrosamides was detected with the Photolysis/Pyrolysis-TEA method in fasting gastric juice samples from subjects in high and low risk areas for stomach cancer (Table 3, 4).

Gastric carcinogenesis is a multistage process. Its precancerous lesions include chronic atrophic gastritis, intestinal metaplasia, and dysplasia. In the case-control study, high levels of total *N*-nitrosamides were detected in the gastric juice samples from patients with chronic gastritis in Putian area, a county along the Chinese southeast coast (Table 3)[13]. The positive rates and mean concentrations

**Table 3 Relationship between presence of total *N*-nitrosamides in gastric juice samples from subjects with pathological changes in gastric mucosa**

| Gastric mucosal status | Positive rate of total *N*-nitrosamides in gastric juice samples (%)** | | |
|---|---|---|---|
| | High risk areas | | Low risk area |
| | Putian[13] | Linqu[28] | Cangshan[28] |
| *N and CSG | 2/12 (16.7) | 1/4 (25.0) | 4/23 (17.4) |
| CAG | 10/14 (71.4)[b] | 38/76 (50.0)[c] | 18/56 (32.1) |
| CAG and IM | 12/13 (92.3) | 17/56 (28.8)[e] | 0/4 (0.0) |
| CAG and DYS | 12/13 (92.3) | 18/44 (40.9) | 5/10 (50.0) |

*N, normal mucosa; CSG, chronic superficial gastritis; CAG, chronic at rophic gastritis; IM, intestinal metaplasia; DYS, dysplasia; **sample was classified as *N*-nitrosamides-positive when more than 184 nmol/L (de tection limit) of the chemicals was detected; [b]Significantly different fr om N and CSG with $P < 0.01$; [c]Significantly different from low risk a rea with $P < 0.05$; [e]Significantly different from CAG with $P < 0.05$.

**Table 4 Presence of total *N*-nitrosamides (NAD) in gastric juice samples (GJ) from low and high risk areas for stomach cancer**

| | High risk area (Linqu) | Low risk area (Chongshan) | *P*-value |
|---|---|---|---|
| No. of GJ samples | 176 | 99 | |
| Proportion, pH≤3 | 48.0% | 84.0% | <0.01 |
| pH≥5 | 45.3% | 13.5% | <0.01 |
| Positive rate, all | 40.9% | 30.3% | = 0.03* |
| GJ, pH≤3 | 46.2% | 27.4% | <0.01 |
| GJ, pH≥5 | 43.3% | 53.9% | |
| Conc. (μmol/L) | 0.91 | 0.73 | |

*after age-adjustment.

in the three groups of patients were positively correlated with the severity of pathological changes in the gastric mucosa. In the populat ion study, the exposure status of total *N*-nitrosamides in stomach of subjects aged 35-68 years from high risk area for stomach cancer was further compared with that in low risk area under the same geographical and socioeconomic conditions in Shangdong Province, China (Table 3)[28]. Similar relationship between presence of *N*-nitrosamides and pathological changes of gastric mu cosa was obtained both in Linqu and Cangshan areas. However, the percentage of *N*-nitrosamides-positive samples was decreased in subjects with chronic atrophic gastritis when intestinal metaplasia was developed. The mechanism of decre ase in concentration of NOC is not clear. *N*-Nitrosamides are alkali-labi le compounds. It is a common step to adjust pH of sample-extractant mixture t o 5.0 with 10% sodium hydroxide in order to accelerate stratification of aqueous phase and organic extractant during extraction of NOC. During extraction of NMU in sample (aqueous solution), it was observed that addition of even one-drop (about 50 μL) of sodium hydroxide solution would destroy all NMU in the sample (Deng *et al*, unpublished data). Gastric mucosa with intestinal metaplasia secretes alkali-mucus. It is necessary to study whether the alkali-mucus catalyzes decomposition (activation) of *N*-nitrosamides and contributes to the dec rease of amount of total *N*-

nitrosamides in fasting gastric juice from patie nts with intestinal metaplasia of gastric mucosa. In addition, development of in testinal metaplasia of gastric mucosa is not suitable for colonization of *H. p ylori* in the alkali-mucus closely adjacent to the surface of gastric epithelium and finally eradicates them from there. It is interesting to study the relati onship between disappearance of *H. pylori* and the decrease in *N*-nitro samides amounts in gastric juice.

In the population study, more *N*-nitrosamides-positive samples in the high risk area (Linqu) were observed than in the low risk area (Chongshan). The diffe rence was significant after age-adjustment (Table 3)[27].

Chemical formation of *N*-nitrosamides and other NOC occur mainly under acidi c conditions. It had observed that 84% of samples was pH≤3 in Chongshan, whe reas only 48% in Linqu (Table 4, $P < 0.001$). That *N*-nitrosamides-positiv e rate is higher in samples with pH≥5 than with pH≤3 in the low risk area indi cates that there are some factors which could catalyze formation of *N*-nit rosamides in the achlorhydric stomach. Colonizations of microorganisms are commo n in the achlorhydric stomach. Some species of bacteria in stomach contain nitra te-reductase and could lead to high concentration of nitrite in gastric juice. It is well known that microorganisms catalyze formation of *N*-nitrosamines. Pan *et al*[29] reported that synthesis of *N*-nitrosamides , NMU, could also be accelerated by *Pseudomonas aeruginosa* at pH of 6-7, simulating achlorhydric stomach conditions. He *et al*[30] reported further that 6 out of 46 strains of bacteria, isolated from patients with gastrit is in Linqu County, promoted formation of NMU at pH 6.0. Biological formation of *N*-nitrosamides mediated by microorganisms in the achlorhydric conditions m ight account for the high concentration of total *N*-nitrosamides in gastric juice sample with pH≥5.

High *N*-nitrosamides-positive rate was observed both in gastric juice sampl es with pH≥5 and pH≤3 from subjects in Linqu, the high risk area. The result s uggests that there is a high chemical formation of *N*-nitrosamides in the acidic stomach. It is supported by the observation that *N*-nitrosamides-posit ive rate in the samples with pH≤3 from Linqu is higher than that from Chongshan , the low risk area (Table 4).

Above results indicate that human intragastric exposure to *N*-nitrosamides is positively correlated to risk of stomach cancer. *N*-Nitrosamides may be synthesized chemically in the acidic stomach and biologically in the ach lorhydric stomach.

## PROSPECTS

Establishment of sensitive methods to detect trace *N*-nitrosamides enabled identification of this sort of NOC in human environments greatly. More natural *N*-nitrosamides need to be discovered in order to understand aetiological role of *N*-nitrosamides in gastric carcinogenesis in populations. Rediscovery of *H. pylori* is an important event in the history of oncology of stomach. It c auses atrophic gastritis, gastric ulcer, and correlates with the occurrence of m ucosa-associated lymphoid tissue lymphoma closely. It might also contribute to the causes of gastric carcinoma. *H. pylori* mediated gastritis induces high levels of nitrogen oxide (NO) in gastric mucosa. Tissue nitrogen oxide could damage DNA directly if it penetrates into cytoplasma during S-phase and further into nucleus. When $Fe^{2+}$ exists in the tissue simultaneously it also nitrosates nitrogenous precursors to form NOC. It is noted that *H. pylori* infection leads to an increase in concentration of nitrite in the stomach lumen, decrease in secretion of ascorbic acid from gas tric mucosa, and might promote formation of *N*-nitrosamides in achlorhydric stomachs. However, relationship between occurrence of *N*-nitrosamides and *H. pylori* is not investigated extensively. Such investigation may bring to light the mechanism of carcinogenesis caused by *H. pylori*.

Because of the instability of *N*-nitrosamides, it is almost impossible for the chemicals to exist in diet. Most of the human exposure might originate from intragastric formation. That provides a good target to prevent stomach cancer by inhibition of nitrosation and elimination of nitrite in stomach. Garlic and related components are ideal candidates for such interventional study. They inhibit proliferation of microorganisms, combine nitrite, block nitrosation, and destroy formed NOC. An interventional study with garlicoil and other chemicals has been undertaken in Linqu area[31].

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