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N-nitrosoproline Excretion in the Presence and Absence of Gastric Disease

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_N_-nitrosoproline (NPRO) excretion, an indicator of endogenous nitrosation, was measured in a group of hospital inpatients who were identified by endoscopy and gastric biopsy as either having gastric lesions or having healthy stomachs. NPRO was assayed in background 24-hour urine samples and samples collected after loading doses of nitrate and L-proline. The presence of gastric lesions was associated with altered gastric pH and concomitant changes in gastric juice nitrate and nitrite concentration. Gastric juice pH increased with increasing severity of gastric disease ($P = 0.031$) and patients with normal stomachs had a lower gastric pH than those with chronic atrophic gastritis (CAG) (3.0 vs. 6.5, $P = 0.017$). The changes in gastric juice nitrate concentration were in the reverse direction ($P = 0.002$ for trend) with normal patients having higher mean levels than CAG patients (12.7 vs. 5.5 μg/ml, $P<0.0001$). Nitrite concentration increased with severity of gastric disease but the results were not significant (normal, 82.9 vs. CAG, 223.4 ng/ml, $P = 0.069$). No association was found between the presence of gastric lesions and increased urinary NPRO excretion. Mutagenic activity was not detected in any of the gastric juice samples.
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INTRODUCTION

THIS STUDY was designed to address the question of whether individuals with chronic gastric disease, who are at a higher risk of developing gastric cancer [1, 2] synthesise more *N*-nitrosoproline (NPRO) *in vivo* than individuals without such disease. NPRO is produced as a result of the nitrosation of L-proline by nitrite ingested or generated from bacterial reduction of nitrate *in vivo*. NPRO synthesis has been used as an indicator of nitrosating ability and a marker of endogenous *N*-nitroso compound formation [3]. Most *N*-nitroso compounds, but not NPRO, are proven animal carcinogens [4] and those *N*-nitroso compounds formed intragastrically (especially the *N*-nitrosamides) have been thought to be a possible cause of GC in humans [5–7].

As secondary objectives the study also sought to determine the interrelationships between NPRO synthesis and gastric juice pH, nitrate and nitrite concentration, and smoking and mutagenicity.

MATERIALS AND METHODS

Subjects

The study was conducted amongst inpatients in the Central Hospital, Siena, Italy and was approved by the local research ethical committee. The consent of all patients was obtained. 81

patients aged 32–69 years (35 females, 46 males), agreed to participate, over the two-year period 1985–1987; 54 were patients attending the gastroenterology department and 27 were patients with non-gastric illness from other departments

Endoscopy

All subjects underwent endoscopic examination after a 12-hour fast. During endoscopy, gastric juice samples were aspirated and biopsy samples were obtained. One endoscopist (G.F.) carried out all biopsies. At least 6 biopsy specimens were taken from each subject, along the lesser curvature; 2 from the antropyloric region, 2 from the corpus and 2 from the fundic region. In addition, biopsies were taken from evident lesions. Biopsy fragments were fixed immediately in 10% (v/v) buffered formalin (pH 7). Gastric juice samples were obtained by aspiration using a sterile teflon catheter passed down the channel of the endoscope, with care being taken to avoid deglutition. Immediately upon aspiration of the gastric juice, pH was measured by glass electrode. One aliquot was then stabilised by disodium tetraborate and stored at −40°C until analysed for nitrate and nitrite [8]. A second aliquot was placed into a sterile glass bottle and stored at −80°C for mutagenicity testing (see below).

The results of the endoscopy and histological examination of the biopsies were all reviewed by one pathologist (C.V.) and on the basis of this review, subjects were categorised into one of four groups representing different degrees of gastric disease from "nothing of pathological significance" (normal controls), through "superficial gastritis (SG)", "chronic atrophic gastritis (CAG)" (with or without intestinal metaplasia or dysplasia), to gastric cancer. Where the biopsy specimens from one individual showed heterogeneity in the histology, the individual was categorised according to the most severe pathology.

Subjects were also categorised into two groups based on gastric juice pH. The cut-off point used was pH 4, below which there would be minimal bacterial colonisation of the stomach lumen due to the acidity [9].

NPRO test

Two consecutive 24-hour urine collections were made, commencing on the morning of the endoscopy; the first was to assess background excretion of NPRO and nitrate and the second followed consumption of beetroot juice (supplying approximately 300 mg nitrate) and, after 30 minutes, a 500 mg dose of L-proline (Forum Chemicals, UK) dissolved in drinking water. Urine samples were collected into polypropylene bottles containing sodium hydroxide (10 g) to avoid artefactual formation of NPRO during collection and storage [10]. Under these conditions, nitrate is also stable [11]. Upon completion of collection, total urine volumes were recorded and two aliquots were taken and stored at −40°C until determination of NPRO and nitrate (in duplicate) using established methods [8, 10, 11]. Multiplication of total urine volume voided over 24 h by NPRO concentration gave total NPRO excreted over this time period.

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Dietary restrictions

All subjects consumed similar, supervised diets during the study period, beginning 2 days prior to endoscopy. These diets excluded foods considered likely to contain preformed NPRO [12–14] and restricted intake of foods usually found to have high levels of nitrate, nitrite [15] or ascorbic acid [16].

Mutagenicity assays

Gastric juice was tested for mutagenicity at three concentrations using two organisms, *Salmonella typhimurium* TA 100 and *Escherichia coli* WP2 *uvrA* pkM101 [17]. Positive (cisplatin and 4-nitroquinoline-N-oxide for *S. typhimurium* and potassium dichromate for *E. coli*) and negative (physiological-saline) controls were run in parallel with each batch of samples. Parallel assays were also run to control for the confounding effects of growth-promoting gastric juice aminoacids [17, 18]. The criteria for a positive result were an increase in colony count above that likely to be due to gastric juice aminoacids, and a significant positive association between colony counts and amount of gastric juice sample per plate. These criteria were to be met independently in tests using both organisms.

Statistical analyses

Subjects undergoing treatment which affected gastric juice pH (e.g. antacids) were not included in analyses. Complete data on gastric juice pH, and nitrate and nitrite concentration were obtained for 74 of the 81 subjects. 53 of these subjects then fully completed the NPRO test for endogenous nitrosation. Data for gastric juice pH were bimodal and, therefore, medians are presented and non-parametric statistical tests were used. Data for age showed a normal distribution. Data for nitrate, nitrite and NPRO were normalised by logarithmic transformation. As current smoking (within 2 weeks of endoscopy) was found to affect nitrate metabolism (see below) results were adjusted, where appropriate, for smoking habit by analysis of covariance. Similarly, gastric juice pH affected gastric juice nitrate and nitrite concentrations (see below) therefore results were adjusted, where appropriate, for the effect of pH by analysis of covariance.

The main analyses compared parameters representing nitrosating ability between those diagnosed as having no gastric disease and those with CAG. Secondary analyses assessed variance and regression of these parameters over the four diagnostic categories.

RESULTS

Table 1 gives details of age, sex and smoking habits of subjects by pathological diagnosis and gastric juice pH. The mean age of subjects did not vary significantly between diagnostic or pH groups. The sexes were evenly distributed between these groups, with the exception of gastric cancer cases where males predominated. Rates of smoking varied from 25.0% to 50.0%.

Table 2 shows the proportion of subjects within each diagnosis group with gastric juice pH values of below 4.0 or 4.0 and above together with the median pH for each group. There was a significant relationship ($P = 0.031$) between disease severity and increased pH and also a significant difference ($P = 0.017$) in median gastric juice pH between the two major comparison groups, i.e. the 26 normal controls (median = 3.0) and the 30 subjects with CAG (median = 6.5).

The mean gastric juice nitrate and nitrite concentrations for these subjects are also shown in Table 2, for the different diagnostic groups and the two gastric juice pH categories. There

Table 1. Mean age (range), sex, and current smoking habit of subjects by diagnostic category and gastric juice pH

	All subjects					NPRO test subjects				
Diagnosis*	*N*	Age		Sex F:M	Smoking S:NS	*N*	Age		Sex F:M	Smoking S:NS
Normal	26	51.1	(32–69)	13:13	9:17	18	52.3	(32–69)	10:8	7:11
SG	8	53.1	(44–63)	5:3	2:6	8	53.1	(44–63)	5:3	2:6
CAG	30	54.2	(36–66)	15:15	8:22	21	54.0	(36–66)	12:9	6:15
GC	10	59.7	(42–69)	1:9	5:5	6	56.5	(42–63)	0:6	3:3
P†		0.096					0.514			
P‡		0.190					0.570			
pH										
<4.0	31	52.9	(32–69)	14:17	11:20	22	54.1	(32–64)	11:11	9:13
≥4.0	43	54.3	(36–69)	20:23	13:30	31	53.3	(36–66)	16:15	9:22
P§		0.490					0.730			

*Normal = nothing of pathological significance, SG = superficial gastritis, CAG = chronic atrophic gastritis, GC = gastric carcinoma.
†Significance of analysis of variance between diagnostic categories.
‡Comparison (*t* test) of normal vs. CAG subjects.
§Comparison (*t* test) of subjects with gastric juice pH < 4.0 vs. pH ≥ 4.0.

Table 2. The effect of gastric pathology on gastric pH (median, 95% CI) and of both on gastric juice nitrate and nitrite concentrations (geometric mean, 95% CI) for all subjects

		Gastric juice pH			Gastric juice	
Diagnosis	*N*	<4.0	≥4.0	Median pH	Nitrate (μg/ml)	Nitrite (ng/ml)
Normal	26	65.4%	34.6%	3.0 (2.3, 4.4)	12.7 (9.9, 16.6)	82.9 (35.3, 193.2)
SG	8	50.0%	50.0%	3.7 (2.0, 7.0)	8.6 (5.4, 13.8)	131.5 (28.3, 610.2)
CAG	30	30.0%	70.0%	6.5 (5.0, 7.0)	5.5 (4.3, 7.0)	223.4 (101.3, 495.8)
GC	10	10.0%	90.0%	6.5 (4.1, 8.0)	6.8 (4.5, 10.4)	250.6 (62.5, 995.9)
*P**				0.031	0.002	0.105
P†				0.017	<0.0001	0.069
Gastric juice pH						
<4.0	31				10.8 (8.3, 13.8)	99.8 (45.9, 217.0)
≥4.0	43				6.5 (5.2, 8.0)	203.7 (105.4, 394.2)
P‡					0.004	0.167

*Significance of Kruskal–Wallis test for variance in gastric pH (unadjusted) between diagnostic groups, and of regression for gastric juice nitrate/nitrite on severity of gastric disease (four disease categories) after adjusting for the effect of smoking.
†Comparison of gastric juice pH (Mann–Whitney test, unadjusted) and gastric juice nitrate/nitrite (*t* test), normal vs. CAG after adjusting for the effect of smoking.
‡Comparison of gastric juice nitrate/nitrite (*t* test), pH <4.0 vs. pH ≥4.0 after adjusting for the effect of smoking.

was a significant association between decreased level of gastric juice nitrate and increased severity of gastric disease ($P = 0.002$). As well, the mean gastric juice nitrate concentrations were significantly different ($P < 0.0001$) between the normal controls (mean = 12.7 μg/ml) and subjects with CAG (mean = 5.5 μg/ml). Mean nitrate concentrations were also significantly lower ($P = 0.004$) in subjects with a gastric juice pH of 4.0 and above. The relationship with gastric juice nitrite was in the reverse direction, increased disease severity and pH being associated with increased nitrite, but these associations were not statistically significant.

The above analyses are repeated in Table 3 for the 53 subjects who completed the NPRO test. The association between disease severity, gastric juice pH and nitrate and nitrite concentrations remained similar with the exception that the increase in gastric juice nitrite concentrations associated with increasing disease severity and gastric juice pH, reached statistical significance; (regression on disease severity, $P = 0.039$; comparison between normal and CAG categories, $P = 0.007$; and comparison between pH groups, $P = 0.034$). Although median gastric juice pH for the diagnostic categories remained virtually unchanged, the trend did not quite achieve statistical significance ($P = 0.195$ for regression on disease severity and $P = 0.083$ for comparison of normal vs. CAG categories). There was a lack of relationship between gastric disease or gastric juice pH and NPRO excretion, either for background levels or for test levels (i.e. after loading doses of nitrate and L-proline) (Table 3). Correcting for background exposure to NPRO by subtraction of background from test NPRO levels did not alter this outcome. These results were similar whether comparing the trend across all four diagnostic groups or the difference between patients with chronic atrophic gastritis and those with normal stomachs.

Background excretion of NPRO was low for all subjects, but after ingestion of the nitrate source and L-proline, geometric mean urinary levels of NPRO increased significantly (3.1 vs. 0.6 μg/24 hours, $P < 0.001$). This increase was apparent in all disease and gastric juice pH groups.

Table 3. The effect of gastric pathology on gastric pH (median, 95% CI) and of gastric pathology and gastric pH on gastric juice nitrate and nitrite concentration and urinary NPRO excretion (geometric means, 95% CI)

		Gastric juice pH			Gastric juice		Urinary NPRO excretion (μg/24 h)		
Diagnosis	*N*	<4.0	≥4.0	Median pH	Nitrate (μg/ml)	Nitrite (ng/ml)	Background	Test	Difference
Normal	18	61.1%	38.9%	3.0 (2.0, 4.4)	12.4 (9.2, 16.6)	49.9 (18.3, 138.5)	0.7 (0.2, 2.2)	3.7 (1.8, 7.9)	0.9 (0.3, 2.4)
SG	8	50.0%	50.0%	3.7 (2.0, 7.0)	8.6 (5.5, 13.2)	119.7 (30.2, 632.6)	0.2 (0.04, 1.1)	1.6 (0.5, 5.0)	0.9 (0.2, 4.2)
CAG	21	28.6%	71.4%	6.5 (5.0, 7.3)	5.7 (4.3, 7.4)	261.8 (102.3, 667.0)	0.6 (0.2, 1.6)	3.4 (1.7, 6.8)	2.0 (0.8, 5.1)
GC	6	16.7%	83.3%	6.4 (1.6, 9.0)	8.2 (4.9, 13.7)	157.8 (27.4, 925.3)	1.9 (0.2, 7.8)	3.0 (0.1, 10.8)	1.4 (0.2, 8.0)
*P**				0.195	0.003	0.039	0.800	0.870	0.306
P†				0.083	<0.0001	0.007	0.709	0.818	0.267
Gastric juice pH									
<4.0	22				11.1 (8.3, 14.5)	60.0 (24.3, 149.4)	0.8 (0.3, 2.2)	4.1 (2.1, 8.0)	1.1 (0.4, 2.7)
≥4.0	31				6.7 (5.3, 8.5)	217.8 (102.2, 471.6)	0.4 (0.2, 1.0)	2.5 (1.5, 4.5)	1.5 (0.7, 3.1)
P‡					0.009	0.034	0.371	0.278	0.648

*Significance of Kruskal–Wallis test for variance in gastric pH (unadjusted) between diagnostic groups, and of regression for gastric juice nitrate/nitrite and urinary NPRO on severity of gastric disease (four disease categories) after adjusting for the effect of smoking.

†Comparison of gastric juice pH (Mann–Whitney test, unadjusted) and gastric juice nitrate/nitrite and urinary NPRO (*t* test), normal vs. CAG after adjusting for the effect of smoking.

‡Comparison of gastric juice nitrate/nitrite (*t* test), pH <4.0 vs. pH ≥4.0 after adjusting for the effect of smoking.

Urinary excretion of nitrate increased significantly after the loading dose of nitrate (geometric mean 16.6 vs. 60.4 mg/24 h, $P < 0.001$). No significant associations between urinary nitrate excretion (background or test) and disease category or gastric juice pH were found (data not shown).

None of the gastric juice samples was identified as being positively mutagenic by our criteria, in the two bacterial test systems used.

DISCUSSION

In this study, a decrease in stomach acidity is associated with a decrease in gastric juice nitrate concentration but an increase in gastric juice nitrite concentration. This effect is almost certainly due to colonisation of the hypochlorhydric stomach by nitrate reducing bacteria and their metabolism [19, 20]. Other studies have also shown a rise in gastric juice nitrite concentration with increasing gastric pH [21, 22]. The rise in nitrite, however, does not equate with the loss of nitrate, presumably due to further reduction of nitrite by the colonising bacteria, to either ammonium or gaseous nitrogen oxides [23]. Indeed, recent work on human nitrate metabolism [24] demonstrated a greater loss of ingested nitrate in achlorhydrics as compared to normochlorhydrics and this was attributed to bacterial metabolism of both nitrate and nitrite in the stomach. Intragastric nitrite concentration is therefore likely to be only a poor representation of the dynamics of nitrate reduction and nitrite flux. It can be used, however, to give an instantaneous picture of the nitrite available for further reaction, including nitrosation. Corresponding with an intragastric nitrite increase, some studies have reported an increase in the concentration of *N*-nitroso compounds in gastric juice under conditions of hypochlorhydria [25–27]. The results of this study, however, are consistent with those of three others in which the specific excretion of urinary NPRO was examined [19, 28, 29], i.e. it is not elevated in patients with low gastric acidity. Here, NPRO excretion was not significantly different in hypochlorhydrics compared to those with normal gastric acidity.

On average, therefore, the mechanism of NPRO formation in the hypochlorhydric group appears to be at least as potent as that in the normochlorhydric group. In the latter, nitrosation would normally be acid catalysed [30]. The formation of *N*-nitroso compounds in hypochlorhydric gastric juice is thought primarily to result either from a non-acid catalysed bacterially mediated pathway [31] or acid catalysed nitrosation occurring locally in the crypts of a diseased stomach [32]. The bacterially mediated reaction is markedly species and strain dependent, and it has been suggested that higher levels of intragastric *N*-nitroso compound formation may be restricted only to a subset of achlorhydric individuals, that is those colonised by significant numbers of nitrosating species of bacteria [31, 33], Thus inconsistencies between studies of hypochlorhydric subgroups may be due to differences in the level of carriage of such organisms.

The trends of decreasing intragastric nitrate and increasing nitrite are also seen across the disease progression from normal through gastritis to carcinoma. Patients with CAG had significantly lower intragastric nitrite concentrations than those with normal stomachs. However, NPRO excretion did not vary significantly between the diagnostic groups (even comparing CAG and normal patients). In the other NPRO studies [19, 28, 29] there was also no elevation of urinary NPRO excretion in patients with gastric precancerous lesions.

Examination of our results by diagnosis groups was complicated by the fact that some of our "normal" patients had high gastric juice pH values and, *vice versa*, some of those with gastric

Table 4. The effect of smoking on gastric juice pH (median, 95% CI), gastric juice nitrate and nitrite concentration, and urinary NPRO excretion (geometric means, 95% CI)

	Current smoker	Current non-smoker*	P†
	n=18	n=35	
Gastric juice pH	4.3 (2.0, 6.5)	5.1 (3.1, 6.8)	0.251
Nitrate‡ (μg/ml)	5.7 (4.2, 7.7)	9.9 (7.9, 12.3)	0.005
Nitrite‡ (ng/ml)	198.6 (71.8, 539.8)	101.91 (50.1, 211.5)	0.301
Urine NPRO Background (μg/24 h)	0.6 (0.2, 1.8)	0.6 (0.2, 1.2)	0.926
Urine NPRO *t* test (μg/24 h)	5.6 (2.8, 11.9)	2.3 (1.3, 3.8)	0.042
Urine NPRO Difference (μg/24 h)	2.6 (1.0, 7.0)	0.9 (0.4, 1.8)	0.090

*Non-smokers includes ex-smokers. The minimum length of time between stopping smoking and participation in the study was two weeks.
†Comparison (*t* test) of current smokers with current non-smokers.
‡These results are adjusted for the effect of gastric juice pH by inclusion of pH as a covariate in the analysis of variance.

disease had low values. Such heterogeneity in gastric acidity was also observed by Crespi [19]. High pH values for "normal" subjects may have occurred due to contamination of gastric juice with saliva or bile. However, in a study by Hall *et al.* [34] in which many precautions were taken to avoid such effects and multiple pH measurements were made, 22.2% of "normal" controls had uniformly high gastric pH (pH > 6). Our corresponding proportion was 19.2% (n = 26). The CAG group, although containing a proportion of acidic individuals, is predominantly hypochlorhydric. Aside from the two possible mechanisms of nitrosation under hypochlorhydric conditions, discussed above which may be operating in this group, there may also be an effect due to the presence of gastritis itself. Activated macrophages present at sites of inflammation have been shown capable of nitrosation catalysis via the generation of nitrosating agents such as dinitrogen trioxide/tetroxide [35].

Another interesting facet of the data is the highly skewed distribution of NPRO results, requiring transformation for statistical analysis. This was apparent in the data from both diseased and healthy, acidic and hypochlorhydric groups and indicates a high degree of interindividual variation in nitrosating ability within these categories. Thus there are likely to be some individuals who, despite major controlling factors such as gastric pH being equal, will synthesise particularly high levels of NPRO due to their specific combination of conditions—i.e. in those with hypochlorhydria, this may be due to the presence of a particular type of bacteria with a significant capacity for nitrosation catalysis. In others, it may be due to a relative excess of nitrosation promoters or deficit of inhibitors [30].

The observed effect of smoking on NPRO synthesis is consistent with three other studies which have demonstrated significant promotion of endogenous L-proline nitrosation due to cigarette smoking [36–38]. These results are attributed to the catalytic effect of thiocyanate ions at higher concentrations in the gastric juice of smokers compared to non-smokers [39]. Higher circulating levels of thiocyanate in smokers may also have resulted in their observed lower levels of gastric juice nitrate due to competition between these two ions for secretion into the saliva [40], a major source of gastric juice nitrate and nitrite.

A problem with the original configuration of the NPRO test as employed here is that the intragastric nitrite concentration is deliberately and considerably elevated as a consequence of the large oral dose of nitrate (equivalent to approximately three times the estimated average *per caput* intake) [15]. This artefactual increase in intragastric nitrite is likely to enhance acid catalysed nitrosation, therefore masking any relatively small differences between normal and hypochlorhydric individuals which may occur under normal circumstances. Indeed, in one recent study of patient groups [41], only with the exclusion of the nitrate loading dose was increased intragastric NPRO synthesis demonstrated in two out of three patient groups (polypartial gastrectomy and postvagotomy) as compared to controls.

However, even with the nitrate challenge, useful information about the nitrosating potential of individuals can be obtained. Indeed, the results of the above study [41] demonstrated non-significantly higher NPRO excretion in some of the presumed achlorhydric patient groups compared to controls even with the nitrate challenge (as is also seen in the present study). This became significant when the challenge was excluded. Perhaps greater numbers in both studies may have increased the significance of the postchallenge NPRO excretion.

The lack of mutagenic activity of the gastric juice sample in this investigation contrasts with the results of two other similar studies which have claimed to have detected directly-acting mutagenicity in nitrite-containing gastric juice. Morris *et al.* [42] reported significantly increased mutagenicity in samples from patients with gastric ulcers, gastric cancer and in previous gastrectomies. In this case mutagenic activity was significantly correlated with gastric pH and total bacterial counts. O'Connor *et al.* [43] reported significant mutagenic activity in 96 out of 123 samples (78%) from gastric patients but there was no significant correlation between this activity and gastric pH, the presence of intestinal metaplasia or bacterial overgrowth. There were no significant differences in mutagenic activity between groups of patients although 96% of samples from patients with gastric ulcers were reported to be positive. Although the disparity between these two positive studies and the present negative one may be real, differences in methods for controlling for the confounding effects of bacterial nutrients in gastric juice, and in the criteria used to define positive results may also have contributed [44].

In summary, our data do not show any clear elevation of NPRO synthesis in patients with chronic gastric disease. It is clear, however, that nitrosation of proline was occurring in both the acidic and the hypochlorhydric stomach, although the mechanism of nitrosation would be different under different pH conditions. Our data also demonstrate a considerable degree of interindividual variation in nitrosating ability within disease and pH groups.

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