Differential Effect of Chronic Ethanol Consumption on the Carcinogenicity of $N$-Nitrosopyrroolidine and $N'$-Nitrosonornicotine in Male Syrian Golden Hamsters

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ABSTRACT

The effect of chronic ethanol consumption on the carcino- 
genicity of $N$-nitrosopyrrolidine (NPYR) and $N'$-nitrosonornico- 
tine (NNN) in male Syrian golden hamsters has been investi- 
gated. Groups of hamsters were maintained on either ethanol- 
containing or control liquid diets for 4 weeks prior to and during 
carcinogen treatment. NPYR or NNN was administered to 
ethanol-consuming or control hamsters by i.p. injection over a 
25-week period in total doses of either 1 or 2 mmol. In the 
group treated with 1 mmol of NPYR and maintained on a control 
diet, we observed 1 of 20 hamsters with a nasal cavity tumor 
and 4 of 20 hamsters with tracheal tumors. In the group treated 
with 1 mmol of NPYR and maintained on the ethanol-containing 
diet, we observed 8 of 18 hamsters with nasal cavity tumors 
and 9 of 18 hamsters with tracheal tumors. The corresponding 
results in hamsters given 2 mmol of NPYR were: nasal cavity 
tumors, 14 of 21 (control) and 16 of 17 (ethanol); tracheal 
tumors, 8 of 21 (control) and 11 of 17 (ethanol). These results 
demonstrate that the carcinogenicity of NPYR is enhanced in 
ethanol-treated Syrian golden hamsters, particularly at the 
lower dose. In the groups treated with 1 mmol of NNN and a 
control diet, we observed 1 of 21 hamsters with a nasal cavity tumor 
and 4 of 21 with tracheal tumors. In the corresponding 
ethanol-treated groups, we observed 1 of 17 hamsters with a 
nasal cavity tumor and 5 of 17 with tracheal tumors. In the 
hamsters given 2 mmol of NNN, the results were: nasal cavity 
tumors, 5 of 21 (control) and 4 of 21 (ethanol); tracheal tumors, 
9 of 21 (control) and 7 of 21 (ethanol). Thus, the carcinogenicity 
of NNN in the Syrian golden hamster was not affected by 
ethanol treatment. These results suggest that the metabolic 
activation of NPYR, but not that of NNN, may be enhanced by 
chronic ethanol consumption in the Syrian golden hamster.

INTRODUCTION

There is now an extensive body of epidemiological evidence 
indicating that the combination of both chronic alcohol and 
tobacco consumption are major risk factors for cancers of the 
head and neck. Positive association of these risk factors for 
cancers of the oral cavity, esophagus, and larynx have been 
noted by numerous investigators (see Ref. 26 for a recent 
review). However, the possible role that each plays in the 
etiology of the disease has remained elusive. In order to study 
the mechanism(s) by which alcohol and tobacco might increase 
the risk for cancer, our working hypothesis assumes that 

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2 To whom requests for reprints should be addressed.

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MATERIALS AND METHODS

NPYR was obtained from Aldrich Chemical Company, Mil- 
waukee, Wis., and NNN was synthesized as described previ- 
ously (19). NIH-07 chow was from Ziegler Brothers, Gordoners, 
Pa.; liquid diets were obtained from Bio-Serv, Frenchtown, N. 
J.; ethanol (95%) was from Publicker Chemical Corporation, 
Cranford, N.J. and IMC Chemical Group, Newark, N. J.

Two hundred ten outbred male Syrian golden hamsters (3 
weeks old) from Bio-Research (Telaco), Cambridge, Mass., 
were housed 3/cage and allowed free access to NIH-07 lab- 
oration chow and water. Cages were solid-bottomed polycar- 
bonate and contained hardwood chip bedding. The laboratories 
were maintained at 21 ± 1°C (S.D.) and 50 ± 10% relative 
humidity, respectively. Animals were kept on light-dark cycles

3 The abbreviations used are: NPYR, N-nitrosopyrroolidine; NNN, N'-nitroso-
nornicotine.
of 12-hr duration starting at 7 a.m. At 8 weeks of age, all animals were placed on an ethanol-containing liquid diet. Both control animals were placed on control liquid diet (Diet 71 supplemented with β-cellulose, 4.14 g/liter). At 9 weeks of age, animals were randomized by weight and one-half (105 animals) were continued on control liquid diet. The remaining animals were placed on an ethanol-containing liquid diet. Both control and ethanol diets were as originally described by DeCarli and Lieber (7). In these diets, ethanol was added as an isocaloric replacement for carbohydrate such that 35% of the total caloric content was as ethanol. The percentage of total calories as protein, fat, and carbohydrate was 18, 35, and 47%, respectively. The ethanol concentration in the diet was 6% (w/v). At 13 weeks of age, each group was again randomized by weight and further subdivided into 5 treatment groups of 21 animals each.

Animals received 0.5-ml i.p. injections 3 times weekly for 25 weeks of: 1.33 mg NPYR (total dose, 1 mmol) (Groups 1 and 2); 2.67 mg NPYR (total dose, 2 mmol) (Groups 3 and 4); 2.37 mg NNN (total dose, 1 mmol) (Groups 5 and 6); 4.75 mg NNN (total dose, 2 mmol) (Groups 7 and 8); and 0.9% NaCl solution vehicle (Groups 9 and 10).

Odd-numbered groups received control liquid diets, and even-numbered groups received ethanol-containing liquid diets. Diet consumption and weight gain were measured once weekly, and consumption by animals on control diet was varied to equal the consumption of the animals on the ethanol-containing diet.

After 16 weeks of injections, a decrease in the weight of all groups on liquid diets was observed. Since the weight loss appeared to be diet related and enhanced by both ethanol and carcinogen, liquid diet consumption and carcinogen administration were suspended for 1 month. All groups were given NIH-07 laboratory chow and water ad libitum. The weights of all groups increased promptly, and the weight differential among groups was abolished. Control and ethanol liquid diets were reinstated, and the carcinogen injection schedule was resumed 1 week later and continued without further interruption. At the end of the 25-week carcinogen administration period, all animals were returned to NIH-07 laboratory chow and water. Animals were sacrificed when moribund. Surviving NPYR animals (Groups 1 to 4) were sacrificed 15 months after the beginning of carcinogen administration, and surviving NNN-treated animals and controls (Groups 5 to 10) were sacrificed 18 months after the beginning of carcinogen administration. All gressly observable lesions were submitted for histological evaluation. Longitudinal sections of all tracheas were also submitted for histopathological examination. Heads were decalcified for 48 hr, cut in at least 3 frontal sections, embedded in paraffin, and stained with hematoxylin and eosin. The Fisher exact probability test was used for statistical analysis.

**RESULTS**

The mortality rates of control and ethanol-consuming hamsters treated with NPYR and NNN are shown in Charts 2 and 3, respectively. Ethanol-consuming animals receiving either 1 or 2 mmol of NPYR exhibited increased mortality compared with NPYR-treated animals on control liquid diet. In contrast, little difference was seen in the mortality of control and ethanol-consuming animals treated with NNN.

The effect of chronic ethanol consumption on the tumor yield in NPYR- and NNN-treated animals is presented in Table 1. With both carcinogens, tumors were observed most frequently in the nasal cavity and trachea. Ethanol-consuming animals receiving NPYR had a higher incidence of both nasal cavity and tracheal tumors compared to NPYR-treated animals on control diet. This difference was greater in animals receiving 1 mmol NPYR than for those receiving 2 mmol NPYR. Seventy % of the nasal cavity tumors were observed in animals sacrificed prior to the termination of the study. The first nasal cavity tumor was observed in an animal sacrificed in Experimental Month 10, but the majority of nasal cavity tumors was observed in animals sacrificed in Experimental Months 14 and 15. The first tracheal tumor was also seen in Experimental Month 10, and approximately one-half of the total tracheal tumors was observed in animals sacrificed prior to the termination of the experiment.

The NNN-treated animals developed more tracheal than did nasal cavity tumors, and no increased tumor incidence was observed in the ethanol-consuming groups. In these animals, approximately 60% of the nasal cavity tumors and 15% of the tracheal tumors were found prior to the termination of the experiment. The first nasal cavity and tracheal tumors were observed in animals sacrificed in Experimental Months 14 and 15, respectively.

Almost one-half of the animals treated with either NPYR or NNN had multiple tracheal tumors, but no difference due to
ethanol consumption was observed. In a few animals, the tracheal neoplasms almost totally obstructed the tracheal lumen. Histological examination showed squamous papillomas, but no malignant transformation was seen. Neoplastic liver nodules were observed only in animals treated with carcinogen and more frequently in NPYR-treated animals. The morphology of these lesions was similar to those described by Stewart et al. (32) for rats.

Benign and malignant tumors of the adrenal gland are among the more common spontaneous tumors found in Syrian golden hamsters (see Ref. 33 for a review). In this study, adrenal tumors were found in both carcinogen-treated and control groups. The incidence of adrenal tumors generally was higher in Groups 5 through 10, which were sacrificed 18 months after the beginning of carcinogen treatment. Fewer adrenal tumors were seen in Groups 1 through 4, which were sacrificed 15 months after the beginning of carcinogen administration. There were marked differences in the number of animals in Groups 1 to 4 which survived until termination (Chart 2). It is possible that the suppression of adrenal tumors (Groups 2 versus 1) is a reflection of animal age at the time of sacrifice.

A detailed examination of the nasal cavity lesions is presented in Table 2. Areas of focal epithelial hyperplasia were observed, with the respiratory epithelia being the major site. However, all of the tumors appeared to arise from the olfactory mucosa (Fig. 1) and ranged from small localized tumors, within a single turbinate, to large invasive neoplasms (Fig. 2). In the advanced stages, the tumors almost completely filled the nasal cavity, destroying bone and infiltrating other tissue (Fig. 3). More than one-half of the tumors in the NPYR-treated animals were malignant invading bone, brain, and surrounding tissues and giving metastases to the cervical lymph nodes. In some cases, tumors appeared to be of multicentric origin. When the tumor was still localized, it was easy to identify its origin in the olfactory mucosa. However, the origin of the more advanced tumors was difficult to determine due to the destruction of normal adjacent structures. Nevertheless, since the tumor morphology of the advanced tumors was nearly identical to that of the more localized lesions, a reasonable presumption of the olfactory origin could be made.

Several tumors displayed a glandular arrangement of relatively small cells (Fig. 4); however, a medullary type image of relatively large tumor cells was found more frequently (Fig. 5). In a few incidences, compact growth in cords of tumor cells with hyperchromatic nuclei was observed (Fig. 6). Some tumors displayed intermingling of different cellular components and patterns. Tumors with a similar morphology were found in hamsters treated with N′-nitrosopiperidine by Hilfrich et al. (17). It was not possible to further classify these tumors as olfactory neuroepitheliomas, olfactory neurocytomas, or olfactory neuroblastomas in the absence of any demonstrable Bodian's staining for neurofibers and Grimelius staining for cytoplasmic argyrophilic granules.

DISCUSSION

The results presented here indicate that chronic ethanol consumption can increase the incidence of nasal cavity and tracheal tumors in hamsters exposed to NPYR. The fact that the enhancing effect of ethanol is more evident at the lower level of NPYR suggests that ethanol consumption in some manner increases the susceptibility of the nasal and tracheal mucosa to NPYR. Our previous studies have shown that ethanol consumption increased liver microsomal metabolism and mutagenicity of NPYR (24). The present observations could be the result of similar changes occurring in the target tissues.

Ethanol by itself does not increase tumor incidence or change the organotropism of either NPYR or NNN, indicating that in this system ethanol is not a carcinogen and that the increased tumor incidence in the target tissues is not caused by decreased incidence at some other site. The observation that chronic ethanol consumption increased the carcinogenicity of NPYR but had no effect with NNN suggests that the mechanism is cocarcinogenic rather than promotional, since the latter mechanism should have resulted in the enhanced carcinogenicity of both nitrosamines.

In view of the structural similarity of NPYR and NNN (Chart 1), the lack of an effect of ethanol consumption on the carcinogenicity of NNN indicates that enhanced metabolism per se is not sufficient to explain increased carcinogenicity. Previous studies have shown that like NPYR, hamster liver microsomal metabolism of NNN is increased by ethanol consumption (22). However, unlike NPYR, the 2 sites of α-hydroxylation of NNN are not equivalent. Although the major site of in vitro hydroxylation (5′) is the site which is increased by ethanol, it is possible that 2′-hydroxylation is involved in carcinogenesis and that 5′-hydroxylation is a detoxification pathway. There are species differences in the response of 5′- and 2′-hydroxylation of NNN to induction. In the hamster liver, it is 5′-hydroxylation which is inducible, while in the rat liver only 2′-hydroxylation is inducible (23). If 2′-hydroxylation is the pathway involved in metabolic activation, the rat may be a more suitable model in which to examine the influences of chronic ethanol consumption on the carcinogenicity of NNN.

The high incidence of nasal cavity and tracheal tumors as well as the increased mortality of NPYR-treated hamsters shows that NPYR is a more potent carcinogen than NNN in hamsters. Except for the brief report by Dotenwill (8), the carcinogenicity of NPYR in hamsters has not been previously reported. Administration (p.o.) of NPYR has been shown to cause hepatocellular carcinomas in several strains of rats (9, 14, 20). The effects of other routes of carcinogen administration have not been examined. The data presented here indicate that the tracheal and nasal cavity mucosa are major target sites, while only early neoplastic changes were found in the liver. Whether these differences are due to species suscepti-
Influence of chronic alcohol consumption on the carcinogenicity of NPYR and NNN in male Syrian golden hamsters

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Animals with tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carcinogen</strong></td>
<td><strong>Upper respiratory tract</strong></td>
</tr>
<tr>
<td></td>
<td>Nasal cavity</td>
</tr>
<tr>
<td><strong>Effective animals</strong></td>
<td></td>
</tr>
<tr>
<td>1 NPYR 1 Control</td>
<td>20</td>
</tr>
<tr>
<td>2 NPYR 1 Ethanol</td>
<td>18</td>
</tr>
<tr>
<td>3 NPYR 2 Control</td>
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<tr>
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<tr>
<td>5 NNN 1 Control</td>
<td>21</td>
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<td>17</td>
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<tr>
<td>7 NNN 2 Control</td>
<td>21</td>
</tr>
<tr>
<td>8 NNN 2 Ethanol</td>
<td>21</td>
</tr>
<tr>
<td>9 None 0 Control</td>
<td>21</td>
</tr>
<tr>
<td>10 None 0 Ethanol</td>
<td>19</td>
</tr>
</tbody>
</table>

* a Animals that survived the carcinogen administration period.
* b Pancreatic islet cell tumor.
* c Significantly different (p ≤ 0.05) from control.
* d Parathyroid adenoma.
* e Forestomach papilloma, follicular adenoma of the thyroid, lymphoma of the mesenteric lymph node, pancreatic islet cell tumor, parathyroid adenoma, and kidney papillary adenoma.
* f Pancreatic islet cell tumor (3 animals), papillary adenoma of the thyroid (2 animals).
* g Pancreatic islet cell tumor, parathyroid adenoma, papillary adenoma of the thyroid, adenocarcinoma, papillary adenoma of the lung.
* h Pancreatic islet cell tumor, lung adenoma.
* i Pancreatic islet cell tumor, lymphoma, hemangioendothelioma of the spleen, forestomach papilloma, s.c. sarcoma.
* j Lymphoma, hemangioendothelioma of the liver, follicular adenoma of the thyroid, parathyroid adenoma, pancreatic acinar cell adenoma, pancreatic islet cell tumor.

Table 2
Detail of neoplastic changes in the nasal cavity mucosa

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Olfactory epithelial tumors</th>
<th>Focal epithelial hyperplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carcinogen</strong></td>
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<tr>
<td><strong>Effective animals</strong></td>
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<td></td>
</tr>
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<td>2 NPYR 1 Ethanol</td>
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<td>3 NPYR 2 Control</td>
<td>21</td>
<td>14</td>
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<tr>
<td>4 NPYR 2 Ethanol</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>5 NNN 1 Control</td>
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<td>1</td>
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<tr>
<td>6 NNN 1 Ethanol</td>
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<tr>
<td>7 NNN 2 Control</td>
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<td>4</td>
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<tr>
<td>9 None 0 Control</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>10 None 0 Ethanol</td>
<td>19</td>
<td>0</td>
</tr>
</tbody>
</table>

* a Animals that survived the carcinogen administration period.

Bility, administration routes, or total dose administered cannot be determined in the absence of strictly comparable studies in the 2 species. The tumor incidence in hamsters treated with NNN at the 2-mmol dose level reported here is similar to the data presented by Hilfrich et al. (17), who administered similar quantities s.c.

Beginning with the investigations by Protzel et al. (27), several studies have shown that ethanol consumption can increase the tumor incidence in animals exposed to benzo(a)pyrene (5, 31) or 7,12-dimethylbenz(a)anthracene (10–12). The role that ethanol plays in increased tumor incidence in polynuclear aromatic hydrocarbon-treated animals is not known. However, the enhanced 3-hydroxylation and mutagenicity of benzo(a)pyrene by subcellular fractions of small intestinal mucosa from ethanol-consuming rats indicates that ethanol can increase microsomal metabolism in at least one extra hepatic tissue (30). Two studies on the effect of alcohol on the carcinogenicity of nitrosamines have been reported. In 1965, Gibel (13) presented evidence that simultaneous administration of ethanol and diethylnitrosamine by gastric intubation increased the incidence of esophageal tumors in rats. Both control and ethanol-treated rats had 100% incidence of liver tumors. Animals treated with dinitrosopiperazine showed no increase due to ethanol. More recently, Schmähl (28) has shown that the carcinogenicity of N-nitrosomethylphenylamine administered either p.o. or s.c. to male and female Sprague-Dawley rats was not increased by ethanol consumption. In none of these studies was there any indication of an increased tumor incidence due solely to ethanol consumption in the absence of carcinogen. A recent study by Schrauzer et al. (29) has shown, however, that chronic ethanol consumption can decrease the latent period and increase the tumor volume of spontaneously occurring mammary adenocarcinoma in female C3H/St mice.

The hamster, in contrast to most commonly used laboratory species, will preferentially consume up to 90% of its total liquid intake from ethanol-water mixtures (1, 2). However, we have observed (25) that as caloric intake from ethanol increased, the caloric intake from diet decreased, making this method of ethanol consumption...
The use of liquid diet preparations in which ethanol isocalorically replaces carbohydrate (7) prevents this disparity in nutrient intake. The effect of consumption of liquid diets per se on the carcinogenicity of NPYR and NNN cannot be determined in the absence of carcinogen-treated chow-fed animals. However, it should be noted that, relative to the usual laboratory chow formulation, these liquid diets have a high fat content.

The data presented here as well as the previous studies on the effect of ethanol on the carcinogenicity of nitrosamines indicate the importance of both the dose and the carcinogen used, since ethanol administration clearly does not increase the carcinogenicity of all nitrosamines. Procedures in which carcinogen administration is decreased with respect to both time and number of injections need to be devised so that the critical time of ethanol consumption can be defined. Further studies are needed to establish whether enhanced metabolic activation is the reason for increased carcinogenicity of NPYR in hamsters. Nevertheless, insight into this aspect as well as other possible mechanisms by which ethanol consumption may increase the risk for cancer can be studied in this model system.

ACKNOWLEDGMENTS

The authors would like to thank Maria Nicolais for her excellent technical assistance.

REFERENCES


Fig. 1. Early tumor arising in the nasoturbinate in a hamster treated with 2 mmol NPYR on ethanol diet. H & E, × 6.
Fig. 2. Fully developed nasal cavity tumor in a hamster treated with 2 mmol NPYR on ethanol diet. The cavity is completely filled by the tumor. H & E, × 6.
Fig. 3. Advanced nasal cavity tumor in a hamster treated with 2 mmol NPYR on control diet. Note invasion and overgrowth of tumor. H & E, × 6.
Fig. 4. One of the representative cellular components and structural pattern of nasal cavity tumors in hamsters. Note glandular pattern of relatively small tumor cells. A hamster treated with 1 mmol NPYR on ethanol diet. H & E, × 100.
Fig. 5. Most frequent nasal cavity tumor morphology in this study. Note the presence of relatively large anaplastic tumor cells. Taken from the tumor shown in Fig. 3. H & E, × 100.
Fig. 6. Less frequently encountered nasal cavity tumor morphology in the present study. Note the streaming-like pattern of hyperchromatic small tumor cells in a hamster treated with 2 mmol NPYR on control diet. H & E, × 100.
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