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# EFFECTS OF ETHANOL CONSUMPTION ON BIOACTIVATION AND HEPATOTOXICITY OF *N*-NITROSODIMETHYLAMINE IN RATS

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**Abstract**—To study the effects of ethanol on the hepatotoxicity of *N*-nitrosodimethylamine (NDMA), 5 mg NDMA/kg body weight was injected intraperitoneally 3 times a week for 6 weeks into rats pair-fed liquid diets containing 36% of energy either as ethanol or as additional carbohydrates. Another group of rats was pair-fed with the same diets but injected with saline instead of NDMA. Co-administration of ethanol and NDMA produced much higher elevations of serum alanine and aspartate aminotransferase and glutamic dehydrogenase activities than the administration of either agent alone. The combined treatment also slightly increased focal necrosis, whereas other liver lesions (steatosis and fibrosis) and the functional impairment of mitochondrial respiration were not affected significantly. Microsomal low $K_m$ NDMA demethylation, as well as NDMA denitrosation, were inhibited markedly by incubation with an antibody against P450IIE1, suggesting the involvement of this alcohol-inducible P450 in both NDMA bioactivation reactions. The addition of ethanol inhibited P450-dependent demethylation and denitrosation of NDMA in liver microsomes, whereas both activities were enhanced markedly by chronic ethanol administration. At ethanol concentrations similar to those prevailing in the blood of alcohol-fed animals at the time of NDMA administration, hepatic microsomal demethylation and denitrosation remained significantly higher in ethanol-fed rats given NDMA than in controls. Our results suggest that bioactivation plays a critical role in the hepatotoxicity of NDMA and its aggravation by chronic alcohol consumption.

*N*-Nitrosodimethylamine (NDMA) and other nitrosamines are potent hepatotoxins and carcinogens present in food, alcoholic beverages (particularly some types of beer), and tobacco products [1, 2]. In addition, they can be formed endogenously by macrophages, by microorganisms of the gastrointestinal tract and, in the stomach, by direct reaction between dietary nitrites and amines [3], including commonly used drugs (such as disulfiram and antibiotics) [4]. The continuous endogenous production of these compounds has been documented *in vivo*, particularly after the administration of ethanol [5], which inhibits their first pass metabolism in the liver [6]. Moreover, intestinal bacterial overgrowth occurs in alcoholics [7].

It has been postulated that the deleterious effects of NDMA are mediated by its microsomal bioactivation to reactive metabolites. Both the microsomal ethanol-oxidizing system (MEOS) and NDMA demethylase and denitrosation activities share cytochrome P450 enzymes [8–12], including one specifically induced by ethanol, P450IIE1, suggesting a likely site for interaction. Addition of ethanol to isolated microsomes decreases NDMA-demethylase activity, whereas chronic alcohol administration enhances demethylation of NDMA [13] and its conversion to a mutagen [14]. Pretreatment with four intragastric doses of ethanol (4 g/kg) over a 48-hr period was reported to increase the acute hepatotoxicity of NDMA [15], whereas chronic pretreatment with alcohol-containing diets almost completely prevented NDMA hepatotoxicity [16–18], with NDMA given after cessation of the alcohol administration and disappearance of ethanol from the blood. In alcoholics, the acute and chronic effects of ethanol on drug-metabolizing enzymes usually coexist. Thus, the purpose of this study was to investigate the net effect of these interactions when large amounts of alcohol and small amounts of NDMA are being consumed together.

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## MATERIALS AND METHODS

*Materials*. NDMA was purchased as a sterile 1% solution in 0.9% NaCl (Sigma Chemical Co., St. Louis, MO) designed for intraperitoneal (i.p.) injections. The detailed composition of the liquid diets used in this study has been reported previously [19]. They contain 1 kcal/mL, 18% of energy as protein, 35% as fat, 11% as carbohydrate and 36% as either ethanol or additional carbohydrate.

*Animal procedures*. All procedures were in compliance with the criteria of the National Research Council for humane care of animals. Twenty-four male weanling rats of a Sprague–Dawley strain [CRL-CR(SD)-BR] were purchased from the Charles River Breeding Laboratories (Wilmington, MA) and were fed a rat chow diet (Ralston Purina Co., St. Louis, MO) and water *ad lib*. When they reached a weight of 130–160 g, rats were placed in individual cages, divided in groups of four animals of similar weight, and fed isocaloric amounts of the liquid diets

described above for 6 weeks. In each group, two rats received the alcohol-containing diet; 3 days each week, one rat was injected with 5 mg of NDMA/kg body weight, i.p., as a 0.5% solution in saline while the other received only saline. This dose of NDMA is one-half of that reported to produce cirrhosis in rats [20, 21]. The other two rats (one injected with NDMA and the other with saline) were limited to consumption of the control diet in amounts providing a caloric intake similar to that of the alcohol-fed rat injected with NDMA, which became the limiting animal in each group. The last NDMA dose was injected 2–3 days prior to sacrifice.

During the last week of treatment, blood samples for ethanol measurements were obtained from the excised tip of the tail at 9:00 a.m., prior to the injection of NDMA. The day preceding killing, at 9:00 a.m., rats received the corresponding diets in amounts equivalent to one-third of their usual daily consumption and, at 5:00 p.m., the remaining two-thirds was given as control diet to ensure the absence of ethanol at the time of killing. The next day, at 9:00 a.m., the animals were anesthesized with pentobarbital (40 mg/kg body wt, i.p.), blood was collected from the aorta, and the liver was removed. Thin slices of liver were fixed in neutral formalin, and embedded in paraffin for staining with hematoxylin and eosin and the trichrome stain of Mallory.

*Analytical procedures.* The liver was homogenized in 3 vol. of ice-cold 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4) and 1 mM EDTA, and centrifuged first at 1100 *g*. The supernatant was used for separation of mitochondria, which were sedimented at 8750 *g* for 10 min at 4° [22]. Mitochondrial respiration and oxidative phosphorylation were measured polarographically with a Clark oxygen electrode (YSI, Yellow Spring, OH) in 3 mL of a medium containing 300 mM mannitol, 10 mM Tris–HCl (pH 7.4), 10 mM potassium phosphate, 2.5 mM $MgCl_2$, and 2 mg of mitochondrial protein, using 1 mM malate and 10 mM glutamate as substrates [23]. State 3 respiration was initiated by the addition of ADP to a final concentration of 266 μM.

The microsomal fraction was isolated by centrifugation of the post-mitochondrial supernatant at 100,000 *g* for 75 min. NDMA demethylation was assessed by the generation of formaldehyde. The incubation mixture contained 100 mM phosphate buffer (pH 7.4), 0.5 mM NADPH, 5 mM NDMA and 0.75 mg microsomal protein in a total volume of 1 mL. The reaction was carried out at 37° for 10 min and terminated by the addition of 0.4 mL of 10% trichloroacetic acid. Formaldehyde formation was measured colorimetrically by the Nash reaction [24] in deproteinized supernatants. NDMA denitrosation was determined in reaction mixtures (1.0 mL) containing 50 mM Tris–HCl buffer, pH 7.4, 0.5 mM NADPH, 10 mM NDMA and 1 mg microsomal protein. Reactions were terminated after 20 min at 37° by the addition of 0.05 mL of 20% $ZnSO_4$ and saturated $Ba(OH)_2$. Nitrite formation was measured according to Amelizad *et al.* [25]. Antibody inhibition experiments were performed by first incubating liver microsomes (0.2 and 0.5 mg protein for NDMA demethylation and denitrosation, respectively) with various amounts of anti-hamster IIE1 IgG (previously shown to cross-react with rats [26]) for 3 min at 37°. Reaction tubes were returned to ice, the remaining incubation components were added, and either NDMA demethylation or denitrosation was assessed as already described.

Ethanol concentrations were measured by head-space chromatography in duplicate 50 μL blood samples collected into heparinized capillary tubes from the wound of the excised tail tip and hemolyzed directly in chromatography vials containing 0.45 mL of ice-cold water [27]. Liver lipids were extracted according to Folch *et al.* [28] and separated by thin-layer chromatography [29]. Triglycerides were then scratched from the plates, extracted with chloroform–methanol (2:1) and measured by the procedure of Snyder and Stephens [30]. Collagen-bound hydroxyproline was determined as reported by Rojkind and Gonzalez [31]. Serum alanine and aspartate aminotransferase activities were measured by the method of Bergmeyer *et al.* [32], while glutamic dehydrogenase activity was determined according to Ellis and Goldberg [33].

*Statistics.* All values are expressed as means ± SEM. The significance of the differences between the four groups was tested by either two- or three-way analysis of the variance (using NDMA, ethanol feeding and ethanol concentration as the factors), followed by *post hoc* Newman–Keuls tests [34]. A probability of less than 5% for the null hypothesis was considered to be significant.

## RESULTS

*Effects of chronic ethanol and NDMA administration on weight gain and blood ethanol levels.* All animals gained weight but, despite similar energy intake, rats fed the ethanol-containing diet tended to gain less weight (2.2 ± 0.4 g/day) than animals pair-fed the control diet (2.5 ± 0.1 g/day). The administration of NDMA to alcohol-fed rats produced a further decrease in weight gain (1.4 ± 0.3 g/day; $P < 0.01$), whereas this treatment did not affect significantly the body weight gain in rats fed the control diet (2.4 ± 0.3 g/day). Thus, either alcohol alone or together with NDMA decreased body weight.

Blood ethanol levels were 28 ± 2 mM in alcohol-fed rats prior to injection with NDMA versus 32 ± 4 mM in those injected with saline (non-significant difference; NS).

*Effects of chronic ethanol and NDMA on the serum levels of hepatic enzymes.* Combined administration of ethanol and NDMA resulted in a marked increase in serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), whereas the administration of either NDMA or ethanol alone produced less striking changes in AST and no significant increase in ALT activities (Table 1). NDMA and chronic alcohol consumption increased serum glutamic dehydrogenase activity 3- and 8-fold, respectively, whereas their combined administration resulted in a 30-fold rise.

*Effects of ethanol and NDMA on liver lesions.* Despite the marked effects of the co-administration

Table 1. Effects of NDMA and ethanol on serum activities of liver enzymes*

| | Control diet | Alcohol diet | Control diet + NDMA | Alcohol diet + NDMA |
|---|---|---|---|---|
| ALT (I.U.) | 20 ± 2 | 21 ± 2 | 24 ± 3 | 138 ± 49† |
| AST (I.U.) | 33 ± 1 | 53 ± 4‡ | 45 ± 2‡ | 153 ± 46† |
| GDH (I.U.) | 2.0 ± 0.4 | 15.8 ± 3.2‡ | 6.6 ± 1.6‡ | 62.6 ± 13.7† |

* Rats were fed isocaloric amounts of either ethanol-containing or control diets for 6 weeks and received either NDMA (5 mg/kg) or saline intraperitoneally 3 days a week. Two-way ANOVA revealed significant effects and interaction of both factors, ethanol and NDMA treatments.
† $P < 0.01$, compared to the other three groups, by *post-hoc* Newman–Keuls test.
‡ $P < 0.01$, compared to control, by *post-hoc* Newman–Keuls test.

Table 2. Effects of NDMA and ethanol on mitochondrial respiration*

| | Control diet | Alcohol diet | Control diet + NDMA | Alcohol diet + NDMA |
|---|---|---|---|---|
| State-4 $O_2$ consumption (nanoatoms/mg protein) | 17.4 ± 1.8 | 13.9 ± 1.1 | 14.6 ± 1.6 | 13.7 ± 1.3 |
| State-3 $O_2$ consumption (nanoatoms/mg protein) | 69.3 ± 11.5 | 44.5 ± 3.4† | 34.4 ± 2.8† | 30.3 ± 3.4† |
| Respiratory control ratio | 3.99 ± 0.40 | 3.28 ± 0.27† | 2.45 ± 0.23† | 2.19 ± 0.16† |

* Rats were fed isocaloric amounts of either ethanol-containing or control diets for 6 weeks and received either NDMA (5 mg/kg) or saline intraperitoneally 3 days a week. Mitochondrial respiration and oxidative phosphorylation were measured using glutamate and malate as substrates.
† $P < 0.01$, compared to control, by *post-hoc* Newman–Keuls test.

of ethanol and NDMA on serum enzymes, the liver lesions were not strikingly different from those produced by either ethanol or NDMA alone. Compared to controls, rats given NDMA and/or the ethanol-containing diet both showed steatosis. This dose of NDMA did not result in fibrosis development, but produced isolated foci of necrosis both in the ethanol-fed and the control rats. Blind assessment by two independent investigators rated focal necrosis as slightly more severe in the ethanol-fed rats given NDMA than in their pair-fed controls given NDMA without alcohol.

*Effects of ethanol and NDMA on functional and biochemical changes of the liver.* The increases noted in serum glutamic dehydrogenase activity were associated with evidence of mitochondrial impairment, as judged by the decreases in the response of oxygen consumption to ADP or in the respiratory control index (Table 2). NDMA or ethanol administration decreased mitochondrial oxygen consumption in response to ADP, but the effects of the co-administration did not differ significantly from those of either NDMA or ethanol alone.

Similarly, both alcohol and NDMA increased liver triglycerides, but their combined effect was not significantly greater than that produced by alcohol alone (Table 3). NDMA administration (but not alcohol consumption) increased liver collagen; this increase was not affected by the concomitant consumption of ethanol.

*Effects of anti-P450IIE1 IgG on NDMA demethylase and denitrosation activities.* Preincubation of microsomes from ethanol-treated rats with increasing amounts of the antibody against ethanol-inducible P450IIE1 markedly inhibited both NDMA demethylation and denitrosation (Fig. 1).

*Effects of co-administration of ethanol and NDMA on NDMA bioactivation.* Rats fed ethanol chronically displayed higher microsomal demethylase and denitrosation activities than the pair-fed controls when tested in the absence of ethanol (Fig. 2). However, in both ethanol-fed rats and their controls, treated with or without NDMA, the *in vitro* addition of ethanol decreased these activities. Nevertheless, at the ethanol concentrations prevailing at the time of NDMA administration to ethanol-fed rats, there was still a net increase in both NDMA demethylation and denitrosation activities compared to controls or to rats given NDMA alone. The administration of NDMA did not change these activities in either ethanol-fed or control rats.

## DISCUSSION

Our findings indicate that the hepatotoxicity of NDMA is enhanced by chronic co-administration of ethanol. Such an enhancement was clearly documented by the increase in serum activities of hepatic enzymes such as glutamic dehydrogenase and alanine and aspartate aminotransferases, compared to the effects of either NDMA or ethanol alone. The lack of a clear-cut aggravation of NDMA-induced lesions in the liver, or significant further impairment of mitochondrial respiration, suggests

Table 3. Effects of NDMA and ethanol on liver composition*

| | Control diet | Alcohol diet | Control diet + NDMA | Alcohol diet + NDMA |
|---|---|---|---|---|
| Liver weight (g/100 g body wt) | 2.43 ± 0.16 | 4.38 ± 0.14† | 3.20 ± 0.08† | 3.84 ± 0.11† |
| Triglycerides (mg/g liver) | 16.6 ± 1.6 | 77.1 ± 15.9† | 34.1 ± 2.3† | 104 ± 40.9† |
| Collagen (mg/g liver) | 0.78 ± 0.05 | 0.82 ± 0.06 | 1.51 ± 0.30† | 1.48 ± 0.37† |

* Rats were fed isocaloric amounts of either ethanol-containing or control diets for 6 weeks and received either NDMA (5 mg/kg) or saline intraperitoneally 3 days a week.
† $P < 0.01$, compared to control, by *post-hoc* Newman–Keuls test.

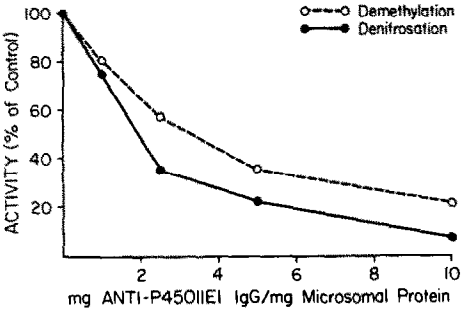


Fig. 1. Inhibition of low $K_m$ NDMA demethylation and denitrosation by anti-P450IIE1 IgG. Microsomes from ethanol-treated rats were preincubated for 3 min at 37° with either anti-hamster IIE1 IgG or preimmune (control) IgG at the ratios given. The amounts of immune-specific and control IgG added were kept constant in order to rule out any non-specific IgG effects. NDMA demethylation and denitrosation were then assayed as described under Materials and Methods. The microsomal demethylation and denitrosation activities in the ethanol-fed rats with preimmune IgG (control) were 4.0 nmol HCHO/min/mg protein and 0.18 nmol $NO_2$/min/mg protein, respectively.

that the hepatocyte membrane may be the primary target for this enhanced toxicity. These effects were associated with enhanced microsomal NDMA demethylation and denitrosation in rats chronically fed ethanol. Both activities were inhibited almost completely by incubation with anti-P450IIE1 IgG, indicating that this P450 enzyme is the major catalyst of both NDMA bioactivation reactions, at least at the low concentrations of NDMA used in this study (10 mM or less). Although microsomal NDMA demethylation and denitrosation were enhanced markedly by chronic ethanol administration, the increase in these activities was less when measured in the presence of ethanol (at concentrations similar to those prevailing in the blood of alcohol-fed animals at the time of NDMA administration) than when measured in the absence of ethanol. Nevertheless, although the well known inductive effect of ethanol feeding on NDMA demethylation and denitrosation was partially offset by an acute inhibitory action of ethanol on this process, a net increase of these activities was still present in the rats receiving ethanol and NDMA together, compared to controls or to those receiving NDMA alone.

Our findings contrast with those of other studies [17, 18, 35] in which a protective effect of chronic ethanol administration on NDMA hepatotoxicity has been suggested. In one study, administration of a 10% ethanol solution as a drinking fluid decreased the centrilobular necrosis produced by administration of 50 ppm NDMA to mice, in association with increased tissue levels of NDMA and decreased demethylase activity [35]. It must be pointed out, however, that this technique of alcohol administration produces very low levels of ethanol in the blood, unlikely to induce microsomal activities; that was made apparent by the lack of a significant increase in NDMA demethylase activity after the chronic consumption of alcohol without NDMA [35]. By contrast, in our study, pair-feeding with liquid diets containing 5% ethanol produced blood ethanol levels more akin to those observed in alcoholics, and more than doubled microsomal NDMA demethylation and denitrosation activities. Under these conditions, co-administration of ethanol resulted in aggravation rather than in attenuation of NDMA-promoted hepatotoxicity. Another group of investigators [17, 18] showed that pretreatment of rats with an alcohol-containing diet similar to ours protects the animals from the NDMA-promoted hepatotoxicity when this nitrosamine is given 2 weeks after cessation of alcohol consumption. However, there was no evidence that the induction of microsomal activities had persisted up to the time of NDMA administration. Whatever the mechanism for the observed protection, that experimental design could be relevant to the exposure to NDMA in abstaining alcoholics. By contrast, our design mimics the situation of NDMA exposure in alcoholics actively drinking, which is known to be associated with induction of microsomal activities [26]. Therefore, the aggravation of the injurious effects of NDMA on the liver is likely to be related to the degree of microsomal induction.

Until recently, most attention was focused on the dealkylating pathway of NDMA metabolism. However, P450IIE1, which is inducible by ethanol consumption and is the major P450 enzyme responsible for low $K_m$ NDMA demethylation, also catalyzes denitrosation of nitrosamines, leading to the formation of nitrite and nitrate via nitrogen

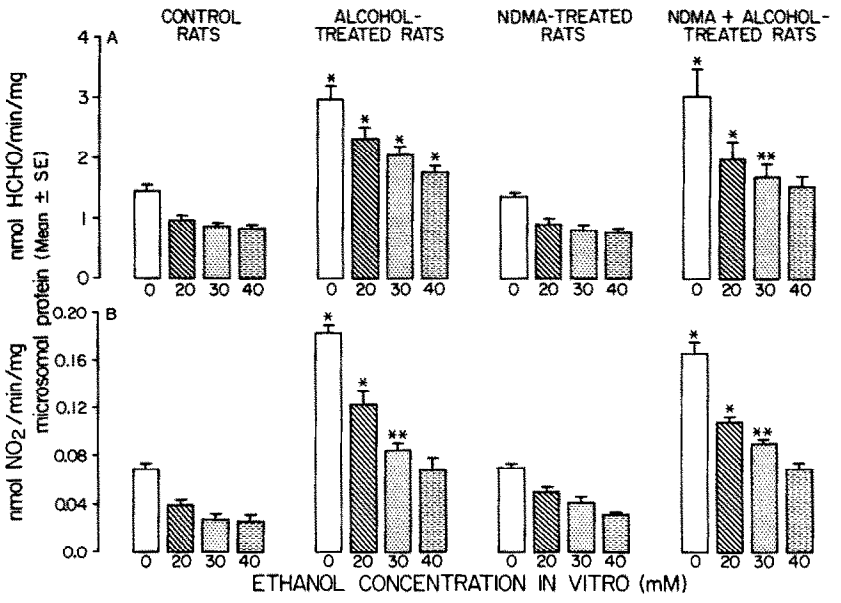


Fig. 2. Effects of chronic administration of ethanol and/or the presence of ethanol on microsomal NDMA demethylation (A) and NDMA denitrosation (B) activities. Twenty-four rats were fed isocaloric amounts of either ethanol-containing (12 animals) or control diets (12 animals) for 6 weeks. Half of the animals in each group received intraperitoneal injections of either NDMA (5 mg/kg) or saline 3 days per week. NDMA demethylation and denitrosation were assayed in microsomes from these rats, with and without *in vitro* addition of ethanol at the concentrations prevailing *in vivo* at the time of NDMA injection. Significance of the differences: * = $P < 0.01$ and ** $P < 0.05$, compared to their respective controls incubated without ethanol.

oxide (NO) production. This process has been shown recently to be enhanced in microsomes from alcoholic patients [25]. It is generally believed that denitrosation leads to the inactivation of NDMA. However, the possibility of intermediary formation of $NO_2$ radicals has been raised [36]. This radical has been shown to promote auto-oxidation of unsaturated fatty acids which could lead to membrane injury [36] and to the production of DNA single-strand breaks [37]. Our finding that NDMA denitrosation and demethylation remained significantly enhanced at the ethanol concentrations prevailing in ethanol-fed animals at the time of NDMA administration (Fig. 2) supports the possibility that NDMA hepatoxicity may be mediated by NDMA bioactivation rather than by NDMA itself. Incubation of microsomes from ethanol-fed rats with a specific antibody raised against purified P450IIE1 virtually abolished both the demethylation and the dinitrosation at low concentrations of NDMA, suggesting that both activities are catalyzed by this ethanol-inducible P450 enzyme.

Alcohol consumption is associated with an increased incidence of several types of cancer [2]. Although carcinogenesis was not assessed in the present study, previous investigations have shown that alcohol can potentiate carcinogenesis elicited by xenobiotic agents. NDMA belongs to the nitrosamine class of proximate carcinogens, all of which require metabolic activation to initiate events (e.g. mutations) leading to carcinogenesis. Most often, however, tumors induced by nitrosamine administration develop in tissues other than the liver. It has been reported that acute ethanol administration changes nitrosamine disposition in rats by decreasing the hepatic capacity to metabolize these compounds, thus leading to increased nitrosamine levels in other target organs [6, 35, 38–40]. However, chronic alcohol administration can also enhance the hepatocarcinogenicity of nitrosamines [41, 42]. Although no enhancement in DNA alkylation (as assessed by $O^6$-methylguanine formation) resulting from NDMA demethylation was noted in rats pair-fed the ethanol-containing liquid diets [43], the possible role of NDMA denitrosation on DNA damage and subsequent carcinogenicity remains to be established. Chronic alcohol consumption could also affect carcinogenesis by mechanisms other than increasing NDMA bioactivation; for instance, an inhibition of the repair of NDMA-induced alkylation of liver DNA has been documented [43].

NDMA can be generated endogenously by microorganisms of the gastrointestinal tract and significant concentrations can be measured in blood and other fluids after acute ethanol administration [5]. Since intestinal bacterial overgrowth and alcoholism are commonly associated [7], it is reasonable to assume that chronic alcohol abusers possess higher endogenous NDMA blood and tissue levels than do non-drinkers. Enhanced NDMA bioactivation in alcoholics due to their increased hepatic P450IIE1 concentrations [26] could, therefore, contribute to the increased incidence of cancer

noted in such individuals. Furthermore, since a low NDMA dose was associated with clear evidence of hepatotoxicity, it is possible that conditions resulting in enhanced NDMA levels (due to increased intake, increased endogenous formation or decreased elimination) or bioactivation of this compound may aggravate the injurious effects of ethanol on the liver.

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