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Research report

Using Swiss Webster mice to model Fetal Alcohol Spectrum Disorders (FASD): An analysis of multilevel time-to-event data through mixed-effects Cox proportional hazards models

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ABSTRACT

Fetal Alcohol Spectrum Disorders (FASD) collectively describes the constellation of effects resulting from human alcohol consumption during pregnancy. Even with public awareness, the incidence of FASD is estimated to be upwards of 5% in the general population and is becoming a global health problem. The physical, cognitive, and behavioral impairments of FASD are recapitulated in animal models. Recently rodent models utilizing voluntary drinking paradigms have been developed that accurately reflect moderate consumption, which makes up the majority of FASD cases. The range in severity of FASD characteristics reflects the frequency, dose, developmental timing, and individual susceptibility to alcohol exposure. As most rodent models of FASD use C57BL/6 mice, there is a need to expand the stocks of mice studied in order to more fully understand the complex neurobiology of this disorder. To that end, we allowed pregnant Swiss Webster mice to voluntarily drink ethanol via the drinking in the dark (DID) paradigm throughout their gestation period. Ethanol exposure did not alter gestational outcomes as determined by no significant differences in maternal weight gain, maternal liquid consumption, litter size, or pup weight at birth or weaning. Despite seemingly normal gestation, ethanol-exposed offspring exhibit significantly altered timing to achieve developmental milestones (surface righting, cliff aversion, and open field traversal), as analyzed through mixed-effects Cox proportional hazards models. These results confirm Swiss Webster mice as a viable option to study the incidence and causes of ethanol-induced neurobehavioral alterations during development. Future studies in our laboratory will investigate the brain regions and molecules responsible for these behavioral changes.

1. Introduction

Despite public health campaigns broadcasting the link between drinking during pregnancy and birth defects, alcohol consumption among pregnant women remains prevalent [1]. The 2006–2010 report from the Behavioral Risk Factor Surveillance System (BRFSS) finds that 7.6% (or 1 in 13) of pregnant women drink and 1.4% binge drink [2]. The prevalence of drinking during pregnancy is likely underestimated because women that have identified their pregnancy status are less likely to self-report alcohol consumption given social stigma [2]. Fetal Alcohol Spectrum Disorders (FASD) affect 1–5% of the population [3,4]. With the increasing incidence of women of childbearing age engaging in alcohol use, particularly in countries with economic development and within reach of global marketing [5], the occurrence of FASD is a growing health problem [6].

Individuals across the FASD spectrum consistently exhibit developmental delays, hyperactivity, inattention, learning and memory defects, and motor impairments [7]. Because affected individuals lacking characteristic signs associated with high levels of alcohol exposure such as retarded growth and facial dysmorphology, mild cases of FASD often go unreported or are not detected until cognitive or behavioral problems become apparent later in childhood, often induced by stress [8]. The neurobehavioral alterations of FASD are recapitulated in numerous animal models from *Drosophila* to non-human primates [9]. Because parameters such as genetic variability and ethanol dosage can be controlled [10], animal models are central to identifying the neurobiological mech-

Abbreviations: FASD, Fetal Alcohol Spectrum Disorders; E, embryonic day; P, postnatal day; DID, drinking in the dark; ppm, parts per million.

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anisms underlying alcohol-induced deficits during development. The utility of animal models to study the impact of prenatal exposure to moderate levels of alcohol is particularly warranted given the contradictions in human epidemiological studies due to confounding differences in methodology, sample demographics, variable and indeterminate drinking patterns, and co-exposures [10]. Most often rodent models are used because they offer a mammalian system with development nearly identical to humans in a smaller package that is amenable to a laboratory setting.

In rodent animal models, variations in routes of ethanol administration can further complicate findings. Initially most rodent animal models used injection or intubation paradigms, but the maternal stress they cause confounds outcomes. Additionally many of the classical studies using these paradigms produce high blood alcohol levels that would only be expected in individuals with high alcohol tolerance, typically alcoholics. Fetal alcohol syndrome resulting from exposure to high levels of alcohol exposure makes up a minority of FASD cases. Thus, there is a need to develop models of FASD that reflect drinking patterns evident in the general population. More recently, voluntary drinking paradigms have been used to more accurately portray FASD resulting from moderate and binge drinking. At this time, most of these studies use inbred mouse stocks, primarily C57BL/6. Because the variability evident in human FASD is likely caused by genotypic differences between individuals, it is important to expand the stocks of rodents used to study this complex disorder.

In our study we set out to validate Swiss Webster mice, an outbred stock, to model FASD. We chose Swiss Webster mice because this stock is frequently used in alcohol dependence studies [11–13]. These reports typically use male mice thus ethanol effects on females have been minimally explored. To administer ethanol to pregnant mice, we used Boehm et al. voluntary drinking paradigm [14] because it accurately portrays human consumption and characteristic FASD neurobehavioral outcomes in offspring. We used the behavioral battery described by Hill et al. [15] to examine offspring prior to weaning. Alterations in the timing to reach these postnatal milestones is suggested to be predictive of future neurobehavioral deficits [16].

For the statistical analyses of the behavioral outcomes, instead of treating time of completion as a standard quantitative variable, we treated time of completion as a time-to-event variable, and used a Cox proportional hazards model. The primary reason for this is because some of the outcomes had censored observations, or in other words, the outcome was not observed within the observation time frame. If one were to proceed by ignoring the fact that observations were censored, then this would result in biased estimates, because it would be implicitly assuming that each pup with a censored observation completed the test at the time that observation ended, which is incorrect. Alternatively, one could discard all censored observations, but this is clearly not ideal either, because the censored observations do indeed carry much information, namely that the individual did not experience an event by the end of the observation time. Thus, under a Cox proportional hazards model, we were able to appropriately handle censored observations to produce unbiased estimates of the hazard ratio.

2. Materials and methods

2.1. Animals

Swiss Webster mice used in this study came from two groups, batch 1 and batch 2.

Batch 1: male (sires) and female (dams) outbred Swiss Webster mice purchased from ace animals were shipped to the animal facility at Ursinus College and bred in house to provide breeders. During the study, mice were individually housed in standard shoebox mouse cages and were approximately 8 weeks old at the start of breeding. To induce pregnancy, dams were placed in the sire's home cage three hours after lights out (at 4:30pm). Dams were removed the next morning, checked for plugs as a sign of intercourse, weighed, and put back in their home cage.

Batch 2: timed pregnant outbred Swiss Webster mice purchased from Charles River were shipped to the animal facility at Ursinus College after a plug was detected. Dams were weighed upon arrival and individually housed.

Dams from both groups began drinking the first day of pregnancy via the drinking in the dark paradigm (see below). Dams were again weighed on the day before birth expected to determine maternal weight gain during exposure. Food and water were freely available to all mice at all times. The mouse facility was maintained on a reverse 12-h light-dark cycle with lights out at 1:30pm. The temperature and humidity of the mouse facility was maintained at $21 \pm 1\,^{\circ}C$ and $60 \pm 5\%$ respectively. All procedures were approved by Ursinus College's Institutional Animal Care and Use Committee (IACUC, permit #A4347-01) and are in accordance with NIH guidelines on the care and use of animals.

2.2. Drinking in the dark paradigm and tissue preparation

To induce FASD, we used the drinking in the dark (DID) paradigm as described by Boehm et al. [14]. Dams were randomly assigned as either control ($n = 9$) or FASD ($n = 8$). Beginning 3 h after lights out (at 4:30pm) each day, water bottles were replaced with a sipper tube of either tap water (control) or 20% (v/v) ethanol diluted in tap water (FASD). After 2 h, sipper tubes were removed and water bottles were replaced. Food was freely available throughout the procedure. DID continued daily until dams gave birth. Each day we weighed the liquid in each sipper tube. On the first and last days of pregnancy, dams were weighed to determine maternal liquid consumption and ethanol intake.

On the day of birth (postnatal day zero, P0), pups were counted. Any pups in excess of 8 were removed, weighed, and euthanized via decapitation. P0 postnatal brains were removed, weighed, and immersion fixed in 4% paraformaldehyde (diluted 20% v/v in phosphate buffered saline, Electron Microscopy Sciences #157-SP). On P21, the pups were removed and weighed.

2.3. Maternal blood ethanol concentration

A separate study was conducted to determine maternal blood ethanol concentration. Four timed pregnant dams were allowed daily access to ethanol via the DID paradigm. Blood ethanol concentration was determined immediately thereafter on embryonic day 12.5. None of the offspring from these dams were used for behavioral testing. For determination of blood ethanol concentration, a trunk blood sample (20 μl) was collected immediately after DID. 15 μl each of 5% $ZnSO_4$ and 0.3 N $Ba(OH)_2$ was added, the sample was centrifuged at 12,000 rpm for 5 min, the supernatant was removed and stored in a $-20\,^{\circ}C$ freezer until the time of assay. Blood ethanol concentration was determined via gas chromatography mass spectrometry (GC–MS). GC–MS was performed by the Proteomics & Mass Spectrometry Facility at the Danforth Plant Science Center. Blood ethanol concentration (in parts per million, ppm) was determined based on comparison to an external calibration curve using certified reference material.

2.4. Postnatal behavioral tests

One hundred seventeen resultant pups underwent postnatal testing ($n = 62$ and 55 pups in the control and FASD groups, respectively). From P1–21, the pups were removed each day to be weighed