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REDUCTION OF ETHANOL-INDUCED OCULAR ABNORMALITIES IN MICE VIA DIETARY ADMINISTRATION OF N- ACETYLCYSTEINE

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Abstract

N-acetylcysteine (NAC) is a derivative of the amino acid L-cysteine that previously has been shown to protect against ethanol (EtOH)-induced apoptosis during early development. Ongoing research is demonstrating that NAC is also proving clinically beneficial in reducing oxidative stress-mediated lung, liver and kidney damage, with protection likely resulting from a NAC-mediated increase in glutathione levels. In the present study, the hypothesis that co-administration of NAC and EtOH via liquid diet on days 7 and 8 of pregnancy in mice would reduce EtOH's teratogenicity was tested. For this work, adult non-pregnant female mice were acclimated to a liquid diet containing EtOH for 16 days, withdrawn from the EtOH, bred and then returned to the liquid diet containing 4.8% EtOH and/or either 0.5 or 1 mg NAC/ml diet on their 7th and 8th days of pregnancy. At the concentrations employed, the mice received NAC dosages of approximately 300 or 600 mg/kg/day and achieved peak blood EtOH levels (BEC) that averaged approximately 200 mg/dl. There was no difference in BEC between the EtOH alone and EtOH plus 600 mg/kg NAC group. Following maternal euthanasia, gestational day (GD) 14 fetuses were removed, fixed, weighed and examined for the presence and severity of ocular abnormalities, a readily assessed endpoint that results from GD 7 and 8 EtOH exposures. While the lower dosage of NAC (300 mg/kg) resulted in a decrease in the incidence of ocular defects in both the left and right eyes, this reduction was not statistically significant. However, doubling the NAC concentration did yield a significant change; as compared to the group treated with EtOH alone, the incidence of ocular abnormalities was diminished by 22%. These results show the potential of an orally administered compound with proven clinical efficacy to reduce EtOH's teratogenic effects and support the premise that oxidative damage plays an important mechanistic role in Fetal Alcohol Spectrum Disorders.

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Keywords

Fetal Alcohol Spectrum Disorders; N-acetylcysteine; Ocular abnormalities

INTRODUCTION

Among the numerous mechanisms that have been proposed for ethanol (EtOH)-induced teratogenesis, the premise that EtOH alters normal development via disruption of reactive oxygen species (ROS) homeostasis has received considerable research attention and support (Henderson et al., 1999; Martinez and Egea, 2007). In this regard, a number of experimental studies have shown that oxidative stress-induced apoptosis of selected cell populations results from EtOH exposure in embryos, with the induced cell death pattern being predictive of the subsequent defects (Chen and Sulik, 1996; Davis et al., 1990; Heaton et al., 2000; Kotch et al., 1995; Spong et al., 2001). Also supporting a significant role for ROS-mediated damage subsequent to prenatal EtOH exposure are studies that have illustrated protective effects of antioxidants (Chen et al., 2004; Cohen-Kerem and Koren, 2003; Dong et al., 2008).

The current investigation is designed to extend this work by examining the potential of N-acetylcysteine (NAC), when administered as a dietary supplement, to reduce EtOH's teratogenesis. NAC is the amino acid cysteine with an attached acetyl group. This acetyl group improves gastrointestinal absorption. Following absorption, NAC is deacetylated (primarily in the liver), producing biologically available cysteine (Cotgreave, 1997). While cysteine's thiol group has anti-oxidant properties, the primary role of this amino acid in reducing oxidative stress is as the rate-limiting factor in the production of glutathione (GSH). As one of the cell's primary mediators of ROS homeostasis (Atkuri et al., 2007; Zafarullah et al., 2003), glutathione scavenges superoxide, nitrous oxide, peroxynitrite and hydroxyl radicals, the latter being scavenged solely by glutathione (Bains and Shaw, 1997; Clancy et al., 1994; Koppal et al., 1999; Winterbourn and Metodiewa, 1994). Because cysteine has the lowest cellular concentration of the three component amino acids of glutathione (along with glycine and glutamate), it is the rate-limiting factor in glutathione synthesis (Dekhuijzen, 2004).

Due to its capacity to reduce oxidative stress-related cellular damage, NAC has current and potential clinical applications. For example, it has been used to prevent oxidative stress-related cellular damage resulting from acetaminophen overdose (Atkuri et al., 2007; Kanter, 2006; Piperno and Berssenbruegge, 1976). Its utility is also being explored for the treatment of cancer, cardiovascular disease, human immunodeficiency virus (HIV) infection, nephrotoxicity resulting from imaging contrast agents, chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis (Decramer et al., 2005; Demedts et al., 2005; Marenzi et al., 2006).

Supporting the potential of dietary NAC treatment to reduce EtOH's teratogenicity are the results of previous studies that have illustrated EtOH-mediated depletion of GSH both *in vivo* and *in vitro* (Green et al., 2006; Watts et al., 2005). Indeed, these researchers have postulated that NAC may improve EtOH-induced abnormal outcomes by replenishing cellular GSH stores. This, along with the fact that NAC is routinely used clinically in pregnant women to prevent acetaminophen toxicity (Wilkes et al., 2005), provides a foundation for the current investigation in which a liquid diet containing teratogenic EtOH concentrations with or without the inclusion of NAC was delivered to pregnant mice. For this work an EtOH treatment paradigm entailing preconceptional acclimation to EtOH was employed. This allowed *in vivo* exposure of mouse embryos to teratogenic EtOH

concentrations during very specific stages of their embryonic development. Previously, this treatment paradigm has been applied to EtOH teratogenesis studies, with EtOH exposure being limited to days 7 and 8 of pregnancy in C57Bl/6J mice (Parnell et al., 2006). EtOH exposure at this time in development results in defects that typically involve the eyes which can be readily assessed both for the overall incidence and severity of insult (Cook et al., 1987; Parnell et al., 2006; Sulik, 1984). Employing ocular defects as a major factor in comparing treatment groups, the current study tested the hypothesis that co-administration of NAC and EtOH via liquid diet on days 7 and 8 of pregnancy in mice will reduce EtOH's teratogenicity.

METHODS

Subjects

C57Bl/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a temperature and humidity-controlled environment on a 12 h/12 h reverse light/dark cycle. Forty adults and 281 fetuses were used in these experiments. The dams were maintained on an *ad lib.* diet of standard laboratory chow and water, except when administered liquid diet as described below. All animal treatment protocols were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Acclimation to the EtOH-Containing Liquid Diet

The female mice in this study were acclimated to a modified Lieber-DeCarli (Lieber and DeCarli, 1975, 1982) liquid diet containing EtOH with 5% sucrose to increase palatability (PMI Micro-Stabilized Alcohol Rodent Liquid Diet; Test Diet, Richmond, IN). As described previously (Parnell et al., 2006), the mice were given a liquid diet containing 2.4% (w/v) EtOH for two days, followed by 14 days during which the concentration of EtOH in the diet was increased to 4.8%. Following this acclimation period, the mice underwent an EtOH deprivation period that entailed removal of the EtOH-containing liquid diet and return to standard chow and water.

Timed Mating, Treatment and BEC Determination Procedures

During the ethanol deprivation period, the mice were allowed to recover for 3-4 days from any potential ethanol withdrawal symptoms. They were then bred by placing 1-2 females with one male for a 1 hour period during the beginning of the dark cycle. The female mice were then examined for a copulation plug. The beginning of the breeding period in which a copulation plug was found was designated as gestational day (GD) 0. The time between the beginning of the deprivation period and successful breeding averaged 13 days.

On GD 7, the mice were assigned to one of five groups and placed back on the liquid diet for two days. The treatment groups and the associated variations in the liquid diet are as follows: EtOH group - 4.8% EtOH; EtOH/High NAC group - 4.8% EtOH plus NAC at a concentration of 1 mg/ml diet; EtOH/Low NAC group - 4.8% EtOH plus NAC at a concentration of 0.5 mg/ml diet; NAC group - NAC alone at a concentration of 1 mg/ml diet; Control group – liquid diet without EtOH or NAC. There were 10 dams in the EtOH group, 8 in the EtOH/High NAC group, 6 in the EtOH/Low NAC group, 8 in the NAC group and 8 in the Control group. To control for the calories derived from the EtOH administered to the EtOH, EtOH/High NAC and EtOH/Low NAC groups, the liquid diet in the control and NAC groups contained an isocaloric amount of maltodextrin. Additionally, to control for possible nutritional effects of altered diet intake, the mice in the EtOH/NAC, NAC and Control groups were matched to the EtOH group on the basis of diet intake (ml) per body-weight (g). The mice were maintained on their respective diet regimen for 48 hrs

(from the beginning of GD 7 to the end of GD 8) and then were returned to *ad lib.* laboratory chow and water until sacrifice.

Blood EtOH concentrations (BEC) were measured in a separate group of mice that underwent an identical EtOH acclimation period as those in the treatment groups, then were bred and placed on either the liquid diet containing EtOH (as in the EtOH group) or the liquid diet containing EtOH and the high dose of NAC (as in the EtOH/High NAC group). BECs were determined by collecting ~35 μ l of tail-blood 10 hrs after the beginning of GD 8. This time point represents the period of approximate peak BEC resulting from this EtOH exposure paradigm (Parnell et al., 2006). BECs were determined from serum using an Analox Alcohol Analyser (Model AM1, Analox Instruments USA Inc., Lunenburg, MA).

Dependent Measures

EtOH-induced ocular dysmorphology was selected as the teratogenic endpoint of interest for this study. Because the eyelids remain open on GD 14, allowing ready assessment of the eyes (Parnell et al., 2006), this was the day on which control and experimental groups of fetuses were collected. At this time, the dams were euthanized via CO₂ asphyxiation and the uterus and fetuses were removed to ice-cold PBS. The number of live fetuses and resorptions were counted and the fetuses were then removed from the uterus and immersion fixed in 10% formalin. After fixation, the fetuses were weighed, measured for crown-rump length and assessed for further morphological anomalies. Both the left and right eyes of each fetus were photographed at 64 X using an Olympus binocular dissecting microscope (Olympus America Inc., Melville, New York). Using these photographs, each eye was examined for the presence of ocular abnormalities by two experienced and independent investigators who were blinded to the treatment groups. Each eye was given a rating of 1 to 5, where each number indicates a distinct category, as illustrated in Fig. 1, and as follows: (1) normal; (2) slightly microphthalmic OR slightly abnormal pupil shape; (3) slightly microphthalmic AND slightly abnormal pupil shape; (4) moderately microphthalmic regardless of pupil shape (although eyes this small usually have abnormally shaped pupils); (5) severely microphthalmic. In the rare cases of disagreement the eyes were reexamined and a rating was agreed upon by both investigators. This procedure has correlated well with computer-based analyses of both eye shape and size as well as palpebral fissure length (Parnell et al., 2006).

Statistical Analyses

The BECs from the EtOH and EtOH/NAC groups were compared using a 2-tailed t-test. Fetal body weights, crown-rump lengths, and litter size were analyzed using a 1-way ANOVA for each dependent measure. Post hoc analyses, when appropriate, were performed using Scheffe's test. The incidence of ocular defects was compared using separate 2-sided Fisher's exact tests (FET). All significance levels were set at $\alpha = 0.05$.

RESULTS

EtOH Intake, BECs, Body Weights and Lengths

There were no differences in EtOH intake among any of the groups. The EtOH group had average EtOH intakes of 23.7 ± 1.0 g/kg (mean $\pm$ SEM) on GD 7 and 24.2 ± 0.7 g/kg on GD 8, the EtOH/High NAC group averaged 23.1 ± 0.7 g/kg on GD 7 and 23.3 ± 0.9 g/kg on GD 8, and the EtOH/low NAC group had average intakes of 25.7 ± 1.1 and 24.1 ± 0.5 g/kg on GDs 7 and 8, respectively. Resulting average BECs were 201 ± 46 mg/dl and 221 ± 29 mg/dl in the EtOH and EtOH/High NAC groups, respectively. These BEC values were not significantly different.

There were no differences among any of the groups in terms of litter size, fetal body weights or crown-rump lengths (Table 1).

Ocular Assessments

The EtOH-exposure paradigm employed for this study has previously been shown to significantly increase the incidence and severity of ocular defects relative to those in paired controls (Parnell et al., 2007; 2006). Similarly, in the current study, the Control group had ocular abnormalities in 6.7% of the right eyes and 2.2% of the left eyes. Compared to these baseline values, EtOH exposure significantly increased the incidence of abnormalities in the right eyes to 42.7% ($p < 0.00001$, FET) and in the left eyes to 24% ($p = 0.0089$, FET). Additionally, utilizing the rating scale described in Fig. 1, it was determined that EtOH exposure increased the severity of ocular defects compared to the Control group, with 18.0% of the eyes in the EtOH group being mildly affected (a rating of 2), while 9.3% of the eyes were both slightly microphthalmic and had abnormally shaped pupils (a rating of 3), 5.3% of the eyes were moderately microphthalmic (a rating of 4), and 0.7% were severely microphthalmic (a rating of 5). Separate values for the right and left eyes are illustrated in Fig. 2.

Concurrent exposure of the pregnant mice to EtOH and NAC via the liquid diet resulted in a lower incidence and severity of ocular abnormalities than that resulting from EtOH alone. The higher NAC dosage (~600 mg/kg) significantly reduced both the incidence and severity of ocular abnormalities (Fig. 2). The lower dosage of NAC (~300 mg/kg) decreased the incidence of ocular defects in both left and right eyes (reductions of 7 and 22%, respectively) as compared to mice that received EtOH alone. However, this reduction was not statistically significant (right, $p = 0.10$; left, $p = 0.63$). The incidence of eye defects in the EtOH/High NAC group was reduced by 27.4% in the right eyes ($p < 0.01$, FET) and 16.7% in the left ($p < 0.05$, FET) as compared to that in the EtOH alone exposure group. In regards to the severity of the ocular defects, there were no fetuses from the EtOH/High NAC group in the most severe rating category (5), and relatively few in the 4th category, while the EtOH group had a higher percentage of eyes that fell into both categories. Additionally, as shown in Fig. 2, no differences were found in the incidence of ocular defects between the Control and EtOH/High NAC groups (right, $p = 0.05$; left, $p = 0.22$), the EtOH/High NAC and EtOH/Low NAC groups (right, $p = 0.62$; left, $p = 0.21$), and the Control and NAC alone groups (right, $p = 0.19$; left, $p = 0.35$). However, there was a significant difference in the incidence of ocular defects between the Control and EtOH/Low NAC groups (right; $p < 0.05$, FET, left eyes; $p < 0.05$, FET).

DISCUSSION

This study demonstrated that in a mouse Fetal Alcohol Spectrum Disorders (FASD) model dietary administration of NAC is effective in reducing EtOH's teratogenesis. This was evidenced by the occurrence of lower incidences of ocular anomalies in fetuses from dams that received a liquid diet containing NAC in combination with EtOH than in those whose mothers' diet contained EtOH, but not NAC. In this study, both the incidence and severity of ocular defects were significantly reduced in the group of mice receiving the higher dosage of NAC (600 mg/kg). While the low dose of NAC (300 mg/kg) slightly decreased the incidence of ethanol-induced teratogenesis, this effect was not statistically significant.

The FASD model employed for this study (drug treatment paradigm, days of treatment, and ocular endpoints) has been used previously for examination of the anti-teratogenic potential of the small peptide SALLRISPA (SAL) (Parnell et al., 2007). As shown for the SAL study, the EtOH exposure strategy, which entails an EtOH acclimation period followed by an EtOH-free mating and early pregnancy period, provides for higher BECs in the dams upon

their return to the EtOH-containing diet than would be achievable in EtOH-naïve pregnant mice. In this study, following return to the EtOH-containing diet, the dam's BECs peaked at just above 200mg/dl. This EtOH concentration is readily achieved in moderate drinkers.

As in the SAL study, employing ocular dysmorphology as the assessed teratogenic endpoint was effective. The defects were not only readily seen, but were present in varying degrees of effect, allowing examination of both incidence and severity. In this study, for the left eyes, the finding of a 7% reduction in defects at the low NAC dose and a 16.7% reduction at the high dose might suggest a dose-response relationship. However, coupled with the finding for the right eyes that the high NAC dosage (600 mg/kg) had only a modest increase in effectiveness relative to that of the lower dosage suggests a threshold effect based on sidedness. These differences in NAC's effects between the left and right sides are most likely a result of inherent asymmetries in development. As in previous studies utilizing this FASD model, the right eyes were affected at a markedly higher incidence than were the left eyes. Similar laterality differences involving the limbs following ethanol exposure at a slightly later point in gestation have also been observed (Chen et al., 2004; Kotch et al., 1992). The reason for the laterality differences, which occur in both control and ethanol-exposed C57Bl/6J mouse eyes remains unclear.

Notably, others have shown that in addition to ocular defects resulting from EtOH-mediated insult on GD 7 and 8 in mice, the brain and face are typically concurrently affected (Cook et al., 1987; Sulik and Johnston, 1983; Sulik et al., 1981; Webster et al., 1980). Given its efficacy in other models of *in vivo* and *in vitro* ethanol exposure, it appears reasonable to expect that the partial protection afforded by NAC to the eyes also extends (via similar mechanisms) to select other embryonic tissues/organs (Chen and Sulik, 2000; Green et al., 2006; Ramachandran et al., 2003; Sheth et al., 2009; Watts et al., 2005; Wentzel and Eriksson, 2008).

Based on previous studies, it is expected that the protective effect that NAC provides is a result of its capacity to boost an antioxidant response. Indeed, other known antioxidants, including vitamins A, C and E, glutathione, lipoic acid and EUK-134 (a superoxide dismutase/catalase mimetic), have all been demonstrated to attenuate the effects of developmental ethanol exposure (Aoto et al., 2008; Chen et al., 2004; Chen and Sulik, 2000; Peng et al., 2005; Reimers et al., 2006; Satioglu-Tufan and Tufan, 2004). In addition to these known antioxidants, SAL, which has some antioxidant properties, has also proven effective when administered in the diet in an identical paradigm as that in the current study (Glazner et al., 1999; Parnell et al., 2007; Steingart et al., 2000).

In contrast to a report showing that a single 600 mg/kg intragastric bolus of NAC is not effective in preventing ethanol-induced defects resulting from exposure during the third trimester equivalent in rats (Pierce et al., 2006), an antiteratogenic effect of NAC following early gestational exposure (during the first trimester equivalent) in mice was found in this study. While, the protection afforded was not complete at the highest dosage (~600 mg/kg/day) tested, these results are in keeping with previous *in vivo* and *in vitro* studies that have shown the capacity of NAC to reduce at least some of ethanol's teratogenesis (Chen and Sulik, 2000; Green et al., 2006; Ramachandran et al., 2003; Sheth et al., 2009; Watts et al., 2005; Wentzel and Eriksson, 2008). Relative to human clinical applications, the ~600 mg/kg dosage is slightly higher than the ~420 mg/kg/day maintenance dose of NAC administered orally after acetaminophen poisoning (Lacy, 2009). However, direct dosage and pharmacokinetic comparisons between mice and humans are difficult due to the inter-species differences in metabolism and administration paradigms.

In conclusion, the results of the current study add to the growing literature that supports ROS-mediated damage as among those involved in ethanol's teratogenicity. Furthermore, it provides new evidence supporting the use of antioxidants to diminish the incidence and/or severity of FASD

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Fig. 1.

Range of ocular defects. The eyes were rated according to the following categories; normal eye (rating of 1) (A), slightly abnormal pupil shape (rating of 2) (B), mild microphthalmia AND slightly abnormal pupil shape (rating of 3) (C), moderate microphthalmia (rating of 4) (D) or severe microphthalmia (rating of 5) (E). Bar in (E) = 10 mm.

Fig. 2.

Incidence and severity of ocular defects. Maternal intake of a liquid diet containing EtOH resulted in significant increases in both the incidence and severity of ocular abnormalities compared to the paired controls (Control) in both the left (*) and right (#) eyes. Concurrent exposure of the pregnant mice to EtOH and NAC resulted in a lower incidence and severity of ocular abnormalities. However, only the higher dose of NAC was statistically significant. The EtOH/High dose NAC group had a significantly decreased incidence of ocular defects in both the left (@) and right (+) eyes compared to mice that received EtOH alone. NAC alone did not alter the incidence of ocular defects compared to the Control group. The severity rating is as described in the Materials and Methods and in Fig. 1.

Table 1

Fetal Weight, Crown-Rump Length and Litter Size

Measurement	Control	Ethanol	Ethanol/ Low Dose NAC	Ethanol/ High Dose NAC	NAC
Fetal Weight (g)	0.13 ± 0.002	0.14 ± 0.003	0.13 ± 0.003	0.13 ± 0.002	0.14 ± 0.003
Crown-Rump Length (mm)	1.01 ± 0.007	1.03 ± 0.008	1.01 ± 0.007	1.01 ± 0.006	1.01 ± 0.007
Litter Size	7.3 ± 0.8	7.5 ± 0.4	6.3 ± 0.7	7.1 ± 0.6	6.6 ± 0.5

Data are reported as the mean ± the standard error of the mean. There were no differences among any of the groups in terms of fetal weight ($p = 0.51$), crown-rump length ($p = 0.14$) or litter size ($p = 0.64$).