Thiocyanate-independent nitrosation in humans with carcinogenic parasite infection

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Infection with the liver fluke, Opisthorchis viverrini, is a causative agent of cholangiocarcinoma. One possible contributing factor in this carcinogenesis is the chronic, local generation of nitric oxide by inflammatory cells expressing inducible nitric oxide synthase and the production of N-nitroso compounds via the reaction between amines and nitrosating agents derived from nitric oxide. Our previous studies provided evidence that nitric oxide synthesis is elevated during human liver fluke infection. Here we present data on the same sample of men which definitively demonstrates increased nitrosation of proline and thioproline (thiazolidine-4-carboxylic acid) among infected men compared to uninfected control subjects on a low nitrate diet. This difference was specifically abolished by co-administration of ascorbic acid with proline and by elimination of parasites by praziquantel treatment. Multivariate statistical models demonstrate the importance of salivary thiocyanate levels to variation in the nitrosation of proline among uninfected individuals, but not among those with current fluke infection. This suggests that considerable generation of nitrosating agents (N₂O₃/N₂O₄) in infected people may be occurring via oxidation of arginine by nitric oxide synthase in inflamed tissue which is thiocyanate insensitive. Analyses revealed positive associations between N-nitrosoprine excretion and nitrate/nitrite levels in urine, plasma and saliva and with usual alcohol intake; with variation in these trends between groups. In conclusion, we have confirmed the relationship between Opisthorchis viverrini infection and enhanced endogenous nitrosation, showing evidence of its extragastric site. New information is also provided on the determinants of N-nitrosamino acid excretion in men on a controlled low nitrate diet without smoking, conditions which reduce exogenous sources of nitrosating agents.

*Abbreviations: NPRO, N-nitrosoprine; NTPRO, N-nitrosothioproline; NMTPRO, N-nitrosomethylthioproline; NDMA, N-nitrosodimethylamine; ELISA, enzyme-linked immunosorbent assay; GC-TEA, gas chromatography-thermal energy analysis; ANOVA, analysis of variance; SD, standard deviation.
chronic infection in the intrahepatic bile ducts and stimulates both cellular and humoral immune responses (32). Bile duct cancer can be induced in hamsters experimentally infected with O.viverrini and its close relative, Clonorchis sinensis, after exposure to low doses of NDMA or its precursors which did not induce tumours in uninfected animals (24–27). Ohshima et al. (28) recently reported the induction of nitric oxide synthase in macrophages, mast cells and eosinophils in inflamed areas surrounding the bile ducts and increased endogenous nitrosation of thiazolidine 4-carboxylic acid (thio-
proline) in O.viverrini infected hamsters. Uncontrolled studies by Srianujata et al. (29) and Srivatanakul et al. (30) suggest that infected people have a higher endogenous nitrosation potential than uninfected people.

We previously demonstrated an increase in endogenous generation of nitric oxide among infected men under strict dietary control as indicated by increased levels of plasma and urinary nitrate and salivary nitrite compared to uninfected men which were abolished by anthelmintic treatment of the fluke (23). This paper details a controlled assessment of endogenous

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**Table I.** Characterization of the sample group with mean values of demographic and biochemical data before and after treatment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity Group</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Background Data:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg count range (eggs/g faeces)</td>
<td>0</td>
<td>1000–6000</td>
</tr>
<tr>
<td>Number of men</td>
<td>31</td>
<td>38</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39.1</td>
<td>38.0</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>56.5</td>
<td>57.0</td>
</tr>
<tr>
<td>Alcohol index</td>
<td>0.63</td>
<td>0.75</td>
</tr>
<tr>
<td>Thiocyanate (µM)</td>
<td>1168</td>
<td>1501</td>
</tr>
<tr>
<td>Creatinine (mg/kg/day)</td>
<td>26.4</td>
<td>27.5</td>
</tr>
<tr>
<td>Nitrate/nitrite:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma nitrate (µM)</td>
<td>40.4</td>
<td>51.1</td>
</tr>
<tr>
<td>Salivary nitrite (µM)</td>
<td>82.6</td>
<td>168.8</td>
</tr>
<tr>
<td>Urinary nitrate (µmol/kg/day)</td>
<td>16.7</td>
<td>20.1</td>
</tr>
</tbody>
</table>

Mean values for nitrate/nitrite and nitrosamine acid measurements are back-transformed geometric means. Nitrosamine acid levels were measured during proline loading. Symbols placed under intensity group indicate statistically significant variation in the log-transformed means of the three intensity groups determined by one-way ANOVA at the level of: **P < 0.01; ***P < 0.001.

**Table II.** Analysis of variance models describing variables associated with amounts of NPRO and NTTPRO (log transformed to normality) per day excreted by the sample

| Variable | Pre-treatment | Post-treatment | | | | |
|----------|---------------|---------------| | | | |
| Covariates | | | | | | |
| Urinary nitrate | 13.4 | 28.5**** | 7.9 | 10.0**** | 0.2 | 0.4 | 1.0 | 1.2 |
| Plasma nitrate | 2.5 | 5.2** | 0.0 | 0.0 | 0.0 | 0.0 | 1.1 | 0.7 |
| Salivary nitrite | 53 | 11.4**** | 0.0 | 0.0 | 9.5 | 17.1**** | 0.0 | 0.3 |
| Alcohol intake | 2.4 | 5.1** | 0.0 | 0.0 | 0.0 | 0.2 | 0.0 | 0.1 |
| Total explained | 62.0 | 22.0**** | 34.0 | 10.8**** | 45.0 | 16.2**** | 20.6 | 2.8*** |

To show the magnitude of each variable as a determinant of nitrosamine acid excretion, the percentage of the total variation in nitrosamine acids between individuals that was found to be associated with the individual variable is given (% variation). The category 'Covariates' includes the sum of variation associated with individual variables and their interactions. The F-ratio indicates the level of variation associated with each degree of freedom of the variable relative to that of the remaining unexplained variation. Symbols placed after the F-ratio indicate the level of significance in the log-transformed means of the intensity groups determined by ANOVA as given in Table I. Intensity of infection was entered first into the model.
Table III. Analysis of variance models describing variables associated with the amount of NPRO (log transformed) excreted per day by uninfected and infected men before and after treatment

<table>
<thead>
<tr>
<th>Intensity group</th>
<th>Covariates</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>Uninfected</td>
<td>Infected</td>
</tr>
<tr>
<td>Moderate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary nitrate</td>
<td>9.2***</td>
<td>7.4***</td>
<td>8.6***</td>
</tr>
<tr>
<td>Plasma nitrate</td>
<td>5.2**</td>
<td>0.3</td>
<td>7.4***</td>
</tr>
<tr>
<td>Salivary nitrite</td>
<td>0.3</td>
<td>0.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Thiocyanate</td>
<td>11.4*****</td>
<td>11.9***</td>
<td>1.2</td>
</tr>
<tr>
<td>Alcohol intake</td>
<td>5.1**</td>
<td>0.2</td>
<td>9.5***</td>
</tr>
<tr>
<td>Total explained</td>
<td>22.0*****</td>
<td>13.6***</td>
<td>22.8****</td>
</tr>
</tbody>
</table>

The numbers given are the F-ratios which indicate the level of variation associated with each degree of freedom of the variable relative to that of the remaining unexplained variation. Symbols indicating the significance levels for the F-ratios are given as defined in Table I.

All variables were allowed to enter into the model simultaneously in order to highlight differences in the important determinants of NPRO excretion.

Fig. 1. Average levels [log_{10} (nanograms per day*10)] and 95% confidence intervals of N-nitrosoproline (NPRO), N-nitrosothioproline (NTPRO) and the sum of N-nitrosamino acids (sum) excreted by men with no (left-most bars), moderate (middle bars) and heavy (right-most bars) infection with the liver fluke, Opisthorchis viverrini. (a) Basal levels recorded with no prior loading with proline; (b) and (d), levels during proline loading; and (c), results measured with co-administration of proline and ascorbic acid. Parts (a), (b) and (c) demonstrate results of the initial assessment (pre-treatment), Part (d) shows levels 4 months after elimination of infection with the anthelmintic drug, praziquantel.

Materials and methods

The rationale, study design and methodology have been detailed previously (23) and were given approval by official ethics committees in Thailand and Australia. Briefly, a total of 148 men, aged 30-50 years, were selected and assigned to three intensity groups on the basis of egg counts (0, 1000-6000 and >6000 eggs/g using the formalin-ethyl acetate concentration method) from a cross-sectional study of cholangiocarcinoma in villages in north-east Thailand (31). The purpose and procedures of the study were explained to each selected individual, followed by an invitation to participate. Those who wished to participate provided written informed consent and stayed together for 5 days in 13 groups of 12-15 men of mixed infection status, eating only the supervised low nitrate diet provided and not smoking. The diet consisted mainly of sticky rice, meat and fish without including green vegetables, fruit or preserved food. After sample collections, the men were treated with the anthelmintic drug, praziquantel, and 134 (90%) attended post-treatment collections 4 months later.

Other potentially important determinants of endogenous nitrosation were also measured as described (23). An alcohol index was calculated as the usual proportion of a 250 ml bottle consumed times the frequency of drinking per month, as recorded during interview. Completeness of 24 h urine specimens were confirmed by urinary creatinine; those containing under 16.9 mg/kg body weight/day were excluded from analysis.
Relative levels of parasite-specific antibody isotypes were determined by ELISA essentially as described (32), with the additional application of isotype-specific monoclonal antibodies (DAKO-PATTS. Denmark) (for IgG1, IgG2, IgG4 and IgE) and avidin-biotin amplification procedures (ABC complex, DAKO-PATTS) (for IgG4 and IgE).

After an initial day of consuming a low nitrate diet without smoking to deplete nitrate from previously high exogenous sources, three 24 h urine specimens were collected. The first was without proline loading. To assess basal amounts of nitrate and N-nitrosamino acid excretion. During the second, 100 mg of proline were given to each subject 1 h after each of three meals (total 300 mg), while during the third, proline was administered after meals with 100 mg of ascorbic acid (total 300 mg proline + 300 mg ascorbic acid).

N-nitrosamino acids were measured in the three pre-treatment urine specimens and in the proline loaded samples collected 4 months after treatment by gas chromatography/thermal energy analysis (GC-TEA) as previously described (16–18). Then 20 ml aliquots of alkalinated urine were treated with 20% ammonium sulphamate and sodium chloride, and 50 ng N-nitroso pipelicolic acid was added as an internal standard. The samples were then extracted with the methylene chloride-methanol, evaporated, redissolved in methylene chloride and derivatized with diazomethane, concentrated by evaporation and subjected to GC-TEA for quantification of N-nitrosamino acids. Values were adjusted according to the percent recovery of the internal standard. In addition, the samples were analysed by GC-TEA in two groups one year apart. A consistent level of loss was observed in the samples, so those analysed in the latter period were adjusted for control to sample deterioration.

Data were analysed statistically after log-transformation to normality using SPSS-PC+. Paired sample t-tests and Pearson’s correlations were used to explore relationships in excretion levels of intensity groups and individuals across nitrosamino acid assessments (e.g. with and without proline loading, pre- and post-treatment). One way analysis of variance (ANOVA) was performed to determine whether nitrosamino acid excretion varied significantly within assessments between the intensity groups before and after praziquantel treatment. Other variables which showed statistically significant associations with nitrosamino acids were then incorporated into multiple variable simple factorial ANOVA models in SPSS. These models were used because they allowed for the greatest control over variable entry. For some analyses (i.e. those presented in Table II), the intensity group variables were forced into the model first, allowing for maximum partitioning of variation to these groups. Log-transformed covariates were then entered using the experimental method (similar to that of stepwise regression where maximum variation is explained) in order to explain much of the remaining variation as possible. In Table III, similar ANOVA models were developed, but with the simultaneous entry of all variables (intensity group and covariates), without initial partitioning of variation by intensity groups. The amount of variation associated with NPRO both before and after treatment with praziquantel and among groups stratified by the presence of infection are given. These detailed analyses are given to best illustrate the most important determinants of NPRO and NTTPRO and to highlight variations in these in the presence and absence of infection.

Results

Three nitrosamino acids were detected in the urine specimens. Nearly every subject excreted NPRO and NTTPRO, while a minority (20%) excreted small amounts of NMTPRO. Back-transformed mean values of the three fluke intensity groups for 24 h urinary output of individual levels, and the sum of all three nitrosamino acids after proline loading and other biochemical measurements, are given in Table I.

Comparisons between assessments

Levels of NPRO excreted by individuals before and after treatment were closely correlated (Pearson’s r correlation coefficient, r = 0.52, P < 0.001). The sum of nitrosamino acids and NTTPRO showed weaker consistency (r = 0.38, P = 0.001 and 0.26, P < 0.05), while NMTPRO levels showed no consistency (r = 0.00) between time points.

In pre-treatment assessments, proline loading was associated with virtually no change in NTTPRO levels (r = 1.2, P > 0.05), but NPRO excretion increased significantly (paired t-test, t = 2.3, d.f. 85, P < 0.05). However, this increase occurred only in the moderately infected group (t = 2.1, d.f. 33, P < 0.05); NPRO levels of the uninfected and heavily infected groups showed almost no variation with and without loading (t = 0.9, 0.8, d.f. 23, 27, P > 0.05, respectively) (compare Figure 1a and 1b).

Post-treatment values of NPRO (P < 0.01), NTTPRO (P < 0.05), NMTPRO (P < 0.001) and the sum of the three (P = 0.05) were significantly increased over pre-treatment levels (Table I and compare Figure 1a and 1d). These increases were statistically significant with similar P values within the groups with no infection and heavy infection. In sharp contrast, no significant variation between pre- and post-treatment levels of individual nitrosamino acids and their sum were observed among those with moderate infection (t = 0.54, 0.05, 1.4, 0.51, respectively; d.f. 29, P > 0.05).

Analysis of variance in nitrosamino acid excretion within assessments

Marginally significant variation between the uninfected and the two infected groups in urinary levels of NPRO, NTTPRO and the sum of the three nitrosamino acids was detected without proline loading (Figure 1a). This variation was markedly enhanced after proline loading (Figure 1b), attaining stronger degrees of statistical significance (NTTPRO: P < 0.05, NPRO and sum: P < 0.01). However, there was no difference in these levels which decreased when ascorbic acid was administered with proline (Figure 1c). Post-treatment assessments of nitrosamino acid excretion after proline loading also demonstrated no variation between former intensity groups (Figure 1d) in levels of NPRO, NTTPRO and the sum of nitrosamino acids.

The models of nitrosamino acid excretion incorporating only intensity group are not very robust due to the large amount of variation not directly associated with liver fluke infection. For example, incorporating only intensity group into the model explains just 10.5% of total variation in NPRO excretion before treatment. The addition of other factors measured in the experiment to the model yielded the explanation of 30–60% of the total variation (Tables II and III) and indicates which other factors are important.

After rigorous testing, the final ANOVA model which was found to best describe variation in excreted NPRO after proline loading, and with initial entry of intensity group, is presented in Table II. Before treatment, this model explained 62.0% of the total variation in NPRO levels, with 13.6% of the variation associated with intensity group and a further 48.4% associated with variation in plasma and urinary nitrate, salivary thiocyanate and usual alcohol intake. These covariates showed strong interactions, i.e. the percent of variation associated with the sum of individual covariates was much less than that of their total, as expected due to the relationship between nitrite and thiocyanate in gastric nitrosation. Moderate infection was associated with markedly higher NPRO excretion than heavy infection; this was particularly apparent after controlling other factors (Table II).

After treatment with praziquantel, a dramatic reduction in the amount of variation (13.6–0.4%) in NPRO associated with initial intensity group was observed. Urinary nitrate, salivary nitrite and thiocyanate levels remained significantly associated with NPRO, and this model was able to explain 45% of the total variation.

The sum of the three nitrosamino acids correlated closely with NTTPRO levels (data not shown), and modelling demon-
strated a similar pattern to that described for NTPRO (see Table II). Before treatment, variation in levels of excretion of NTPRO and the sum were mainly determined by the liver fluke intensity group (8.3–13%) and levels of saliva nitrite and urinary nitrate. Salivary thiocyanate was not associated with significant variation in NTPRO. After treatment, 1.1% of variation in NTPRO and the sum was explained by intensity of infection, while urinary nitrate levels retained a strong association.

Analysis of variance without forced initial entry of intensity group variables was also performed; this allowed for comparisons of the basic determinants of NPRO excretion among the initially uninfected and infected groups (Table III). Although a weakly significant association with moderate infection remained, it is clear that levels of urinary and/or plasma nitrate and salivary nitrite were more closely related to NPRO excretion than infection status, both before and after treatment with praziquantel.

The relationship between salivary thiocyanate and NPRO levels was highly significant within the uninfected group both before and after treatment (F = 11.9, 18.4; P < 0.01) and in the previously infected group tested after treatment (F = 7.8, P < 0.01). In sharp contrast, the entry of thiocyanate into the model among those with current infection explained little of the variation in NPRO levels (F = 1.2, P > 0.05). Usual alcohol intake patterns were only associated with NPRO among those with current fluke infection.

Other factors tested in ANOVA of both pre- and post-treatment levels of NPRO, NTPRO and their sum were not found to explain the significant variation after controlling the above variables. These were infection with other parasites (hookworm, Taenia, intestinal flukes, echinostomes), urinary tract infection, hepatitis B surface antigen carriage, self-reported smoking frequency, height, weight, age, hepatobiliary status determined by ultrasound. Weak positive associations approaching on statistical significance (P < 0.10) were found between parasite-specific IgE and IgG1 levels and NPRO excretion in infected men.

Discussion

Data from two previous studies (29,30), plus this investigation, strongly suggest that liver fluke infection leads to increased rates of endogenous nitrosation. This indicates an enhanced potential for the endogenous formation of carcinogenic N-nitroso compounds, most likely NDMA, which induces bile duct cancer in liver fluke infected animals (24–27). The elevation in NPRO excretion associated with infection reported here is approximately two-fold; considerably smaller than the ten-fold increase reported by Srivatanakul et al. (30) in a study which did not control dietary intake during assessments. Furthermore, much larger increases in NPRO excretion have been observed in studies which co-administered nitrate with proline (21,22). The majority of NPRO measured in these studies is likely to have been generated in the intragastric site, thus masking the contribution of NPRO produced outside the stomach, e.g. in inflamed tissue. Thus, the demonstration of extragastric nitrosation of proline during chronic infection, is possible only while gastric nitrosation is minimized by limiting the intake of exogenous nitrate and nitrite.

As also observed for levels of plasma, urinary nitrate and salivary nitrite (21), there does not appear to be a direct dose-dependent increase (i.e. heavy > moderate infection) in endogenous nitrosation potential with intensity of liver fluke infection as had been expected. In fact, after controlling the other important determinants of nitrosation, the statistical model indicated that the heavily infected group excreted less NPRO than the moderate group. This may be the result of a decrease in nitric oxide generation due to a modulation of parasite-specific immune responses in heavy infection; which has been noted in this group using T-cell proliferation assays to fractionated parasite antigens (manuscript in preparation). Modulation of cellular immune responses during heavy infection and those of long duration is a commonly observed phenomenon and is thought to protect the host against immunopathology (33,34). Since helminth infections are cumulative and liver fluke infections are acquired at relatively early ages (35), it is likely that people with heavy infections at a given time also experienced moderate infections with more active immune responses over long periods during their life.

In our study, proline loading had little effect on NPRO excretion except in the moderately infected group. Thus, when the contribution of nitrogen oxides from dietary sources and inflammation were minimized, basal levels of proline and thioproline were apparently sufficient to capture the relatively small amount of endogenously generated nitrosating agents which are ultimately excreted in urine as nitrosamino acids. This has also been reported for non-smokers, but not for smokers allowed to smoke during urine collections (36). This observation further confirms the tight control on both dietary- and smoking-associated intake of nitrogen oxide, as maintained here.

A method for assessment of the level of endogenous nitrosation that occurs in the tissue, as opposed to the stomach, is not yet available. However, differences in sensitivity to thiocyanate may be a useful indicator, since thiocyanate is required for the generation of nitrosating agents, e.g. N₂O₃/ N₂O₄, from the inert precursor, nitrite, under acid conditions in the stomach (37,38). In contrast these nitrosating agents are independently produced during oxidation of arginine by nitric oxide synthase in the tissue and favourably react with amines at neutral or alkaline pH without thiocyanate catalysis (10,12,39). Our data suggest that under controlled conditions, liver fluke infection increases the relative contribution of extragastric nitrosation as indicated by variation in the sensitivity to thiocyanate levels in the presence and absence of infection. This is the first in vivo evidence of variation in the relative importance of the two compartments of nitrosation.

Another example of variation in sensitivity to thiocyanate level was observed between NPRO and NTPRO. Thioproline is one thousand times more effective at scavenging nitrosating agents than proline, such that the presence of thiocyanate is not required for the catalysis of thioproline nitrosation in any compartment (14,18). Thus the complete absence of association between NTTPRO and salivary thiocyanate was expected.

The variables entered into the statistical models are of two types, namely those totally independent of parasites (e.g. thiocyanate, alcohol intake) and those partly determined by infection (salivary nitrite, urinary and plasma nitrate) [see Table I and (23)]. Control of the latter variables was therefore associated with a reduction in the variation attributed to infection status (e.g. as in Table III), but this does not indicate a reduced importance of the parasite as a determinant of nitrosation. The fact that moderate infection maintains significance even after controlling nitrate levels suggests that the parasite not only increases the biosynthesis of nitric oxide, but
also that the intermediate between nitric oxide and its end product (nitrate) among infected men may be more likely to participate in the nitrosation reaction than nitrate precursors available from non-inflammatory sources.

The relationship between usual alcohol consumption and NPRO excretion is difficult to explain. Reported alcohol intake was largely moderate and similar between intensity groups, but was associated with NPRO only among those currently infected. Similar, but weaker, associations between NPRO and parasite-specific antibodies of IgE and IgG1 before treatment may suggest a link between these antibodies and nitrosation. Although such a link has not been previously described, binding of mast cells with antigen-bearing IgE and opsonization of macrophages with IgG1 may enhance their response to cytokines secreted by parasite-specific T-cells upon stimulation. Macrophages and mast cells in the bile duct of fluke infected hamsters are known to express nitric oxide synthase (28).

Finally, the increase in both NPRO and NTPRO associated with assessments at the later time point (4 months post-treatment) may reflect problems in correcting for sample activity loss through storage. Since the samples were collected over a period of months, the amount of decay between groups would not be equal. Furthermore, there may have been a true increase in background variation in the levels of nitrogen oxides, as previously reported (23). Although the controlled diet contained as little nitrates as possible, exogenous sources of nitrogen oxides cannot be completely eliminated and seasonal differences in these, as well as other infections, might be expected. However, because this study utilized a block design (i.e. individuals with no, moderate and heavy infection, were assessed at the same time and under exactly the same conditions, in probable contrast to previous geographic studies), it is highly unlikely that either problem (seasonal variation or correction for sample decay) would introduce bias associated with infection. Although this background variation prevented the observation of a clear decrease in the infected groups post-treatment, the lack of increase in the moderately infected group may reflect a balance between reduction due to loss of parasites and increases in background variation.

In summary, the dramatic reduction in the amount of variation (13.6-0.4% in NPRO and 8.3-1.1% in NTPRO) associated with the initial intensity group is strong evidence that liver fluke infection is an important determinant of total body nitrosation. Since a direct dose-response was not observed and carcinogenesis is a multi-stage process, there are likely to be many factors involved in the relationship between immune responses, endogenous nitrosation and the high risk of bile duct cancer associated with fluke infection (22,23,28,31,32). We are now investigating other determinants and events in human nitrosamine carcinogenesis which the parasite may also influence.

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References

24. Flavell, D.J. and Lucas, S.B. (1983) Promotion of N-nitrosodimethylamine-
Endogenous nitrosation in liver fluke infection

initiated bile duct carcinogenesis in the hamster by the human liver fluke, *Opisthorchis viverrini*. *Carcinogenesis*, 4, 927-930.


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