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Alcohol exposure on postnatal day 5 induces Purkinje cell loss and evidence of Purkinje cell degradation in lobule I of rat cerebellum

Youngki Lee[a], Julie Rowe[b,†], Kyle Eskue[b,†], James R. West[b], Susan E. Maier[b,*]

[a]*Department of Histology, College of Medicine, Cheju National University, Jeju-si-690-756, Republic of Korea*
[b]*Department of Neuroscience and Experimental Therapeutics (formerly known as Department of Human Anatomy and Medical Neurobiology), Room 228 Reynolds Medical Bldg., College of Medicine, 1114 TAMU, The Texas A&M Health Science Center, College Station, Texas 77843-1114, USA*

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Abstract

The reduction in neuron number in specific brain regions is one of the most destructive aspects of alcohol-induced developmental brain injury, and its occurrence depends on the timing, pattern, and dose of maternal alcohol consumption during pregnancy. The purpose of this investigation was to quantify the dose–response aspect of Purkinje cell loss and rapid cellular degradation indicative of Purkinje cell loss following a single alcohol exposure on postnatal day 5 in lobule I, a lobule that has been shown to be vulnerable to alcohol-induced injury during cerebellar development. Fluoro-Jade B was used to identify Purkinje cell degeneration in 2-h intervals during the first 24 h following the single alcohol exposure. At the end of 24 h, stereology cell counting techniques were used to estimate the number of Purkinje cells in lobule I of the cerebellum. Significant Fluoro-Jade B labeling of lobule I Purkinje cells began at 12-h postexposure in the 6.0-g/kg group with continued significant expression of the marker at the 16- and 18-h time points. Notably, the magnitude of Fluoro-Jade B expression in the 6.0-g/kg group remained high during the period between 12 and 24 h even though the difference between the 6.0-g/kg group and other groups did not reach statistical significance at the 14-, 20-, and 24-h time points. On postnatal day 6, 24 h following the alcohol exposure, rats exposed to the highest alcohol dose (6.0 g/kg) had lost significantly more Purkinje cells than those in the nutritional or caloric control to the highest dose of alcohol group. These results are suggestive of a unique relationship among the quantity of alcohol, the onset and duration of cell degradation, and the degree of eventual cell loss. Given that cerebellar Purkinje cells (and many developing neurons) are vulnerable to alcohol-induced neuronal loss within hours of a single alcohol insult, women should be counseled to avoid drinking alcohol in a manner that significantly increases blood alcohol levels during pregnancy (e.g., binge drinking).

Keywords: Fetal alcohol syndrome; Third trimester; Cell death; Ethanol; Stereology; Fluoro-Jade B

Introduction

Significant brain damage occurs as a function of fetal exposure to alcohol during various stages of development. One of the most detrimental outcomes of exposure to alcohol during gestation is the permanent loss of cells that make up various organs in the human body, including the loss or damage of neurons that make up the central nervous system (CNS). In experimental animal model systems of gestational-equivalent alcohol exposure, neuronal loss in various brain regions results in disorders of CNS such as movement, balance, gait disturbance (Thomas et al., 2004), and impairments of learning and memory (O'Leary-Moore et al., 2006), especially spatial learning (Iqbal et al., 2006; Popovic et al., 2006). The clinical condition called Fetal Alcohol Syndrome represents the most severe manifestation of heavy maternal alcohol consumption during pregnancy among surviving offspring (Jones & Smith, 1973) and includes somatic growth deficiencies, microcephaly (small head for body size), craniofacial dysmorphology, and CNS deficits such as behavioral and cognitive problems (Stratton et al., 1996). Less specific, but consequential deficits, fall under the umbrella term Fetal Alcohol Spectrum Disorders, and include deficits in somatic growth, brain growth, and CNS function. Risk factors such as pattern of alcohol exposure (binge drinking), timing of exposure relative to critical fetal developmental windows (temporal vulnerability), and alcohol dose influence the nature and occurrence of the offspring deficits (Bonthius &

* Corresponding author. National Institute on Alcohol Abuse and Alcoholism, 5635 Fishers Lane, Room 2017, Bethesda MD 20892-9304, Tel.: +1-301-451-7583; fax: +1-301-443-7043.
E-mail address: maiers@mail.nih.gov (S.E. Maier).
† Both authors contributed equally to this study.

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West, 1990; Livy et al., 2003; Maier et al., 1996, 1999; O'Leary-Moore et al., 2006; Tran & Kelly, 2003). Using animal model systems increase the specificity with which the nature and severity of the CNS deficits can be determined under controlled conditions by viewing the underlying neuropathology, including cell loss and the cellular or molecular events preceding such loss that arises from time and dose-specific developmental alcohol exposure.

The rat cerebellum is an excellent model for evaluating anatomical deficits, as well as cellular and molecular events resulting from developmental alcohol exposure. Cerebellar Purkinje cells are vulnerable to alcohol-induced cell death during very specific periods of their development and there is a strong relationship between developmental alcohol-induced Purkinje cell loss and behavioral deficits (Goodlett & Lundahl, 1996; Lalonde & Strazielle, 2003a, 2003b; Thomas et al., 1998; Tran et al., 2005). Importantly, alcohol exposure during the period of extensive connectivity among Purkinje and other neurons, either from postnatal days (PD) 4–6 (Goodlett & Eilers, 1997; Hamre & West, 1993; Light et al., 2002; Pierce et al., 1999) or only on PD4 (Goodlett et al., 1990), results in a significant and permanent loss of Purkinje cells. Purkinje cells within the different lobules of the cerebellum are differentially vulnerable to the third trimester equivalent exposure to alcohol; lobules I, II, III, and IX are the most vulnerable and lobules VI and VII are the least vulnerable (Bauer-Moffet & Altman, 1977; Hamre & West, 1993; Light et al., 2002). In addition to the developmental specificity of timing to observable alcohol-induced Purkinje cell loss, many cellular and molecular events have been identified that occur in close proximity to the loss of cells, and these cellular and molecular events have been assumed to drive the loss of Purkinje cells. For example, changes in the levels of proapoptotic genes (bad, bax) and antiapoptotic genes (Bcl-2) have been shown to correlate in time with specific periods of Purkinje cell vulnerability (Ge et al., 2004), whereas several by-products of apoptotic cell death signaling pathways (e.g., cytochrome c, cleaved caspase-3) have been localized to Purkinje cells in vulnerable lobules of neonatal cerebellum following time-specific, developmental alcohol exposure (Light et al., 2002). In addition, manipulations of pro- and antiapoptotic genes through selective gene knockout (Heaton et al., 2006) or overexpression (Heaton et al., 1999) have been shown to alter the sensitivity of the neonatal cerebellum to ethanol exposure. Therefore, in general, the cerebellar Purkinje cells can be used to evaluate the extent and severity of alcohol-induced pathologies given their unique vulnerability to time-specific alcohol exposure and their gradient of alcohol-induced vulnerability within the cerebellar lobules (Bauer-Moffet & Altman, 1977).

The purpose of this investigation was to quantify the dose–response aspect of Purkinje cell loss (by counting surviving Purkinje cells using stereology) and rapid cellular degradation indicative of Purkinje cell loss (through fluorescent label) following a single alcohol exposure on PD5 in lobule I, one of the most vulnerable lobules of the developing cerebellum. Fluoro-Jade B (FJB), a polyanionic fluoroescein derivative that binds to degenerating neurons and colabels with apoptotic markers (Scallet et al., 2004; Schmued & Hopkins, 2000; Ye et al., 2001), was used to identify Purkinje cell degeneration in 2-h intervals during the first 24 h following the single alcohol exposure. At the end of 24 h, stereology cell counting techniques were used to estimate the number of surviving Purkinje cells in lobule I of the rat cerebellum.

Materials and methods

Subjects. Male and female rat pups derived from timed matings of adult Sprague–Dawley rats served as subjects in this study. The Institutional Animal Care and Use Committee at Texas A&M University approved all procedures prior to the start of the experiment. Skilled laboratory animal technicians cared for all animals on a daily basis and on-site, full-time veterinarians monitored animal health routinely. A conscientious, ongoing effort was made to minimize or eliminate the potential for pain or discomfort. All animal care issues were performed under the minimal standards set forth by the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23).

Timed mating. Evidence of pregnancy was determined by the observation of sperm in vaginal samples taken from the female rats on the morning following pairing with a male. If sperm were detected on the slide, then the female was moved to individual housing until delivery. The day sperm were detected was considered gestational day (GD) 1 and delivery was expected 22 days later. The pregnant rats were monitored carefully from GD22–23; only subjects born on GD23 were used in the study. On the day following delivery, the litter was culled to 10 pups (equal male and female if possible) and left with the dam. Litters containing fewer than eight pups had additional extra pups from other litters fostered in to make litter size 10.

Treatment groups. The treatment groups consisted of alcohol (2.5, 4.5, 6.0 g/kg, abbreviated ALC 2.5, ALC 4.5, ALC 6.0, respectively), nutritional or caloric control to the highest dose of alcohol (abbreviated GC), and untreated control pups (abbreviated SC). Because the end point of the fluorescent labeling involved timed-sacrifice, there were multiple sacrifice times with specific subjects within each group assigned to a specific time point, making the experimental design completely between-subjects. To minimize the potential influence of litter effects, all treatment groups were represented within one litter when possible. Pups in the SC group remained with their mothers from PD4 until the time of sacrifice. Pups randomly assigned to the alcohol treatment groups received a single 20-min infusion of milk diet containing 2.5 g/kg (11.3% v/v), 4.5 g/kg (20.4% v/v), or 6.0 g/kg (27.2% v/v) of ethanol. Pups assigned to the GC group received the identical feeding regimen as alcohol-treated pups except that ethanol diet was replaced by