PREGNATAL ALCOHOL EXPOSURE AND THE NEUROAPOPTOSIS WITH LONG-TERM EFFECT IN VISUAL CORTEX OF MICE

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(Received 4 January 2007; first review notified 15 February 2007; in revised form 24 February 2007; accepted 6 March 2007; advance access publication 30 May 2007)

Abstract — Aims: The prenatal ethanol exposure induced neuroapoptosis and neuron loss in visual cortex would be investigated in mice at P0, P7 and P14. Methods: Intubating pregnant mice ethanol daily began on E5 and continued through the pup’s birth. The neuroapoptosis in visual cortex was visualized by the caspase 3 immunocytochemistry, and the neuron loss was observed with Nissl method as well. Results: With prenatal ethanol exposure, the dose-dependent neuroapoptosis and neuron loss in visual cortex could be found at P0 and even at P7 and P14 as well. Conclusions: The prenatal ethanol exposure induced neuroapoptosis and neuron loss will persist into postnatal stage, and the long-term effect of neuroapoptosis might be one of the causes of postnatal neurobehavioural disturbances associated with fetal alcohol syndrome.

INTRODUCTION

Alcohol abuse causes injuries of multiple organs and tissues affecting the brain, liver, cardiovascular system, immune system (Fillmore, 2003; Frank et al., 2004). Prenatal ethanol exposure can potentially jeopardize both mother and embryo. Fetal alcohol syndrome (FAS) is a serious injury to fetus and child caused by maternal prenatal exposure (Jones and Smith, 1973; Sulik et al., 1981). FAS is characterized with abnormal facial features, growth deficiencies and the dysfunction of central nervous system (CNS) (Jones and Smith, 1973, 1975; Abel and Sokol, 1986). The dysfunction might cause the children some problems in learning, memory, attention span, communication, vision, hearing and so on (Ahveninen et al., 2000; Sampson et al., 2000). Because prenatal ethanol exposure has become a widespread social problem, the scientists are studying the alcohol-related cortical disorders with great interest.

Alcohol-related cortical abnormalities fall into several categories: neuron loss (Miller, 1995a; Fakoya and Caxton-Martins, 2006), indistinct lamination in cortical plate (CP) (Miller, 1986, 1993), disturbance of postmitotic neuron migration (Miller, 1995b), dendritic arborizing and elongating (Stoltenburg-Didinger and Spohr, 1983; Pentney et al., 1984; Qiang et al., 2002). On the other hand, ethanol has been implicated in triggering apoptotic neurodegeneration in recent years. With administering ethanol subcutaneously to postnatal rat pups, the cerebral cortices showed several typical symptoms of neuroapoptosis. The activated caspase-3 positive cells could be detected in the cortex within 2 h of the first injection, and reached a peak at 12 h (Han et al., 2005). Acute ethanol causing neuronal cell death via a caspase-3-dependent pathway has been demonstrated (Young et al., 2005). The evidences from transgenic mice also indicate that caspase 3 activation is the key for ethanol-induced neuroapoptosis. With ethanol treatment in both homozygous caspase-3 knockout mice and wild-type mice on P7, the brains were examined for the evidence of apoptotic neurodegeneration. In caspase-3 knockout mice, the cell death process evolved more slowly than in wild-type mice (Young et al., 2003, 2005). Although the effect of ethanol-induced neuroapoptosis is certain, the study is still incomplete, for example, the influence of prenatal ethanol exposure induced neuroapoptosis and its long-term effect to CNS development. In present study, the prenatal ethanol exposure was carried out in mice, and the dose-dependent neuron loss and neuroapoptosis with their long-term effect in postnatal mice were investigated in visual cortex. Certainly, the data will provide more detail to understand how prenatal ethanol exposure causes the injury of CNS in infants and children through neuroapoptosis. Meanwhile, understanding the long-term effect of neuroapoptosis will be meaningful for us to carry out the prevention and treatment of the mental disorder in infants and children associated with prenatal ethanol exposure.

MATERIALS AND METHODS

Animals model for prenatal ethanol exposure
C57BL/6 mice were used in the study. All experiments were carried out in accordance with institutional guidelines for animal welfare. Adult male and female were placed in breeding cages in a standard laboratory animal housing environment with the light cycle of 12 h on and 12 h off. The females were checked each morning for the presence of a vaginal plug. The day that a positive plug was present was defined as E0. Embryonic (E = day of conception; E0 = the day a vagina plug is found in mated females) or postnatal (P = days postnatal; P0 = the first 24 h after birth) offspring were produced from timed pregnancies.

The animal model of prenatal ethanol (EtOH) exposure was made according to Deng and Elberger (2003). The pregnant females were housed singly and assigned to one of two groups: (i) EtOH treatment groups: females received
a daily intragastric gavage of 25% (w/v) ethanol at a dose of 2.0 g/kg or 4.0 g/kg, beginning on E5 and continuing through the pup’s birth. To allow the stomach to empty and facilitate the absorption of the EtOH, all food was removed 4 h before EtOH dosing. The EtOH was gavaged at the same time each day. (ii) Control group: The chowfed females were allowed free access to food and water, except in the morning when food was removed for about 2 h prior to weighing. The paired females were intubated with same quantity of isocaloric and isovolumetric maltose-dextrin as EtOH treatment groups. Due to no meaningful differences for neuroapoptosis and neuron loss between paired and Chowfed groups, the data of control were combined with both Chowfed and paired groups, therefore, the statistical tests would be simplified (see also Deng and Elberger, 2003). Usually the control groups gave birth after E19, but the EtOH treatment delayed birth by 1–2 days to E20 or E21. As expectation, the spontaneous resorption of fetuses and malformations, such as microcephaly, anencephaly and myeloschisis with spina bifida, would be found in the newborns received prenatal EtOH exposure (see also Deng and Elberger, 2003). A total of 90 pups at P0, P7 and P14 were used for the study.

**BAC measurement**

On E10 and E15, the tail tip of the pregnant EtOH treatment mothers was nicked with a scissors at 60 and 120 min after gavage, and 10 µl of blood was collected into heparinized hematocrit tubes. To separate blood cells from serum, the blood was centrifuged. Approximately 5 µl of serum was obtained, and tested using an ANALOX model GL5 Analyser for blood alcohol concentration (BAC) analysis following a standardization procedure. Thus, every dam had four BAC values, two each for treatment on E10 and E15. The highest of the four values was defined as each dam’s (and her offspring’s) peak BAC value. The peak BAC of the 4 g/kg dose group was in the range of 300–500 mg/dl, and the peak BAC of the 2 g/kg group was in the range of 150–300 mg/dl. Accordingly, these groups were designated as high and moderate EtOH treatment groups, respectively.

**Hematoxylin and eosin (H.E.) staining and Nissl method**
Pups at P0, P7 and P14 were overdosed with sodium pentobarbital (80 mg/kg, i.p.) and then perfusion-fixed transcardially (80 mg/kg, i.p) and then perfusion-fixed transcardially at 7.2). Whole brains of pups were removed from the skulls for blood alcohol concentration (BAC) analysis following a standardization procedure. Thus, every dam had four BAC values, two each for treatment on E10 and E15. The highest of the four values was defined as each dam’s (and her offspring’s) peak BAC value. The peak BAC of the 4 g/kg dose group was in the range of 300–500 mg/dl, and the peak BAC of the 2 g/kg group was in the range of 150–300 mg/dl. Accordingly, these groups were designated as high and moderate EtOH treatment groups, respectively.

**Caspace 3 immunohistochemistry and hematoxylin counter-staining**
Activated caspace 3, an apoptotic enzyme, is often used as a specific marker of apoptosis cells (Yaginuma et al., 2001). With caspace 3 (activated) immunohistochemistry, the apoptotic neurons have been visualized in the study. Section, deparaffin and hydration were preformed as H. E. staining above. Three percent of H2O2 was used to block the activity of intrinsic peroxidase. After that, coronal sections of 7 µm thickness were incubated for 30 min in 0.5% normal goat serum to block unspecific binding. Then, the primary antibody (1:100, Promega Corporation G7481) was added, and sections were incubated overnight at 4°C. HRP conjugated goat anti-rabbit IgG (1:500, MaxVision™, Kit-5001/5002/5003) was used as secondary antibody. Finally, Diaminobenzidine (DAB) was administered as substrate of HRP to visualize the caspase 3 positive neurons in visual cortex. After immunohistochemistry, some of sections were counterstained with hematoxylin. In this way, the apoptotic neurons would be double-labeled with both hematoylin in nucleus and DAB in cytoplasm as well. The normal neurons just stained with only hematoxylin in nucleus.

**Statistic tests**

**Measurement of neuron loss and apoptotic cell.** In order to investigate the ethanol-induced neuroapoptosis and neuron loss, the neurons in CP of visual cortex were used as targets for observation and measurement. The neuron loss and apoptotic neurons were measured at P0, P7 and P14 respectively. Although both neuron and neuroligia had been stained with immunolabeling and hematoxylin, we could recognize them easily according to their morphological features. Some parameters were accepted to quantify the neuroapoptosis and neuron loss. (i) Apoptotic index (AI): AI = apoptotic neurons/total neurons (both apoptotic neurons and un-apoptotic neurons). Virtually, AI represents the rate of neuroapoptosis in CP of visual cortex. (ii) Neuron density (ND): ND = neurons in CP/area of CP. It describes the number of neurons in unit area of CP in visual cortex. (iii) Cortical thickness (CT). All parameters were available for high EtOH dose, moderate EtOH dose, and control. Five sections were measured for each animal, and the mean values of each animal were calculated for further statistic tests. Total 10 animals were used for each treatment group. In the meantime, the parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>CT (µm)</th>
<th>ND (neurons/mm²)</th>
<th>AI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>10</td>
<td>306 ± 16.25</td>
<td>3834.88 ± 208.70</td>
<td>24.31 ± 1.71</td>
</tr>
<tr>
<td>M EtoH</td>
<td>10</td>
<td>213 ± 24.35*</td>
<td>3189.76 ± 181.99*</td>
<td>54.44 ± 3.29*</td>
</tr>
<tr>
<td>H EtoH</td>
<td>10</td>
<td>198 ± 9.80*</td>
<td>2869.76 ± 165.00**</td>
<td>66.44 ± 5.88***</td>
</tr>
<tr>
<td>P7:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>518 ± 41.18</td>
<td>2339.52 ± 223.45</td>
<td>21.64 ± 1.11</td>
</tr>
<tr>
<td>M EtoH</td>
<td>10</td>
<td>464 ± 8.00*</td>
<td>1584.68 ± 279.29*</td>
<td>46.48 ± 5.17*</td>
</tr>
<tr>
<td>H EtoH</td>
<td>10</td>
<td>374 ± 14.97**</td>
<td>1267.62 ± 190.24*</td>
<td>56.66 ± 3.07***</td>
</tr>
</tbody>
</table>

Mark: CT, cortical thickness; ND, neuron density; AI, apoptotic index; M EtoH, moderate EtOH treatment; H EtoH, high EtOH treatment. ** and *** mean P < 0.01, if EtoH treatments versus control. * means P < 0.05, and ** means P < 0.01, if H EtoH versus M EtoH.
above were prepared for P0, P7 and P14 respectively, therefore, the long-term effect of ethanol-induced neuron loss and neuroapoptosis would be studied.

Statistical analyses. Groups were defined according to treatments (High EtOH, Moderate EtOH, and Control) at various ages (P0, P7 and P14). The comparisons were tested among various treatments. Initially, AI, ND and CT were recorded in a Microsoft Excel spreadsheet, according to their treatments and ages. Then, the comparisons among them were made by \( q \) test.

RESULTS

Prenatal ethanol exposure and development of cortical plate

In order to understand the neuroapoptosis well, the development of neocortex should be described briefly. Visual cortex belongs to the neocortex or isocortex. It has typical six layer lamination, and the CP is regarded as the anlage of cortical development. CP will develop into layer II–layer VI of cortex. The word, ‘inside-out’, is often used to describe the pattern of neurons migration and histogenesis of CP. It says that the newly migrating postmitotic neurons will penetrate through the deep CP and settle down in superficial CP. Therefore, CP matures in proper sequence from layer VI to layer II. Temporally, the neocortex is not matured until P7 in mouse (Bayer et al., 1991a, b). Figure 1 shows the lamination of visual cortex at P14. With prenatal ethanol exposure, the developmental retardation was found in the developing visual cortex. For instance, the cortices in EtoH groups thinned with disorder of the lamination (Fig. 1A–F), and their neurons lost with irregularity of the cell polarity (Fig. 1D–F). These histological alterations above were dose-dependent (Fig. 1A–F). In order to quantify the CT and neuron loss, the ND and CT of visual cortex were measured in the study. The Table 1 shows the mean values and standard errors of ND and CT among different groups at P0, P7 and P14. The data show that the control has higher ND and thicker CT than treatment groups. With \( q \) test, the \( P \) values among various groups at P0 or P7 and P14 are also displayed in the Table 1. The difference is meaningful among them.

Prenatal ethanol exposure and neuronal apoptosis

Caspase 3 is called an ‘apoptotic’ enzyme existing in cytoplasm of apoptotic neurons. With immunohistochemistry and DAB visualization, the cytoplasm of apoptotic neuron was stained with brown color. Neuroapoptosis would even be found in normal development of CNS. In order to understand ethanol-induced neuroapoptosis well, it is helpful to describe the neuroapoptosis in the normal developmental CP with a few words. In our observation, the neuroapoptosis in visual cortex showed an uneven process or temporospatial variation. It meant that the process of neuroapoptosis was changeable in quantity with the location and age alteration. For instance, neuroapoptosis was more common in young animals than old ones, and it was rich in proliferative zone (including subventricular zone and intermediate zone) and superficial CP. This is probably because there are more neuroblasts or new postmitotic neurons in young animal and in proliferative zone and superficial CP, and these cells are usually susceptible in the process of apoptosis (Blaschke et al., 1996). In present study, as CP laminated with various degrees of mature neuron, the neuroapoptosis decreased gradually from layer II to layer VI, coinciding with the 6 layer lamination of cortex. Figure 2(A and B) just show the temporospatial variation of neuroapoptosis in visual cortex. At P7, the activated caspase 3 positive cells distributed either in superficial CP (Layer II–IV) or in deep CP (layer V and VI) widely. However, at P14, the caspase 3 positive neurons concentrated mainly in layer II, remaining immuno-positive cells free in layer III–VI. In order to recognize the apoptotic neuron from the normal, hematoxylin counterstaining was carried out after immunohistochemistry. The apoptotic neurons would be double-stained with DAB in cytoplasm and hematoxylin in nucleus, and the normal neurons was stained only with hematoxylin in nucleus (Fig. 2C). In the study, the prenatal ethanol exposure induced
the neuroapoptosis showed dose dependent (Fig. 2D–F). AI was ever introduced to quantify the neuroapoptosis in visual cortex. CP was targeted for the measurement instead of proliferative zone, due to its structural consistency in the postnatal stage. The data had been catalogued, according to their treatments and ages. The Table 1 lists the values of AI among different groups at P0, P7 and P14. Then, the comparisons were made with q test, and P values were meaningful, indicating dose-dependent difference between EtoH groups and control. Temporally, the higher AI in treatment groups would be found not only at P0 but also at P7 and P14 as well, suggesting the ethanol-induced neuroapoptosis had long-term effect.

DISCUSSION

The animal model of prenatal ethanol exposure and marker for apoptotic neuron

Animal models of prenatal ethanol exposure are often used to study the alcohol toxicity to developing CNS (Cudd, 2005). The model in present study was ever used in our previous research (Deng and Elberger, 2003). It, in a sense, probably might be presumed as an alternative for FAS model. The prenatal ethanol exposure is done through daily intragastric gavage to imitate the pregnant drinking with a chronic process of ethanol exposure during almost whole pregnancy. The injuries are extensive to embryos, including spontaneous reabsorption and teratogeny. No live births were obtained in ethanol treatments (either high or moderate doses) with gavage from E0. With the intragastric intubation from E5, some embryos would survive until birth, even though the spontaneous reabsorption including complete reabsorptions and selective terminations still occurred time to time in the EtoH treatment groups. The teratogenies were also seen in the model, for example, microcephaly, anencephaly and myeloschisis with spina bifida (Deng and Elberger, 2003).

For sure, the animal model will help us to understand the neuroapoptosis with prenatal ethanol exposure, even, in a sense, the neuroapoptosis in FAS for human being.

Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. A family of proteins, caspases, is typically activated in the early stages of apoptosis. It is a group of cysteine proteases that exist within the cell as inactive zymogens. These zymogens can be cleaved to form active enzymes which breakdown or cleave the cellular substrates that are required for normal cellular function, such as the structural proteins in the cytoskeleton and nuclear proteins for DNA repair. In caspase family, caspase 3 plays important role in cell apoptosis. At the onset it proteolytically cleaves polymerase and activates caspase 6, caspase 7 and caspase 9 (Doseff, 2004). Therefore, caspase-3 is proposed as an ‘apoptotic’ enzyme, and it is used widely as marker for apoptotic cell especially the apoptotic neuron (Yaginuma et al., 2001; Yakovlev and Faden, 2001). Ethanol induces apoptotic neuronal death at specific developmental stages, particularly during the brain-growth spurt, which occurs from the beginning of third trimester of gestation and continues for several years after birth in humans, whilst occurring in the first two postnatal weeks in mice.
Neuroapoptosis with temporospatial variation in visual cortex

Neuroapoptosis is important in the developmental sculpting of a normal nervous system. As apoptosis susceptibility occurs in neuroblasts and young postmitotic neurons, an adult neuron must survive from neuroapoptosis during their neuroproliferation or early postmitotic migration (Chun and Schatz, 1999). Data from Blaschke et al. (1996) have revealed the rule of neuroapoptosis in prenatal and postnatal mice. The neuroapoptosis would appear as early as at embryonic day 10, and by embryonic day 14, 70% of cortical cells were found to be dying. This number declined to 50% by embryonic day 18, and only few dying cells were observed in the adult cerebral cortex. One of potentially important role of neuroapoptosis is to eliminate these neurons without function, for example, without synaptic formation or with synaptic formation in wrong location during embryonic or newborn period.

In present study, the neuroapoptosis in CP proceeds with uneven process, or with temporospatial variation. Due to the neuroblasts and young postmitotic neurons’ susceptibility to neuroapoptosis, the neuroapoptosis often occurred spatially within proliferative zones and the superficial PC, and on the other hand, the neuroapoptosis would decrease temporally with age progression. The newly postmitotic neurons migrate to the superficial PC with ‘inside-out’ pattern. Therefore, the developing CP contained the neurons with various degree of maturity, coinciding with the cortical lamination from layer II–VI. In our study, the gradual decreasing of neuroapoptosis from layer II to layer VI just reflects the process of neuron’s maturity and apoptosis in the lamination of CP. With age increasing, the young postmitotic neurons would differentiate into mature neurons, and the caspase 3 positive cells in visual cortex would decline gradually. For instance, in control group, AI at P0, P7 and P14 are 24.31, 21.64 and 18.61% respectively ($P < 0.05$). The finding harmonizes with the observation from Blaschke et al. (1996) and Chun and Schatz (1999).

Dose-dependent neuroapoptosis with long term effect

It is known that ethanol is an inductor of neuroapoptosis particularly for the developing nervous system (Nowoslawski et al., 2005). Prenatal exposure during the last trimester of gestation can produce a broad spectrum of neuroapoptosis. Even once prenatal exposure could cause detectable neuroapoptosis in cortex and cerebellum of mice (Farber and Olney, 2003; Dikranian et al., 2005), and the Bax-dependent caspase-3 was expressed extensively in apoptotic neurons (Sari and Zhou, 2004). The mechanism that alcohol causes the damage of CNS, for example, neuron loss, is not clear (Butterworth et al., 1993). N-methyl-D-aspartate (NMDA) glutamate receptors and GABA$_A$ receptors are suggested to be involved in the alcohol toxicity to CNS. Blockade of NMDA glutamate receptors and excessive activation of GABA$_A$ receptors can trigger widespread apoptotic neuroapoptosis in the developing neocortex potentially. In our previous study, prenatal alcohol exposure would induce the dendritic prolonging and branching of pyramidal cell in wild type mice, but not in NMDA receptor knockout mice. It implies that NMDA receptor is involved in ethanol-induced toxicity in the neuronal development (Deng and Elberger, 2003). The vulnerability to ethanol toxicity coincides with the period of synaptogenesis, last third trimester of pregnancy and the first 2 weeks of postnatal mice (Ikonomidou et al., 2000).

In the present study, the neuroapoptosis and neuron loss in visual cortex caused by prenatal ethanol exposure were investigated. The long-term effect of neuroapoptosis was also demonstrated. At P0, the AI in visual cortex is 66.44% in high dose, 54.44% in low dose and 24.31% in control. Q test shows the dose-dependent differences among the three treatment groups ($P < 0.01$). In the meantime, AI at P7 and P14 was tested as well, and the neuroapoptosis would persist until 2 weeks postnatal, with dose-dependency. The long-term effect of ethanol-induced neuroapoptosis was demonstrated here, as Sari and Zhou (2004) and Fakoya and Caxton-Martins (2006) found the neuroapoptosis in adult, following prenatal ethanol exposure. In our opinion, significantly, the long-term effect of neuroapoptosis probably indicates one of the causes of lifelong mental retardation of FAS in childhood.

In addition, the neuron loss and cortical thinning induced by ethanol were found in the present study as well. Decreasing of neuronal density has ever been reported by others in isocortex (Tran and Kelly, 2003), and the neuronal loss would have continued for 2 months after ethanol withdrawal in mice (Mitra and Ghosh, 2003). In our study, the number of neuron and CT in the visual cortex were measured in different postnatal ages. The results show that treatment groups have neuron loss and cortical thinning with dose dependency, not only at P0 but also at P7 and P14 as well. It suggests that ethanol-induced neuron loss and cortical thinning are also with long-term effect.

In summary, in the present study, the neuroapoptosis and neuron loss at P0, P7 and P14 was studied after prenatal ethanol exposure. The main points are concluded in the following. (i) The neuroapoptosis occurs with temporospatial variation during cortical development. (ii) The prenatal ethanol exposure induces neuroapoptosis with dose-dependency and long-term effect. (iii) The long-term effect of neuroapoptosis and neuron loss is probably one of the causes of postnatal neurobehavioural disturbances associated with FAS.

Acknowledgements — This work was supported by the Natural Science Key Grant of Henan University (04DDZB012), the International Cooperation Grant of Science & Technique Department of Henan Province (0646630014) and the National Natural Science Foundation of China (No. 30670688).

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