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Mutagens, *N*-Nitroso Compounds and their Precursors in Gastric Juice from Patients With and Without Precancerous Lesions of the Stomach

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This study examined whether elevated risk of gastric cancer is associated with high levels of total *N*-nitroso compounds (NOC), their precursors and nitrosation-dependent genotoxins in gastric juice (GJ). An improved method for quantifying total NOC was used and genotoxicity was assayed in *E. coli*. Results from patients (n = 210) with or without precancerous lesions of the stomach and living in three areas with up to 8-fold variations in gastric cancer risk (U.K., France, Colombia) were compared. The level of nitrite (range <1–472 μmol/l) was found to increase with the pH of GJ from the three countries and was dependent on country of collection. The levels of NOC (range: ≤0.01–8.0 μmol/l) in GJ were not affected by stomach histology and country of collection. NOC levels increased linearly with nitrite concentrations, but the slope of the regression line was greater for acidic GJ (pH ≤4). These data together suggest that chemical nitrosation contributes at least as much as other nitrosation pathways to the intragastric formation of NOC. Acid-catalysed nitrosation of GJ *in vitro* increased the NOC concentration (range: 7–1332 μmol/l) up to several 1000-fold but this increase was not predictive of gastric cancer risk either by country or by stomach histology. After acid-catalysed nitrosation, direct genotoxicity (SOS-inducing potency) was significantly higher in GJ with original pH > 4 and highest in samples from Colombia. The results (a) provide no support that intragastric total NOC levels are elevated in subjects with precancerous stomach lesions or living in a high risk area for stomach cancer; (b) confirm that a high nitrite level and elevated pH in GJ are strongly associated, the level of nitrite being associated with precancerous stomach conditions only in Colombia; (c) reveal the presence of precursor compounds in GJ, that after nitrosation yield direct mutagens that probably contain NOC and other substances. As their concentrations were significantly higher in achlorhydric subjects and highest in Colombian patients, these data together provide support for a role of intragastrically formed nitrite-derived direct mutagens in gastric cancer aetiology.
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INTRODUCTION

PATIENTS WITH chronic atrophic gastritis, intestinal metaplasia, dysplasia or pernicious anaemia and those who have undergone gastric surgery are at high risk of stomach cancer [1]. Endogenous formation of *N*-nitroso compounds (NOC), mediated by bacteria in the achlorhydric stomach through their nitrate-reducing activity and/or their catalytic effect on nitrosation, has been implicated in stomach cancer aetiology [1–5]. We have examined whether this elevated risk is associated with high levels of NOC determined by a recent sensitive method, their precursors and nitrosation-dependent genotoxins in gastric juice (GJ) by comparing patients with or without precancerous lesions of the stomach. These were inhabitants of three areas with variations of up to 8-fold in risk of gastric cancer [1, 6–8].

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MATERIALS AND METHODS

Chemical and reagents

All reagents used were of analytical reagent grade. Ethyl acetate was treated with 20% sulphamic acid with repeated shaking, kept for at least 3 days and filtered immediately before use. Hydrogen bromide was prepared as described by Walters *et al.* [9].

Subjects

Patients (21–86 years of age, fasted and without medication on the day of the examination), living in France, the U.K. or Colombia were selected from those undergoing gastroscopy without premedication, altering the gastric juice volume or pH as part of a routine diagnostic procedure at E. Herriot Hospital (Lyon, France), Tuquerres Hospital (Nariño, Colombia), and the General Infirmary (Leeds, U.K.). They were classified according to histologically confirmed diagnosis (Table 1). For each individual a simple questionnaire containing basic demographic data, smoking habits and clinical symptoms was completed. The incidence of gastric cancer in the Colombian population of Nariño is among the highest in the world [7, 8], while

Table 1. Distribution of patients according to country of origin and gastric status

Group	Diagnosis	Number of patients from: U.K.	France	Colombia
I	Normal gastric mucosa (NGM)	12	23	0
	Superficial gastritis (SG)	6	4	32
II	Reflux gastritis (RG)	9	0	0
III	Diffuse interstitial gastritis (DIG)	12	17	0
IV	Chronic atrophic gastritis with or without intestinal metaplasia (CAG)	17	32	6
	Dysplasia (DYS)	0	5	32
	Cancer	0	3	0
	Total	56	84	70

French and English populations have similar lower risks [1, 6]. Patients were examined and samples taken after overnight fasting.

Histopathology

Biopsies taken from the antrum (n = 2 or 3) and fundus (n = 2) were assessed by histopathologists blind to other patient data. The diagnosis of the severity of gastritis was based on at least three biopsies. On the basis of histological assessments, patients were grouped into the categories shown in Table 1, according to commonly agreed criteria reported previously [8, 10]. Only British patients were diagnosed with reflux gastritis; this group had changes of reflux gastritis in antrum or body with no evidence of chronic gastritis [11].

Collection and preparation of GJ samples

GJ was collected during gastroscopy (French and British samples). Immediately on passage of the endoscope into the stomach, as much GJ as possible was aspirated up a teflon catheter passed down the suction/biopsy channel and visually directed into the gastric juice puddle. Suction was applied with a 10 ml syringe or a pump. Colombian GJ samples were obtained via a sterile naso-gastric tube in fasting patients for whom gastroscopy had been previously performed. GJ was aspirated into a sterile 50 ml plastic syringe; while performing the aspiration, the patients were asked to take different positions to ensure complete aspiration of the gastric contents.

The pH of GJ was measured after thorough mixing, using a pH meter with a glass electrode (French and British samples) or pH-sensitive indicator paper (Colombian samples) with a precision of ± 0.2–0.3 pH units. Aliquots for the different analyses to be performed at the International Agency for Research on Cancer were immediately prepared as follows:

(a) Bacteriology. A 0.5 ml aliquot was added to glycerol infusion broth (4.5 ml), immediately vortexed and frozen at −80°C.

(b) Nitrate and nitrite. A 1.5 ml aliquot was transferred to a vial containing 0.5 ml of 0.5 NaOH to give a pH > 6 and frozen immediately at −20°C.

(c) Total NOC. A 2.5 ml aliquot was transferred to a glass/teflon joint-stoppered vial containing 50 mg of solid sulphamic acid, immediately vortexed (30 s), kept at room temperature with protection from light for a further 4 min and then stored in the dark at −20°C until analysis within 15 days.

(d) Genotoxic activity. A 5 ml aliquot was transferred to a vial and frozen immediately at −20°C.

Bacteriology

In order to count total bacteria, nitrate-reducing bacteria, aerobic/anaerobic microorganisms and yeasts, GJ was vortexed, diluted in sterile saline and serial dilutions were plated on the following media: chocolate agar plates supplemented with vitamins, incubated under 5–10% CO_2; 5% horse blood agar incubated under anaerobiosis; and Sabouraud agar plates supplemented with chloramphenicol. Each distinct type of bacteria was tested for nitrate reductase activity. The dominant bacterial species were identified using classical bacteriological tests. For less abundant microorganisms, only the genus was determined.

Genotoxic activity

GJ samples were rapidly thawed at 37°C, centrifuged (10 min at 12 000 g) and sterilised by pressure filtration before assaying for genotoxicity. Eight hundred microlitres of GJ were mixed with 800 µl of demineralised water containing sufficient $NaNO_2$ to give 80 mmol/l after adjustment to pH 1.5 with 1–4 N HCl. The mixtures were then incubated at 37°C for up to 1 h. After destruction of residual nitrite with 8.5 mg (i.e. to give 88 mmol/l) sulphamic acid, a 100 µl aliquot of the latter mixture was added to 100 µl of an aqueous solution containing 2 mg of sulphamic acid and analysed for total NOC. After adjustment to about pH 6 with 2–4 N NaOH and filter-sterilisation, aliquots of the mixture were added immediately to buffered medium containing *E. coli*.

The SOS-chromotest was carried out using a procedure adapted from Quillardet and Hofnung [12]. Log-phase culture of *E. coli* PQ37 (2×10^8 bacteria/ml) was diluted with an equal volume of L-medium buffered with 0.4 mol/l Tris pH 7.4. Up to 100 µl of sterile GJ or nitrosated GJ were added to 100 µl of Tris-buffered bacterial suspension (neutral pH was maintained). After incubation for 1 h at 37°C, with shaking, the assay medium was diluted by adding 0.8 ml of L-medium buffered with 0.2 mol/l Tris, pH 7.4, and incubated for a further 2 h. One hundred and twenty microlitres aliquots were removed for enzyme assays. β-Galactosidase and alkaline phosphatase assays were performed as described by Quillardet and Hofnung [12]. The optical density at 405 nm was measured just after addition of the enzyme substrates using an enzyme-linked immunosorbent assay (ELISA) plate reader.

Microtitre trays for β-galactosidase assays and alkaline phosphatase assays were further incubated for 2 h and 15 min, respectively, and the absorbance measured at 405 nm. Enzyme activities were calculated from the difference between the absorbance before and after incubation. This double absorbance reading avoids interference by yellowish test samples in the determination of enzyme activity. Parallel assays carried out in the absence of bacteria permitted the measurement of enzyme activities already present in the GJ samples. The ratio of β-galactosidase units to alkaline phosphatase units reflects the induction of *sfiA* gene (one SOS gene). SOS-inducing potency (SOSIP) was calculated as described previously [12]. Concurrent positive controls with 0.59 µmol methyl methanesulphonate per assay accompanied each experiment: SOSIP for this concentration ranged from 3.4 to 7.8 (mean 4.7) and from 3.4 to 7.3 (mean 4.1) for series of experiments with GJ from Colombia and France, respectively. For comparative purposes, the SOSIP per

ml GJ were adjusted taking into account the activity of methyl methanesulphonate in concurrent experiments and the mean value obtained for the series of experiments which were performed.

Determination of nitrate and nitrite

Nitrate and nitrite were analysed according to a method described by Green *et al.* [13] after 1:3 dilution for nitrite analysis and 1:9 dilution for nitrate analysis. Nitrite was determined colorimetrically by diazotisation of sulphamic acid followed by coupling to *N*-(1-naphthyl)-ethylenediamine after removal of interfering substances on an anion exchange column. Nitrate was analysed by the same method following reduction to nitrite on a cadmium column. The limit of detection was 1 μmol/l.

Determination of NOC

Total NOC in GJ samples before and after *in vitro* nitrosation ($NaNO_2$ 80 mmol/l, pH 1.5, 1 h at 37°C as detailed above, see genotoxic activity) were determined by a group-selective method as previously described [14]. Briefly, the GJ sample, treated with sulphamic acid to remove nitrite, was injected directly into refluxing ethyl acetate containing either acetic acid–0.1% (v/v) HCl for determining thermo- and acetic acid-labile thermal energy analyser-responsive compounds (TAC) or hydrogen bromide for the determination of TAC plus NOC. The amount of nitric oxide released in each case was measured through chemiluminescence detection using a thermal energy analyser, the difference between the two determinations representing the concentration of total NOC in the sample. The limit of detection was 0.01 μmol/l.

Statistical methods

Histograms, box-plots and bivariate plots were used for the exploratory analysis of the continuous variables. The non-parametric Mann–Whitney or Kruskal–Wallis test was applied to test the equality of distributions of the continuous response variables on their original scale. These distributions were compared according to diagnosis, smoking status, sex, origin and pH of GJ. To eliminate the skewness of the continuous variables, a transformation was sought in the Box–Cox family. Multivariable linear regression was used to select the best predictors of response variables and interactions among them. Forward and backward selection and all subset regression techniques were employed. The adequacy of the model was checked by residual analysis. Contingency tables were constructed for the categorical variables (total flora, nitrate-reducing flora and yeast flora). The hypothesis of equality of proportions was tested by the Fisher's exact test. BMDP and MINITAB computer programs were used for the statistical analysis [15]. The critical level of significance chosen was 5%.

RESULTS

Distribution of GJ samples according to pH, bacterial flora and histology

A total of 210 subjects classified according to histological diagnosis (Table 1) were investigated. Grouping of GJ samples by gastric pH levels and histological diagnosis (Table 2) revealed that the percentage of GJ samples with pH > 5, which permits bacterial proliferation, increased with the presence and severity of gastric lesions in subjects from each of the three countries. Prevalance of acidic GJ (pH ≤ 4) for the patients from the three countries diagnosed as having normal gastric mucosa (NGM) or superficial gastritis (SG) (i.e. no precancerous lesions of the stomach) is shown in Table 3. Consistent with earlier findings, a low gastric pH was strongly associated with normal gastric mucosa or superficial gastritis (*P* value ranging from 0.004–0.027) in the subjects from each of the three countries. For NGM/SG patients, the frequency of acidic GJ was lower, while that of GJ with pH > 7 was much higher in Colombia as compared with the other countries (Table 2).

Table 2. Distribution of GJ samples based on gastric diagnosis, country of origin of patients and gastric pH

Diagnosis*	Country of origin	% of gastric juice samples with pH ≤ 4	4–5	5–6	6–7	> 7
NGM/SG	U.K.	88.9	11.1	0	0	0
	France	85.2	7.4	0	3.7	3.7
	Colombia	56.3	9.4	3.1	3.1	28.1
RG	U.K.	77.8	11.1	0	0	11.1
DIG	U.K.	91.7	0	0	8.3	0
	France	70.6	5.9	0	11.8	11.8
CAG	U.K.	52.9	5.9	11.8	23.5	5.9
	France	56.3	3.1	3.1	12.5	25
	Colombia†	50.0	16.7	0	16.7	16.7
DYS	France†	40.0	0	0	0	60.0
	Colombia	25.0	9.4	3.1	15.6	46.9
Cancer	France†	0	0	66.7	0	33.3
All	U.K.	76.8	7.1	3.6	8.9	3.6
	France	65.5	4.8	3.6	8.3	17.9
	Colombia	41.4	10.0	2.9	10.0	35.7

*For abbreviations, see Table 1. †Small number of samples tested (see Table 1).

French GJ samples (for logistic reasons, no British and Colombian GJ samples) were analysed for total bacterial content and nitrate-reducing bacteria. The frequencies of French individuals classified by pH of the GJ, type and abundance of microflora as well as the *P* values for the equality of proportions between pH groups (pH ≤ 4 versus pH > 4 groups) are shown in Table 4. In agreement with literature data, most GJ samples with pH > 4 contained at least 300 microorganisms per ml. However, only nine (one acidic, eight with pH > 4) out of 23 (39%) GJ samples contained above 10^5 (maximum 5.4×10^7) microorganisms per ml. Among GJ samples with pH > 4 containing more than 300 microorganisms per ml, 14 out of 21 (66%) displayed nitrate-reducing capacity.

Levels of nitrate, nitrite, total NOC and genotoxins in GJ

Mean concentrations of nitrate, nitrite and total NOC in GJ, standard errors of the means, ranges, sample sizes and univariate *P* values for the hypothesis of equality of distributions are shown in Table 5. Colombian and French GJ samples from patients with precancerous lesions of the stomach showed higher levels of nitrate ($P = 0.0085$ and $P = 0.0191$, respectively) as compared to those with normal stomach or superficial gastritis. Among French samples, acidic GJ (pH ≤ 4) showed higher levels of nitrate ($P = 0.0013$), but this difference was not significant in either Colombian or British samples (Table 5b). The nitrite concentrations were significantly higher in GJ of Colombian patients with precancerous lesions of the stomach (Table 5a) and in GJ with pH > 4 (France, $P = 0.0001$; Colombia, $P < 0.0001$;

Table 3. Prevalence of acidic GJ in patients without precancerous conditions of the stomach

Country	Diagnosis	Frequency* GJ pH ≤ 4	Frequency* GJ pH > 4	*P* value†
U.K.	NGM‡ or SG	16/25 (64%)	2/10 (20%)	0.0275
France	NGM or SG	23/43 (53%)	4/24 (17%)	0.0041
Colombia	SG	18/29 (62%)	14/41 (34%)	0.0191

*Number of GJ samples from patients with NGM or SG divided by the total number of samples, excluding those from patients with DIG or RG. †Comparison of pH ≤ 4 vs. pH > 4 (see Materials and Methods). ‡For abbreviations, see Table 1.

Table 4. Prevalence of gastric flora as a function of the pH of GJ from French patients

Flora* Type, number of cells per ml	Frequency GJ pH ≤ 4	Frequency GJ pH > 4	*P* value†
All, > 300	2/54 (4%)	21/28 (75%)	< 0.0001
Nitrate-reducing, > 0	1/54 (2%)	14/28 (50%)	< 0.0001
Yeast, > 300	6/54 (11%)	12/28 (43%)	0.0017

*Maximal number of microorganisms per ml were as follows: all flora 5.4×10^7; nitrate-reducing organisms 4.3×10^6; yeast 3×10^4. †Comparison of pH ≤ 4 vs. pH > 4 (see Materials and Methods).

U.K., $P < 0.0022$). The distributions of total NOC were not significantly different between GJ samples grouped according to histopathological diagnosis (Table 5a) or according to a pH below or above 4 (Table 5b) for the three countries. Genotoxicity (expressed as SOSIP per 100 μl of GJ) was detectable with values ranging from 0.6 to 7 in only 18% of GJ from France (12/68) but in none from Colombia (0/46).

Levels of total NOC and genotoxic activity in GJ after acid-catalysed nitrosation

Mean concentrations of total NOC and mean genotoxic activities of nitrosated GJ, standard errors of the means, ranges, sample sizes and univariate *P* values for the hypothesis of equality of distributions are shown in Table 6. For logistic reasons British GJ samples could not be analysed. Acid-catalysed nitrosation of GJ (with excess $NaNO_2$ at pH 1.5 for 60 min at 37°C *in vitro*) increased the concentration of total NOC by up to several 1000-fold, with a maximum of 1330 μmol/l (Table 6). Levels of total NOC after nitrosation were significantly higher in GJ with an original pH above 4 as compared to acidic GJ among French samples ($P = 0.0001$). There was no such significant difference in the case of Colombian GJ.

After acid-catalysed nitrosation as detailed above, all GJ samples from France and Colombia exhibited genotoxic activity (Table 6). The levels of SOSIP were significantly higher in GJ with an original pH above 4 than in acidic GJ for both France ($P = 0.0003$) and Colombia ($P = 0.0059$).

Predictors for the levels of nitrate, nitrite, total NOC and genotoxic activities, before and after nitrosation, analysed by the multivariable linear regression

Table 7 summarises the results of the multivariable linear regression analysis. Levels of nitrate, nitrite, total NOC and genotoxic activities both before and after nitrosation were transformed to obtain normally distributed residuals and stabilise variances (see legend to Table 7). To perform the regression analysis, the qualitative predictors were also transformed as detailed in the legend to Table 7. Table 7 shows for each of these variables, the sample size, the measure of the overall association (R^2), the corresponding statistical significance (*P*), the significant predictors with their coefficients, standard errors and *P* values for the coefficients. All qualitative and quantitative predictors in square brackets refer to the corresponding transformed value. The pH of GJ appeared as a predictor for all variables tested, but the diagnosis was not. Indeed, although there is a strong association between pH and diagnosis (Tables 2 and 3), for a given response variable, the difference between GJ samples with pH > 4 and those with acidic pH (≤ 4) is larger than between groups I (absence of precancerous lesions) and IV (presence of precancerous lesions) (Table 1), making pH a better predictor than diagnosis.

Differences in geographical origin were significant for transformed levels of nitrite ([nitrite]), total NOC ([NOC] after nitrosation) and genotoxic activity ([SOSIP after nitrosation]) after acid-catalysed nitrosation (Table 7, Figs 1 and 2). The predictors for levels of NOC ([NOC]) are levels of nitrite ([nitrite]) and interaction between this variable and pH categorised in two classes ([pH]) (Table 7, Fig. 3). NOC levels ([NOC]) increased linearly with nitrite concentrations ([nitrite]), the slope of the regression line being greater for acidic GJ (Fig. 3). The levels of NOC after nitrosation ([NOC] after nitrosation) may be predicted in France by the pH of GJ (Fig. 1). No other significant association was seen for this variable. This result confirms the fact that the difference between pH groups was significant in France but not in Colombia, as already inferred in Table 6. For nitrate concentration ([nitrate]), the significant predictor was pH and interaction between pH and smoking status. Thus, this variable decreases linearly as pH increases, the rate of decrease being higher in GJ of smokers. No other variables were affected by smoking. The independent predictors of the levels of nitrite ([nitrite]) are listed in Table 7: (i) untransformed pH for which [nitrite] increases with pH; (ii) [sex], for which [nitrite] is higher in females than in males; (iii–iv) origin, [France] and [Colombia]: the levels of [nitrite] are the highest in France, followed by Colombia (intermediate), with U.K. the lowest; (v) [nitrate]: after adjustment of the other covariates, [nitrite] increases proportionally with [nitrate]. The predictors of [SOSIP after nitrosation] were pH and [France] (Fig. 2). The levels of [SOSIP after nitrosation] increase with pH, confirming the results shown in Table 6, and are significantly higher in Colombian than in French GJ samples ($P < 0.001$).

Table 5. (a) Concentrations of nitrate, nitrite, total NOC in human GJ samples in relation to histopathological status of the stomach and country of collection

	Concentration (μmol/l) in GJ*								
	U.K.			France			Colombia		
Group†	I	IV	*P* value‡	I	IV	*P* value‡	I	IV	*P* value‡
Nitrate	322 ± 57 (44–843) *n* = 16	358 ± 90 (84–1485) *n* = 16	0.6242	531 ± 76 (102–1461) *n* = 26	346 ± 60 (15–1647) *n* = 33	0.0191	265 ± 34 (19–1005) *n* = 30	528 ± 96 (74–1677) *n* = 22	0.0085
Nitrite	16.0 ± 3.4 (5–48) *n* = 16	25.0 ± 5.5 (5–78) *n* = 16	0.3461	29.0 ± 3.8 (7–88) *n* = 26	32.2 ± 5.4 (0–151) *n* = 33	0.9817	30.0 ± 28 (4–110) *n* = 30	81.0 ± 23 (7–472) *n* = 22	0.0181
NOC	0.67 ± 0.25 (0–4.5) *n* = 18	0.47 ± 0.18 (0–3.0) *n* = 17	0.4772	0.39 ± 0.10 (0–2.3) *n* = 27	0.35 ± 0.06 (0–1.6) *n* = 37	0.9295	0.26 ± 0.05 (0–1.1) *n* = 32	0.77 ± 0.21 (0–8.0) *n* = 59	0.3910

*Values are given as mean ± S.E., with range in parentheses. †Group I: patients with NGM or SG; group IV: patients with precancerous lesions of the stomach, see Table 1. ‡Univariate *P* value for equality of distributions (see Materials and Methods). *n* = number of patients.

Table 5. (b) Concentrations of nitrate, nitrite, total NOC in human GJ samples in relation to gastric pH and country of collection

	Concentration (μmol/l) in gastric juice*								
	U.K.			France			Colombia		
Group	pH ≤ 4	pH > 4	*P* value†	pH ≤ 4	pH > 4	*P* value†	pH ≤ 4	pH > 4	*P* value†
Nitrate	360 ± 32 (111–898) *n* = 41	334 ± 135 (44–1485) *n* = 10	0.1478	446 ± 44 (96–1647) *n* = 51	322 ± 77 (15–1405) *n* = 25	0.0013	389 ± 61 (139–1345) *n* = 24	366 ± 73 (19–1677) *n* = 28	0.1522
Nitrite	13.0 ± 1.3 (4–48) *n* = 41	36.7 ± 7.3 (5–78) *n* = 10	0.0022	21.4 ± 1.6 (0–51) *n* = 51	48.0 ± 7.0 (13–151) *n* = 25	0.0001	20.5 ± 3.8 (4–76) *n* = 24	78.1 ± 18.0 (11–472) *n* = 28	< 0.0001
NOC	0.46 ± 0.13 (0.02–4.5) *n* = 43	0.39 ± 0.12 (0.02–1.4) *n* = 13	0.7191	0.34 ± 0.06 (0–2.3) *n* = 54	0.34 ± 0.07 (0–1.6) *n* = 28	0.9961	1.11 ± 0.40 (0–8) *n* = 29	0.27 ± 0.05 (0–1.5) *n* = 41	0.0838

*Values are given as mean ± S.E., with range in parentheses. †Univariate *P* value for equality of distributions (see Materials and Methods). *n* = number of patients.

Table 6. Concentration of total NOC in and SOS-inducing potency of human GJ in relation to pH range after acid-catalysed nitrosation

	Concentration and genotoxic activity of nitrosated GJ samples*					
	France			Colombia		
	pH ≤ 4	pH > 4	*P* value†	pH ≤ 4	pH > 4	*P* value†
NOC (μmol/l)	231 ± 21 (7–603) *n* = 43	448 ± 59 (62–1332) *n* = 21	0.0001	130 ± 11 (43–291) *n* = 23	135 ± 24 (17–564) *n* = 26	0.2293
Genotoxic activity (SOS-inducing potency per 100 μl)	8.23 ± 1.52 (0–74.5) *n* = 48	13.12 ± 1.83 (4.6–43.6) *n* = 22	0.0003	11.33 ± 0.88 (3.7–23.3) *n* = 23	17.23 ± 1.86 (7.5–50.4) *n* = 26	0.0059

*Values are given as mean ± S.E, with range in parentheses. †Univariate *P* value for equality of distributions (see Materials and Methods). *n* = number of patients.

Table 7. Summary of multiple stepwise regressions for determining predictors of levels of nitrate, nitrite, total NOC and genoxotoxicity before and after nitrosation*

Transformed† variable	Number of samples	Model R^2	Model *P* value	Predictor‡	Coefficient‡	S.E.	*P* value
[Nitrate conc.]	177	0.14	< 0.0001	Intercept	0.495	0.007	< 0.01
				pH	−0.006	0.002	< 0.01
				[smok] × pH	−0.004	0.002	0.026
[Nitrite conc.]	179	0.44	< 0.0001	Intercept	1.176	0.185	< 0.001
				pH	0.077	0.008	< 0.001
				[sex]	0.096	0.038	0.011
				[France]	0.212	0.045	< 0.001
				[Colombia]	0.146	0.049	0.004
				[Nitrate conc.]	0.733	0.370	0.049
[NOC conc.]	178	0.07	0.0017	Intercept	−0.839	0.180	< 0.001
				[Nitrite conc.]	0.225	0.084	0.008
				[Nitrite conc.] × [pH]	0.102	0.034	0.003
[NOC conc. after nitrosation]	113	0.38	< 0.0001	Intercept	6.961	0.170	< 0.001
				[France] × pH	0.435	0.053	< 0.001
[SOSIP after nitrosation]	119	0.27	< 0.0001	Intercept	2.673	0.176	< 0.001
				pH	0.125	0.029	< 0.001
				[France]	−0.608	0.155	< 0.001

*In order to normalise quantitative variables/predictors (x), the following power transformation was applied: $[X] = [(x + 0.5)^{\lambda} - 1]/\lambda$ with $\lambda = -0.3$ for nitrite and NOC concentrations, $\lambda = 0.15$ for NOC concentration after nitrosation, $\lambda = 0.16$ for SOSIP after nitrosation and $\lambda = 0$ for nitrate concentration, i.e. the normalisation function was log (nitrate conc. + 0.5). Qualitative predictors were transformed as follows: [pH]: value 1 when pH ≤ 4, otherwise zero; [sex]: value 1 when sex is female, otherwise zero; [France]: value 1 when origin is France, otherwise zero; [Colombia]: value 1 when origin is Colombia, otherwise zero; smoking status [smok]: value 1 when patients were smokers, otherwise zero. †Untransformed variables were expressed in μmol/l except SOSIP after nitrosation that was expressed as SOS induction factor per 100 μl of gastric juice. ‡The general equation for response variable (RV) as a function of predictor (P) was as follows: [RV] = intercept + coefficient 1 [P1] + coefficient 2 [P2] . . . + coefficient i [Pi].

DISCUSSION

Correa *et al.* [2] and Correa [5] have proposed an aetiological hypothesis for multistage gastric carcinogenesis, according to which the achlorhydric environment of the stomach found in patients with CAG may provide a suitable milieu for proliferation of bacteria. Because many of these can convert nitrate to nitrite and catalyse nitrosation of amines, intragastric formation of NOC, in particular of the nitrosamide type, has been implicated in gastric carcinogenesis [16].

Numerous studies provided support for several steps of this hypothesis. Most of them have reported a positive correlation between the prevalence of CAG, increased gastric pH, and

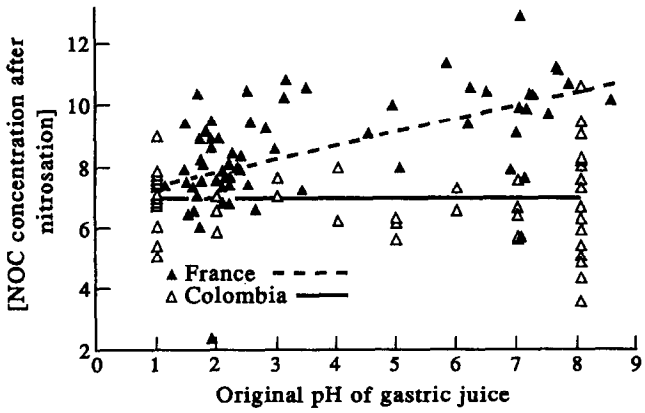

Fig. 1. Association between concentrations of NOC after *in vitro* acid-catalysed nitrosation of GJ and their original pH for French and Columbian samples. Linear regression analyses were performed using transformed concentration of NOC as follows: transformed values: [NOC conc.] = [(NOC conc. + 0.5)$^{0.15}$ − 1]/0.15. [France] = 1 when origin is France, otherwise zero; n = 113. Equation of the lines: [NOC conc. after nitrosation] = 6.96 + 0.435 pH × [France].

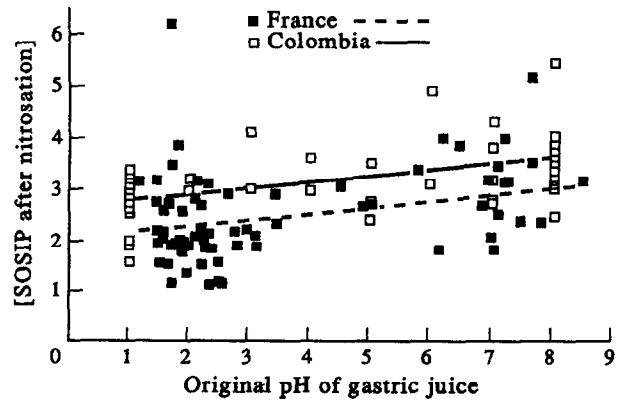

Fig. 2. Association between SOS-inducing potency of nitrosated GJ and their original pH for French and Colombian samples. Linear regression analyses were performed using transformed SOS-inducing potency as follows: transformed value [SOSIP] = [(SOSIP + 0.5)$^{0.16}$ − 1]/0.16. [France] = 1 when origin is France, otherwise zero; n = 119. Equation of the lines: [SOSIP after nitrosation] = 2.67 + 0.125 pH − 0.608 [France].

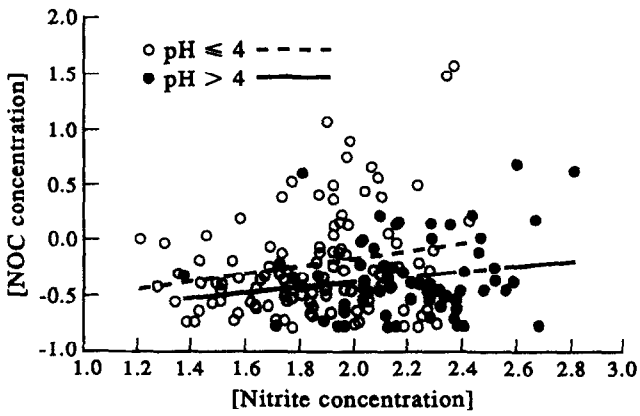

Fig. 3. Association between concentrations of NOC and nitrite in acidic (○) and neutral/basic (●) human GJ. Linear regression analyses were performed using transformed variables as follows: transformed values: $[x] = [(x + 0.5)^{-0.3} - 1]/-0.3$. x = nitrite concentration or NOC concentration; $n = 178$. [pH] value 1 when pH ≤ 4, otherwise zero. Equation of the lines: [NOC conc.] = −0.839 + 0.225 [nitrite conc.] + 0.102 [nitrite conc.] × [pH].

counts of both total and nitrate-reducing bacteria [17–19]. It has been shown that various common bacterial strains (including some of those isolated from human GJ) can catalyse nitrosation of secondary amines at neutral pH *in vitro* by an enzymatic reaction [20–25]. However, it is still not clear whether the concentration in GJ of endogenously formed NOC in subjects with an achlorhydric stomach or precancerous conditions is higher than in asymptomatic subjects. Six out of seven studies using the nitrosoproline (NPRO) test indicated that the urinary excretion of NPRO was not increased in subjects with precancerous conditions as compared with healthy subjects [26–32]. Other studies showed contradictory results concerning levels of NOC in GJ and achlorhydria [18, 19, 26, 33–37]. Such discrepancies have arisen from the methods applied to determine total NOC [38, 39]. We have, therefore, developed an improved procedure for total NOC analysis in GJ which has overcome major technical problems for which the previous methods were criticised [14] that was subsequently applied to GJ samples from patients with or without precancerous stomach conditions.

In the present study, we found that levels of total NOC in fasting GJ samples from the three countries were not dependent on either their pH or gastric histology (Table 5). The observation that NOC concentrations were not different in achlorhydric and acidic stomach may be due to the fact that bacterial activity for nitrosation and nitrite production compensated for the lack of acidity required for chemical nitrosation. However, multivariate analysis revealed that levels of NOC are predictable not only based on nitrite concentrations but on an interaction between nitrite concentrations and pH (Fig. 3). This association shows the relevance of covariate adjustment, as in the univariate analysis (Table 5b) the distributions of NOC levels for the two pH groups were not significantly different. The fact that levels of NOC increased with nitrite concentrations at a greater rate in acidic GJ than in those with pH > 4 suggests that chemical nitrosation contributes at least as much as bacterial nitrosation to the intragastric formation of NOC. Consistently with these findings, Maragos *et al.* [40] reported a low yield of nitrosotrimethylurea was formed in the porcine stomach at elevated pH and in the presence of substantial nitrite levels.

In keeping with previous studies (reviewed in [18] and [41]), nitrite concentrations were found to be significantly higher in GJ with pH > 4 than in acidic GJ from French, British and Colombian patients; the highest value was found for GJ of Colombian patients with precancerous lesions and whose pH was above 4 (Table 5). The mean nitrite concentrations we have found in acidic GJ were at least three times higher than most of those reported previously [18, 41]. This discrepancy may be attributable to our samples being made alkaline just after collection and before freezing, thus preventing an acid-catalysed degradation of nitrite during thawing.

We have further examined whether the levels of nitrosatable amino/amido precursors in GJ may constitute a better marker of gastric cancer risk than those of NOC. The very high levels of NOC after *in vitro* nitrosation, ranging from 7 to 1330 μmol/l, were not associated in the study with gastric cancer risk as they were higher in French than in Colombian GJ samples (Table 6). Furthermore, they were positively related to the original gastric pH only for the French group (Table 7, Fig. 1). These findings suggest that both the level and probably the nature of some of the substances are different between the two groups.

As Gatehouse and Tweats [42] have shown that nitrosation of GJ produced mutagens, we have also examined whether the levels of such nitrite-dependent mutagens reflect the gastric cancer risk. Genotoxicity expressed as SOSIP was found to be dependent on the original pH of GJ for both French and Colombian groups (Fig. 2). Gastric cancer risk was reflected by the levels of these genotoxins, that were higher in Colombian than in French GJ (Fig. 2) although the magnitude of the difference (~ 2-fold) does not match the difference in risk (~ 8-fold). This suggests that carcinogenic effect is supralinear and/or that other factors contribute to gastric cancer risk. Based on data shown in Figs 1 and 2, we conclude that nitrosation of precursors in GJ leads to mutagens including not only NOC but also possibly diazo compounds that are not detected by our method for NOC analysis. Accordingly, Kyrtopoulos [43] has shown that nitrosation of GJ leads to the formation of O^6-methyl- and ethyl-deoxyguanosine and nitrosation of phenols/indoles leads to diazonium mutagenic compounds (reviewed in [44, 45]), some of which were carcinogenic for glandular stomach of rat [46]. Isolation and characterisation of major precursors of genotoxic NOC and other nitrosation-dependent genotoxins are being attempted in our laboratory. Other types of DNA damage than those measured may contribute to this risk, such as chronic *Helicobacter pylori* infection, possibly causing oxidative damage through activated macrophages [47], which has emerged as a potential aetiological factor (reviewed in [48, 49]). We have recently demonstrated that in patients, the presence of premalignant conditions such as CAG was highly associated with *H. pylori* infection, lower ascorbic acid level indicative of oxidative stress and elevated pH but not with total NOC in fasting GJ [11].

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Study of the Role of Breast Self-examination in the Reduction of Mortality from Breast Cancer

The Russian Federation/World Health Organization Study.
Vladimir Fedorovich Semiglazov, Vsevolod Nicolaevich Sagaidak, Vladimir Michaelovich Moiseyenko and Eduard Alexaevich Mikhailov

The protocol of a study, sponsored by the World Health Organization, of the role of breast self-examination (BSE) in reduction of mortality from breast cancer is presented. The major objective of the study is to determine the effect of a BSE programme on mortality from breast cancer. A population of over 193 000 women aged 40 to 64 has been defined in Moscow and St Petersburg and randomised to study and control groups. In Moscow the education programme is based on a two-way communication principle allowing efficient person-to-person education in groups of up to 20 individuals and feedback information through specially designed personal calendars. In St Petersburg, class and individual instruction is carried out. After a 1-year feasibility study the project is planned to last for 15 years. It consists of an aggressive education programme, during and following which, all newly diagnosed breast cancers will be registered and treated, and followed up for 3 to 15 years. A key issue of the study is compliance of the population with BSE. The frequency and competence of BSE practice has been defined in subsamples of 400 randomly selected women by means of surveys at 6 months, 1, 2 and 3 years after the start of the project. The study is expected to result in the accrual of more than 1470 new breast cancer cases and 778 deaths from breast cancer. The power of the study is expected to permit detection of a 30% reduction in cumulative breast cancer mortality, assuming that 50–70% of the women in the study group practise BSE.
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INTRODUCTION

Background

SINCE THERE is no practical method of primary prevention of breast cancer immediately available at present, screening, resulting in the diagnosis and treatment of breast cancer at an early stage, seems to be a rational approach to reducing mortality from this common tumour. Poor prognosis has been repeatedly associated with an advanced stage of the disease. Thus, screening that detects cancer at an early stage when treatment may be more effective, is likely to become a major contributor to strategies for control of breast cancer.

Screening of limited groups of the population (on an experimental basis), health demonstration programmes to promote early breast cancer detection, and—more recently—national policies for breast cancer screening have been established in a number of countries during the last two decades. Different technologies to meet this goal have been developed: mammography, thermography, diaphanography, ultrasound, physical examination by health professionals and self-examination by women. The reported results indicate that screening by mammography with or without breast physical examination can reduce mortality from breast cancer in woman age 50–69 [1, 2]. However, breast cancer screening programmes involving imaging technologies can be expensive and for this reason cannot be adopted in many countries as a routine public health approach [2].