Video Article

Construction of Vapor Chambers Used to Expose Mice to Alcohol During the Equivalent of all Three Trimesters of Human Development

Russell A. Morton[1], Marvin R. Diaz[1], Lauren A. Topper[1], C. Fernando Valenzuela[1]

[1]Department of Neurosciences, School of Medicine, University of New Mexico Health Sciences Center

Correspondence to: Russell A. Morton at ramorton@salud.unm.edu

URL: http://www.jove.com/video/51839
DOI: doi:10.3791/51839

Keywords: Medicine, Issue 89, fetal, ethanol, exposure, paradigm, vapor, development, alcoholism, teratogenic, animal, mouse, model

Date Published: 7/13/2014

Citation: Morton, R.A., Diaz, M.R., Topper, L.A., Valenzuela, C.F. Construction of Vapor Chambers Used to Expose Mice to Alcohol During the Equivalent of all Three Trimesters of Human Development. *J. Vis. Exp.* (89), e51839, doi:10.3791/51839 (2014).

Abstract

Exposure to alcohol during development can result in a constellation of morphological and behavioral abnormalities that are collectively known as Fetal Alcohol Spectrum Disorders (FASDs). At the most severe end of the spectrum is Fetal Alcohol Syndrome (FAS), characterized by growth retardation, craniofacial dysmorphology, and neurobehavioral deficits. Studies with animal models, including rodents, have elucidated many molecular and cellular mechanisms involved in the pathophysiology of FASDs. Ethanol administration to pregnant rodents has been used to model human exposure during the first and second trimesters of pregnancy. Third trimester ethanol consumption in humans has been modeled using neonatal rodents. However, few rodent studies have characterized the effect of ethanol exposure during the equivalent to all three trimesters of human pregnancy, a pattern of exposure that is common in pregnant women. Here, we show how to build vapor chambers from readily obtainable materials that can each accommodate up to six standard mouse cages. We describe a vapor chamber paradigm that can be used to model exposure to ethanol, with minimal handling, during all three trimesters. Our studies demonstrate that pregnant dams developed significant metabolic tolerance to ethanol. However, neonatal mice did not develop metabolic tolerance and the number of fetuses, fetus weight, placenta weight, number of pups/litter, number of dead pups/litter, and pup weight were not significantly affected by ethanol exposure. An important advantage of this paradigm is its applicability to studies with genetically-modified mice. Additionally, this paradigm minimizes handling of animals, a major confound in fetal alcohol research.

Video Link

The video component of this article can be found at http://www.jove.com/video/51839/

Introduction

Drinking during pregnancy can harm the fetus, causing persistent alterations in many organs and systems that significantly decrease the quality of life for the affected individuals and their families. It is estimated that approximately 10-30% of women drink during pregnancy in the U.S., with 1-8% drinking in a binge pattern[1,2]. The range of effects produced by ethanol exposure during fetal development is collectively known as fetal alcohol spectrum disorders (FASDs). Recent estimates indicate that FASDs are a major public health problem with a prevalence as high as 2-5% in the U.S.[3]. The more severe manifestation of FASDs is Fetal Alcohol Syndrome (FAS), which is characterized by growth retardation, craniofacial abnormalities, and neurobehavioral deficits, including learning disabilities. The prevalence of FAS has been estimated to be 0.2-0.7% in the U.S.[3]. The currently available treatments for FASDs are only partially effective and development of more effective treatments is limited by the poor understanding of the cellular and molecular underpinnings of this complex spectrum of disorders.

Data from the National Birth Defects Prevention Study (NBDPS) indicate that pregnant women most commonly drink during the 1st trimester, before pregnancy has been detected, followed by abstinence during later stages of gestation [2]. The NBDPS also found that the second most common pattern of ethanol consumption during gestation involves drinking throughout all trimesters of pregnancy[2]. The reasons for this include lack of awareness about the potentially harmful effects of fetal ethanol exposure (even at low doses), limited access to prenatal care, positive history for neuropsychiatric disorders, and abuse of or dependence on ethanol[4]. Interestingly, the NBDPS reported that the third most common pattern of consumption involved abstinence during the 1st and 2nd trimesters followed by consumption during the 3rd trimester, when it is often assumed that drinking is safe because organogenesis has been mostly completed. However, the 3rd trimester is a period of high susceptibility to ethanol-induced nervous system damage because this is a period when neuronal circuits undergo profound refinement[2]. The NBDPS also identified other, less frequent patterns of alcohol consumption that occur during pregnancy, including consumption throughout the 1st and 2nd trimesters followed by abstinence during the 3rd trimester[2].

In an attempt to model the different patterns of ethanol consumption observed in pregnant women, a number of developmental ethanol exposure paradigms have been established using diverse animal species, with rats and mice being most common[5,6]. The duration of pregnancy in these animals typically lasts approximately 3 weeks, which corresponds to the 1st and 2nd trimesters of human pregnancy. Many rodent studies have assessed the impact of various doses and patterns of ethanol exposure during this period. Examples of the methods frequently used to administer ethanol to pregnant mice and rats include administration via liquid diets[7,8], addition of ethanol to the drinking water[9,10], voluntary drinking of saccharin-sweetened solutions[11], gastric gavage[12], vapor inhalation[13], and subcutaneous or intraperitoneal injection[14]. Results of

these studies have recapitulated several of the deficits observed in humans with FASDs, demonstrating that exposure during early stages of pregnancy is sufficient to damage neuronal circuits across the brain (reviewed in [6,15]).

Experiments with rodents have also demonstrated that exposure during the equivalent to the 3rd trimester of human pregnancy, which approximately corresponds to the first 1-2 weeks of postnatal life in rats and mice, can significantly impair brain development. Exposure during this period has been modeled by administering ethanol to neonatal rats or mice. Ethanol has been administered to these animals using a variety of methods, including feeding via gastrostomy in artificially-reared animals[16-18], intragastric intubation[19], subcutaneous injection[20], and vapor inhalation[21,22]. These studies have convincingly demonstrated that the brain growth spurt is a period of high vulnerability to the developmental effects of ethanol[6].

As mentioned above, drinking during all trimesters of pregnancy is a common pattern of ethanol consumption in women[2]. However, comparatively few studies have assessed the impact of this pattern of exposure using animal models. Some of these studies have taken advantage of large animals where the 3rd trimester-equivalent occurs *in utero* rather than the neonatal period as in the case of rats and mice. These animal models include non-human primates[23,24] and sheep[25-27]. However, these animal models have not been widely used in FASDs research, in part, because of high cost and the need for specialized care facilities. Rodents have been more commonly used to characterize the effect of all-trimester ethanol exposure on fetal development[5]. Guinea pigs have been particularly advantageous in this regard given their extensive prenatal development and similarities in brain maturation to that of humans[28,29]. With guinea pigs, it has been possible to characterize the effect of ethanol exposure *in utero* that includes the equivalent development period of the human 3rd trimester. The comparatively high cost of these animals, as well as the relatively long duration of their pregnancy (~67 days), has limited their use to a few laboratories working on FASDs research.

Because of their cost-effectiveness and wide use in biomedical research, investigators have used rats to model exposure to ethanol during all trimesters of pregnancy. In initial studies, rats were exposed during pregnancy via liquid diets followed by administration of ethanol via gastrostomy to artificially reared neonates (postnatal days (P) 1-10) resulting in peak blood ethanol levels (BEC) in the dams of 0.08 g/dl and in the pups 0.16 g/dl. This paradigm caused long-lasting alterations in optic nerve myelination and reduced the number of Bergmann glia fibers in the cerebellum[30-32]. Similarly, Maier and collaborators using artificial rearing conditions administered ethanol to pregnant rat dams in a binge-like manner via intragastric intubation followed by neonatal administration during part of the 3rd trimester equivalent (P4-9)[33,34]. Peak maternal and pup BECs were 0.3 g/dl at both gestational day 20 and P6. This all-trimester exposure paradigm resulted in growth retardation that was significantly greater than that observed in pups exposed during selected periods of gestation[33]. In addition, rats exposed to ethanol during the equivalent to all trimesters exhibited a reduction in the number of cerebellar Purkinje and granule cells that was greater than that observed in animals exposed during other periods[34]. Reductions in hippocampal cell numbers were also reported with this paradigm, but these effects appear to be primarily a consequence of exposure during the 3rd trimester-equivalent[35]. A method that involves ethanol administration via intragastric gavage to both pregnant rats and neonatal mice has also been used to model all trimester exposure[36]. This method, which yielded BECs of 0.13 g/dl in the dams (gestational day 17) and 0.24 g/dl in P6 pups, induced long-lasting alterations in monoamine neurotransmitter levels in the hippocampus and hypothalamus, and increased expression of DNA methyltransferases and methyl CpG binding protein 2 in the hippocampus[37,38]. Using a similar exposure paradigm (BEC = 0.14-0.2 g/dl in dams and 0.2 g/dl in pups), Gil-Mohapel *et al.*[39] detected an increase in the number of new immature neurons in the dentate gyrus of adult rats that may represent a compensatory mechanism to ethanol-induced neuronal damage or an alteration in the maturation of adult-born neurons. Investigators have also attempted to model all trimester ethanol exposure by exposing dams via liquid diets or drinking water during both pregnancy and lactation[9,40]. However, the utility of exposing the pups via their mother's milk is limited because it typically results in low pup BECs (*e.g.*, 0.002-0.05 g/dl; [41,42]).

Mice have also been used extensively to characterize the effects of developmental ethanol exposure. This animal model shares many of the strengths described above for the rat animal model, with the additional advantage that many genetically modified mouse strains are available[5]. Mice have been successfully used to characterize the effects of ethanol during the 1st, 2nd or 3rd trimesters of pregnancy[43,44]. However, the impact of all trimester exposure on these animals has not been well characterized because it is technically more difficult to expose mice during the equivalent to all trimesters of human pregnancy. For instance, artificial rearing and gastric gavage, which have been used successfully in rats, require more specialized procedures in mice[45]. To the best of our knowledge, only one study to date has attempted to study the effect of all trimester ethanol exposure using mice; these animals were exposed to ethanol solution in drinking water during pregnancy and lactation[46]. Maternal BECs were 0.07 g/dl and pup BECs were not determined, but expected to be a fraction of those in dams.

Here, we describe a new model for all-trimester ethanol exposure of mice where alcohol is administered to both pregnant dams and neonates via vapor inhalation chambers. Vapor chambers were built based on a previous design[47]. We provide detailed instructions on how to build the inhalation chambers and carry out the exposure procedures. We also provide information on the BECs that can be achieved and the impact of exposure on pup survival and growth.

Protocol

All Animal procedures were approved by the University of New Mexico-Health Sciences Center Institutional Animal Care and Use Committee.

1. Vapor Chamber Assembly

1. Cut polycarbonate sheets with a circular saw or jigsaw to the dimensions provided in the video for the top, bottom, front, back, sides, and door (**Figure 1** and **Table 1**).
2. With a circular saw or jigsaw, cut an opening 8 inches high by 16 inches wide in the middle of the front panel.
3. Measure and mark the holes for the piano hinge onto the 18 inch by 10 inch polycarbonate sheet that will become the door of the chamber.
4. In the door, drill counter sink holes with a 5/16 inch drill bit, and with a 3/16 inch drill bit, drill the hole for the screws. Make sure to drill counter sink holes on the inside of the door for the screw heads.
5. Prepare the polycarbonate sheets for welding.
6. Assemble front, back, and side panels onto the bottom panel using Weld-on #16.

7. Seal any gaps between panels with Weld-on #16.
8. Attach the top panel with Weld-on #16.
9. Use bar clamps or heavy text books to hold everything in place while the welds cure.
10. Attach a 1 inch by a 12 inch piece of polycarbonate onto the front panel 1 inch below the opening for the attachment of the door hinge.
11. Allow at least 24 hr for Weld-on to cure.
12. Cut 2 pieces of PEX tubing 12 inches long and 1 piece of 1 inch long PEX tubing.
13. With a 5/16 drill bit, drill a hole in each of the 12 inch PEX tubing approximately 1 to 2 inches from one end.
14. Attach 2 x 12 inch PEX tubing to a 3/8 inch T-connector with the holes located away from the connector.
15. Cap the open ends of the PEX tubing with 3/8 inch plugs.
16. Attach the piano hinge to the front panel and door using 4-40 machine screws and nuts.
17. Using the door as a guide, mark the holes needed for the piano hinge onto the 1 inch spacer on the front panel of the chamber.
18. With a 3/16 inch drill bit, drill the holes into the front panel for the piano hinge.
19. Attach the door and piano hinge to the front panel using 4-40 machine screws and nuts.
20. Assemble the toggle clamps as shown in the video with a washer and nut on either side of the side arm.
21. Mark and drill 3/16 inch holes for the toggle clamps on the front panel and attach toggle clamps with 4-40 machine screws and nuts.
22. Add 3/8 inch rubber bulb seals to the inside of the door.
23. With a 5/8 inch boring drill bit, drill a hole in the center of the top panel for the inlet port.
24. Drill a 1/2 inch hole in bottom / center of the back panel for the exit port.
25. Assemble the exit port by pushing the threaded part of the 3/8 inch through-wall adaptors through the back wall of the vapor chamber in the exit port hole. Attach the nut from the inside of the chamber to hold in place.
26. Remove the protective covering from polycarbonate sheets.
27. Attach the 1 inch piece of PEX tubing to the T-connector and force the PEX tubing through the hole in the top of the chamber from the inside.
28. Attach a 3/8 inch 90 elbow to the top of the 1 inch PEX tubing from the outside.
    NOTE: Drill a 1/2 inch hole in the door and insert a 1/2 inch seal septa into the hole.
29. Repeat steps 1-22 for the air only control chamber.

2. Rack and Air Delivery Assembly

1. If placing the chambers on the cart/rack mentioned in the material list, assemble the cart according to the manufacturer's instructions.
2. With spare nuts and bolts, attach a scrap piece of polycarbonate to the rack to hold the airflow regulators.
3. Anywhere on the scrap piece of polycarbonate, mark and drill 3/4 inch holes for the airflow regulators and attach regulators with the nuts provided.
4. Attach 3/8 inch through-wall adapter to the inlet and outlet ports of the air flow regulators.
5. Assemble the ethanol flask with the aeration stone, #8 stopper and quick release in-line connectors.
6. Using 3/8 inch TYGON tubing, connect the flow regulators to the air pump and ethanol flask as shown in **Figure 1**.
7. For the air only chamber, attach the exit port of the airflow regulator to the inlet 3/8 inch 90 elbow with 3/8 inch TYGON tubing as shown in **Figure 1**.

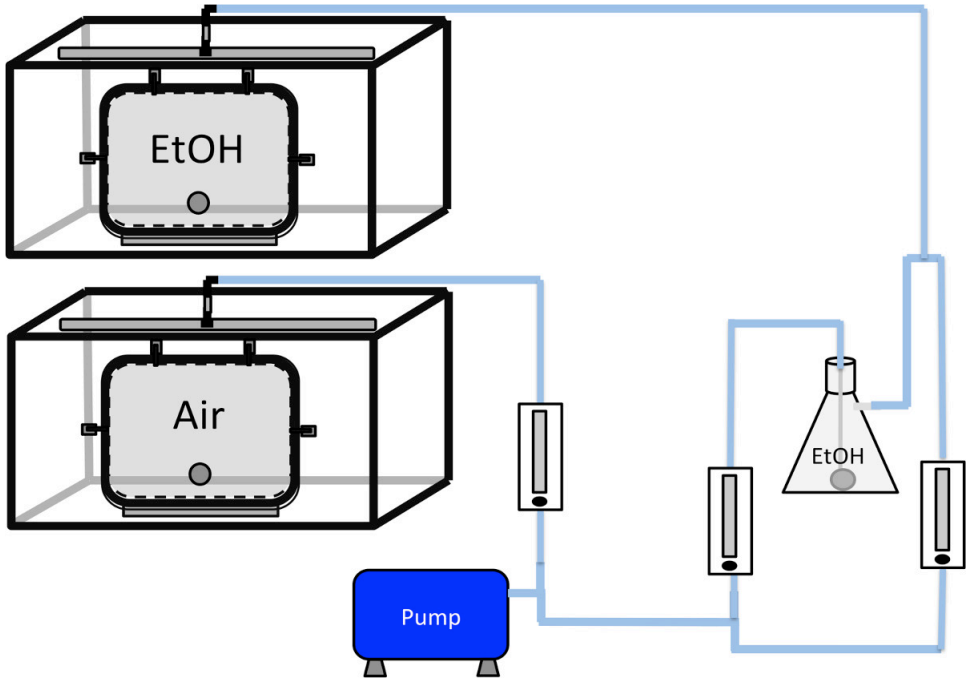

Figure 1. Schematic representation of the configuration of the vapor chambers. A T-connector is attached to the low noise air pump. One side of the T-connector is directly connected to an airflow regulator for the air only vapor chamber. The other side is split again and attached to 2 different airflow regulators, one for air and one for ethanol. The air chamber is directly connected to the regulator as shown. For the ethanol vapor chamber, one airflow regulator is connected to the aerator stone immersed in liquid ethanol in the filter flask. The side arm port of the filter flask is connected with the output of the air regulator as indicated. The merged ethanol vapor and air is then connected to the inlet of the ethanol vapor chamber. The outlet tubes (not shown) are connected to a ventilation outlet in the room.

3. Test Vapor Chambers and Adjust Ethanol Levels

1. Add 600 ml of 190 proof ethanol to the filter flask, insert the aeration stone, and hook up to inlet tube to the side arm of the flask.
2. Close chamber doors and turn on air pump.
3. Adjust airflow regulators so that approximately half as much air flows through the liquid alcohol as the air it is mixed with. Adjust the air only airflow to the combined flow of the alcohol and air of the ethanol chamber.
4. Allow chambers to equilibrate for at least 30 min before measuring the air ethanol concentration.
5. Measure air ethanol concentration by extracting 5 ml of air with an 18G needle and a 60 ml syringe through the septum. Dilute that sample with room air by drawing the plunger back to 60 ml (1:12 dilution). Measure air ethanol level using a breathalyzer in accordance to manufacturer's directions. The dilution of the chamber air is needed to achieve alcohol vapor levels within the detection range of the breathalyzer.
   NOTE: If using different levels of alcohol exposure the dilution may need to be adjusted.
6. As a starting point, adjust the airflow regulators as needed to achieve an air alcohol concentration of approximately 4.5-5 g/dl (g of vaporized alcohol per dl of air).

4. Animal Breeding

1. Group house females C578BL/6 mice (2-3 months old) for at least 1 week to synchronize ovarian cycles.
2. Individually house male C57BL/6 mice (2-5 months old) for at least 2 weeks.
3. After synchronizing, put a single female in with a single male for 5 days to allow for mating.
4. Following mating, remove males and individually house females and place them into chambers.

5. Pre- and Post-natal Ethanol Vapor Exposure

1. Expose pregnant dams to ethanol vapor for 4 hr per day starting at 10 am during the light cycle (lights on from 6 am-6 pm) except the day of birth to prevent pup death.
2. Weigh dams on gestational day (G) 5, G13-G14, and G18-G20 to monitor pregnancies; bedding was changed on days that females were weighed to minimize handling.
3. Each day replace the food for the ethanol exposed groups to avoid consumption of pellets with any ethanol absorbed into them.
4. On the day of birth do not expose animals. After birth expose the dams and pups for 4 hr per day starting at 10 am from P1-P12.
5. Weigh pups on P2, P8, P12, and P25; and change bedding on P8 and P12 to minimize additional handling.
6. Immediately after the last exposure (P12), transfer the cages to a standard animal housing room.

Representative Results

Figure 2A shows that both pregnant mice and neonatal offspring were exposed to relatively stable ethanol vapor concentrations in the chambers. These ranged between 4-6 g/dl. **Figure 2B** shows the BECs achieved in the pregnant mice as a function of time. BECs were measured using a standard alcohol dehydrogenase based assay[48]. At G5, BECs quickly rose to ~60 mM 2 hr after the start of exposure and peaked at the end of the 4 hr exposure period. BECs gradually decreased to ~12 mM after an additional 4 hr following the end of the exposure. By G13-14, there was a dramatic decrease in BECs to approximately 60% of levels detected at G5. In addition, BECs levels rose more slowly and decreased more rapidly, resulting in a shorter presence of ethanol in the blood of the pregnant mice. At near term (G18-20), BECs were further reduced to approximately 30% of levels detected at G5. These findings are consistent with the development of rapid metabolic tolerance to ethanol in the pregnant mice. **Figure 2C** shows that neonatal offspring were exposed to BECs near 30 mM. BECs gradually rose in these animals, reaching a peak at the end of the 4 hr exposure period and gradually decreasing to baseline levels 8 hrs after the end of the 4 hr exposure paradigm. In contrast to the pregnant dams, there was no difference between BECs measured in neonates that were exposed early (P2) vs. late (P7-P12) in the neonatal period. These findings indicate that the neonatal mice did not develop metabolic tolerance to ethanol.

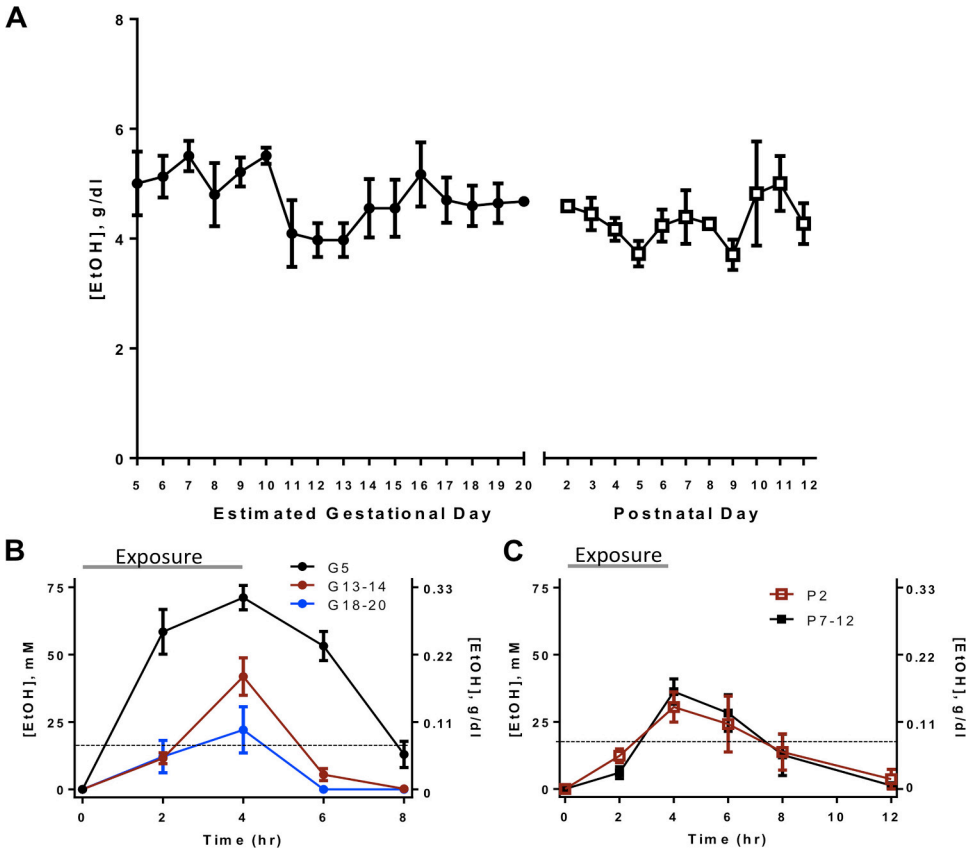

Figure 2. Characterization of ethanol levels. A) Ethanol vapor chamber levels remained relatively constant throughout the gestational and postnatal phases of the exposure paradigm. To measure these levels, chamber air was withdrawn with a syringe through the rubber septum, diluted with ambient air, and expelled into the inlet port of a breathalyzer (see video for details). Values were obtained from 5 and 4 different exposure rounds for the gestational and postnatal phases, respectively. **B)** Blood ethanol levels measured at different time points for the indicated estimated gestational days in pregnant dams (*n* = 5-7 dams). The legal intoxication limit (17.4 mM or 0.08 g/dl) is indicated by the dotted line. The gray bar indicates the time the dams were exposed to ethanol. **C)** Same as in B but for neonatal mice (*n* = 5-9 pups from different litters).

Figure 3 shows that the exposure paradigm did not significantly affect weight gain in the dams or the pups. **Table 2** shows that ethanol exposure did not significantly affect the number of viable fetuses, number of resorbed fetuses, fetus weight, and placenta weight (measured at near term). **Table 2** also shows that the number of pups per litter and pup mortality were not significantly affected by ethanol exposure. Please click here to view a larger version of this figure.

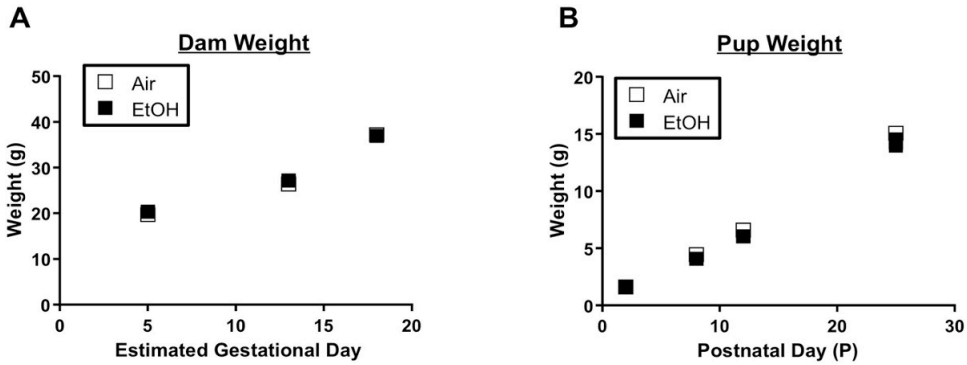

Figure 3. Lack of an effect of ethanol exposure on dam and pup weights. A) Dam weight gain as a function of the estimated gestation day (*n* = 8-12). The weight at estimated gestational day 5 corresponds to the weight measured on the first day of exposure. **B)** Pup weight gain as a function of age (*n* = 7-9). For both panels, the error bars are smaller than the symbols. Please click here to view a larger version of this figure.

Dimensions (inches)	Height	Width
Top	32	22
Bottom	32	22
Front	32	14
Back	32	14
Side 1	21.5	14
Side 2	21.5	14
Door	18	10

Table 1. Dimensions of polycarbonate sheets.

	Air	EtOH
Number of Fetuses: ~E18	7.50 ± 1.08, n = 6	7.33 ± 1.52, n = 6
Average Fetus Weight (g): ~E18	1.04 ± 0.09, n = 6	0.82 ± 0.09, n = 6
Average Placenta Weight (g): ~E18	0.12 ± 0.003, n = 6	0.14 ± 0.01, n = 6
Number of Re-absorbed Fetuses: ~E18	0.50 ± 0.34, n = 6	0.50 ± 0.50, n = 6
Number of pups/litter	7.11 ± 0.67, n = 9	6.89 ± 0.42, n = 9
Number of dead pups/litter	0.11 ± 0.11, n = 9	0.66 ± 0.24, n = 9

Table 2. Pre- and post-natal characterization of mouse vapor exposure paradigm.

Discussion

Here, we describe in detail the methods for the construction of vapor inhalation chambers. The materials and tools required to build the chambers are readily available from a number of commercial suppliers and the steps for the construction of the chambers are relatively straightforward. The system that we describe here does not contain any in-line check valves to prevent back-flow and mixing. We were unable to measure any detectable ethanol in the air only chambers suggesting that we do not have any mixing or back flow of ethanol into the air only chambers. The air only chambers should be checked for ethanol vapor periodically (it should be noted that the air chamber should always be tested prior to the ethanol chamber to avoid detection of residual ethanol vapor present in the breathalyzer and/or syringe). Ideally, the chambers should be placed in dedicated rooms at the animal care facility where mouse cages can be continuously housed for the duration of the exposure paradigm, eliminating the need to transport the mice, thereby reducing stress. A standard room with a ventilation outlet is all that is needed for this paradigm. However, if the room is shared with other investigators, it may be necessary to place the chambers in a room with separated cubicles to minimize exposure of other animals to ethanol odor. Each chamber can accommodate up to 6 standard mouse cages, making it a less labor intensive method of exposure than, for example, intragastric gavage.

The exposure paradigm can be easily modified according to the requirement of a specific experiment. Animals can be exposed to ethanol during the equivalent to all trimesters of human pregnancy. It should be noted that the rodent developmental equivalent to human development can vary depending on what brain region is considered and what developmental process one is interested in (neurogenesis, synaptic integration, etc). In this study, we defined the third trimester-equivalent as the brain growth spurt period. Researchers are encouraged to consult the website translating time[49]. Based on the results shown in **Figure 2B**, we recommend that exposure be started at a lower level of ethanol vapor (*e.g.*, ~3 g/dl) and gradually increased to compensate for the development of metabolic tolerance. During the implementation of the procedure, investigators should closely monitor BECs at different gestational days to determine if this results in more stable levels in the pregnant dams. An inhibitor of alcohol dehydrogenase has been used to prevent the development of tolerance in mice exposed to ethanol in vapor chambers[50]. However, it is not recommended that this agent be used in pregnant mice because studies suggest it has potentially teratogenic effects that

could complicate the interpretation of results[51,52]. Furthermore, injections of any form will cause additional stress to the pregnant dam, potentially confounding the experiment[53].

In contrast to pregnant dams, vapor chamber exposure during the neonatal period did not result in the development of metabolic tolerance. BECs were not different between P2 and P7-12 mice. Peak BECs were similar to those detected in the dams at G13-14 and slightly higher than those detected at G18-20. However, BECs took longer to return to baseline in the pups. It remains to be determined if ethanol exposure in vapor chambers during the gestational period alters the capacity of neonatal offspring to metabolize ethanol. However, based on the literature, it would be expected for pups that were exposed during gestation to ethanol to exhibit a slightly decreased or unchanged capacity to metabolize ethanol[54,55]. In our studies we did not observe a difference in average pup weight, and our data show that by the end of the gestation, the maternal BECs barely rise above the legal intoxication for no more than 2 hr. These data suggest that the mothers are not significantly affected from the alcohol exposure during the post-natal period. Nonetheless, this should be explored further especially if animals are exposed to higher ethanol levels.

This exposure paradigm has some limitations. Female mice were not exposed during the first five days of pregnancy because they are bred with the males during this period. A shorter breeding interval can be attempted (*e.g.*, 2-3 days) but this may result in a reduction in the number of females that become pregnant. Alternatively, animals can be bred for a shorter time and females checked for copulation plugs. Another limitation is that all the mice in a given chamber can only be exposed to a single concentration of ethanol vapor. Moreover, some aspects of the ethanol vapor chamber exposure paradigm are stressful, such as the fact that dams are singly housed throughout the majority of gestation and are exposed to strong ethanol odor. It is also possible that ethanol vapor exposure causes some alterations in the respiratory system of dams and/or pups. In addition, exposure to neonatal rats is not a perfect model of human exposure during the third trimester (*e.g.*, the placental-fetal unit is not present in this model). Nevertheless, we argue that the advantages of this paradigm outweigh its weaknesses, and that it can be a useful model to characterize the mechanisms involved in the pathophysiology of FASDs.

Disclosures

The authors have nothing to disclose.

Acknowledgements

Supported by National Institutes of Health grants R01-AA015614, R01-AA014973, T32-AA014127 and K12-GM088021. The authors thank Samantha L. Blomquist for technical assistance and Drs. Kevin Caldwell and Donald Partridge for critically evaluating the manuscript and video.

References

1. Centers for Disease Control and Prevention (CDC). World Health Organization Who. Alcohol use and binge drinking among women of childbearing age--United States, 2006-2010. *MMWR Morb Mortal Wkly Rep.* **61**(28), 534-538 (2012).
2. Ethen, M. K. *et al.* Alcohol consumption by women before and during pregnancy. *Matern Child Health J.* **13**, 274-285, doi:10.1007/s10995-008-0328-2 (2009).
3. May, P. A. *et al.* Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Dev Disabil Res Rev.* **15**, 176-192, doi:10.1002/ddrr.68 (2009).
4. Wendell, A. D. Overview and epidemiology of substance abuse in pregnancy. *Clin Obstet Gynecol.* **56**, 91-96, doi:10.1097/GRF.0b013e31827feeb9 (2013).
5. Cudd, T. A. Animal model systems for the study of alcohol teratology. *Exp Biol Med (Maywood).* **230**, 389-393 (2005).
6. Valenzuela, C. F., Morton, R. A., Diaz, M. R., & Topper, L. Does moderate drinking harm the fetal brain? Insights from animal models. *Trends Neurosci.* **35**, 284-292, doi:10.1016/j.tins.2012.01.006 (2012).
7. Sliwowska, J. H., Song, H. J., Bodnar, T., & Weinberg, J. Prenatal Alcohol Exposure Results in Long-Term Serotonin Neuron Deficits in Female Rats: Modulatory Role of Ovarian Steroids. *Alcohol Clin. Exp. Res.*, doi:10.1111/acer.12224 (2013).
8. Sutherland, R. J., McDonald, R. J., & Savage, D. D. Prenatal exposure to moderate levels of ethanol can have long-lasting effects on hippocampal synaptic plasticity in adult offspring. *Hippocampus.* **7**, 232-238, doi:10.1002/(SICI)1098-1063(1997)7:2<232::AID-HIPO9>3.0.CO;2-O (1997).
9. Naassila, M., & Daoust, M. Effect of prenatal and postnatal ethanol exposure on the developmental profile of mRNAs encoding NMDA receptor subunits in rat hippocampus. *J Neurochem.* **80**, 850-860 (2002).
10. Servais, L. *et al.* Purkinje cell dysfunction and alteration of long-term synaptic plasticity in fetal alcohol syndrome. *Proc Natl Acad Sci U S A.* **104**, 9858-9863, doi:10.1073/pnas.0607037104 (2007).
11. Brady, M. L., Allan, A. M., & Caldwell, K. K. A limited access mouse model of prenatal alcohol exposure that produces long-lasting deficits in hippocampal-dependent learning and memory. *Alcohol Clin Exp Res.* **36**, 457-466, doi:10.1111/j.1530-0277.2011.01644.x (2012).
12. Bake, S., Tingling, J. D., & Miranda, R. C. Ethanol exposure during pregnancy persistently attenuates cranially directed blood flow in the developing fetus: evidence from ultrasound imaging in a murine second trimester equivalent model. *Alcohol Clin Exp Res.* **36**, 748-758, doi:10.1111/j.1530-0277.2011.01676.x (2012).
13. Cuzon, V. C., Yeh, P. W., Yanagawa, Y., Obata, K., & Yeh, H. H. Ethanol consumption during early pregnancy alters the disposition of tangentially migrating GABAergic interneurons in the fetal cortex. *J Neurosci.* **28**, 1854-1864, doi:10.1523/JNEUROSCI.5110-07.2008 (2008).
14. Godin, E. A. *et al.* Magnetic resonance microscopy defines ethanol-induced brain abnormalities in prenatal mice: effects of acute insult on gestational day 7. *Alcohol Clin Exp Res.* **34**, 98-111, doi:10.1111/j.1530-0277.2009.01071.x (2010).
15. Gil-Mohapel, J., Boehme, F., Kainer, L., & Christie, B. R. Hippocampal cell loss and neurogenesis after fetal alcohol exposure: insights from different rodent models. *Brain Res Rev.* **64**, 283-303, doi:10.1016/j.brainresrev.2010.04.011 (2010).
16. Diaz, J., & Samson, H. H. Impaired brain growth in neonatal rats exposed to ethanol. *Science.* **208**, 751-753 (1980).

17. Stanton, M. E., & Goodlett, C. R. Neonatal ethanol exposure impairs eyeblink conditioning in weanling rats. *Alcohol Clin Exp Res.* **22**, 270-275 (1998).
18. West, J. R., Hamre, K. M., & Pierce, D. R. Delay in brain growth induced by alcohol in artificially reared rat pups. *Alcohol.* **1**, 213-222 (1984).
19. Tran, T. D., Stanton, M. E., & Goodlett, C. R. Binge-like ethanol exposure during the early postnatal period impairs eyeblink conditioning at short and long CS-US intervals in rats. *Dev Psychobiol.* **49**, 589-605, doi:10.1002/dev.20226 (2007).
20. Ikonomidou, C. *et al.* Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science.* **287**, 1056-1060 (2000).
21. Heaton, M. B., Paiva, M., Madorsky, I., Siler-Marsiglio, K., & Shaw, G. Effect of bax deletion on ethanol sensitivity in the neonatal rat cerebellum. *J Neurobiol.* **66**, 95-101, doi:10.1002/neu.20208 (2006).
22. Ryabinin, A. E., Cole, M., Bloom, F. E., & Wilson, M. C. Exposure of neonatal rats to alcohol by vapor inhalation demonstrates specificity of microcephaly and Purkinje cell loss but not astrogliosis. *Alcohol Clin Exp Res.* **19**, 784-791 (1995).
23. Kraemer, G. W., Moore, C. F., Newman, T. K., Barr, C. S., & Schneider, M. L. Moderate level fetal alcohol exposure and serotonin transporter gene promoter polymorphism affect neonatal temperament and limbic-hypothalamic-pituitary-adrenal axis regulation in monkeys. *Biol Psychiatry.* **63**, 317-324, doi:10.1016/j.biopsych.2007.07.017 (2008).
24. Schneider, M. L. *et al.* Moderate-level prenatal alcohol exposure alters striatal dopamine system function in rhesus monkeys. *Alcohol Clin Exp Res.* **29**, 1685-1697 (2005).
25. Ramadoss, J., Hogan, H. A., Given, J. C., West, J. R., & Cudd, T. A. Binge alcohol exposure during all three trimesters alters bone strength and growth in fetal sheep. *Alcohol.* **38**, 185-192, doi:10.1016/j.alcohol.2006.06.004 (2006).
26. Ramadoss, J., Lunde, E. R., Pina, K. B., Chen, W. J., & Cudd, T. A. All three trimester binge alcohol exposure causes fetal cerebellar purkinje cell loss in the presence of maternal hypercapnea, acidemia, and normoxemia: ovine model. *Alcohol Clin Exp Res.* **31**, 1252-1258, doi:10.1111/j.1530-0277.2007.00422.x (2007).
27. Ramadoss, J., Tress, U., Chen, W. J., & Cudd, T. A. Maternal adrenocorticotropin, cortisol, and thyroid hormone responses to all three-trimester equivalent repeated binge alcohol exposure: ovine model. *Alcohol.* **42**, 199-205, doi:10.1016/j.alcohol.2007.12.004 (2008).
28. Byrnes, M. L., Reynolds, J. N., & Brien, J. F. Brain growth spurt-prenatal ethanol exposure and the guinea pig hippocampal glutamate signaling system. *Neurotoxicol Teratol.* **25**, 303-310 (2003).
29. Catlin, M. C., Abdollah, S., & Brien, J. F. Dose-dependent effects of prenatal ethanol exposure in the guinea pig. *Alcohol.* **10**, 109-115 (1993).
30. Phillips, D. E., & Krueger, S. K. Effects of combined pre- and postnatal ethanol exposure (three trimester equivalency) on glial cell development in rat optic nerve. *Int J Dev Neurosci.* **10**, 197-206 (1992).
31. Phillips, D. E., Krueger, S. K., & Rydquist, J. E. Short- and long-term effects of combined pre- and postnatal ethanol exposure (three trimester equivalency) on the development of myelin and axons in rat optic nerve. *Int J Dev Neurosci.* **9**, 631-647 (1991).
32. Shetty, A. K., Burrows, R. C., Wall, K. A., & Phillips, D. E. Combined pre- and postnatal ethanol exposure alters the development of Bergmann glia in rat cerebellum. *Int J Dev Neurosci.* **12**, 641-649 (1994).
33. Maier, S. E., Chen, W. J., Miller, J. A., & West, J. R. Fetal alcohol exposure and temporal vulnerability regional differences in alcohol-induced microencephaly as a function of the timing of binge-like alcohol exposure during rat brain development. *Alcohol Clin Exp Res.* **21**, 1418-1428 (1997).
34. Maier, S. E., Miller, J. A., Blackwell, J. M., & West, J. R. Fetal alcohol exposure and temporal vulnerability: regional differences in cell loss as a function of the timing of binge-like alcohol exposure during brain development. *Alcohol Clin Exp Res.* **23**, 726-734 (1999).
35. Livy, D. J., Miller, E. K., Maier, S. E., & West, J. R. Fetal alcohol exposure and temporal vulnerability: effects of binge-like alcohol exposure on the developing rat hippocampus. *Neurotoxicol Teratol.* **25**, 447-458 (2003).
36. Kelly, S. J., & Lawrence, C. R. Intragastric intubation of alcohol during the perinatal period. *Methods Mol Biol.* **447**, 101-110, doi:10.1007/978-1-59745-242-7_8 (2008).
37. Perkins, A., Lehmann, C., Lawrence, R. C., & Kelly, S. J. Alcohol exposure during development: Impact on the epigenome. *Int J Dev Neurosci.* **31**, 391-397, doi:10.1016/j.ijdevneu.2013.03.010 (2013).
38. Tran, T. D., & Kelly, S. J. Alterations in hippocampal and hypothalamic monoaminergic neurotransmitter systems after alcohol exposure during all three trimester equivalents in adult rats. *J Neural Transm.* **106**, 773-786 (1999).
39. Gil-Mohapel, J. *et al.* Altered adult hippocampal neuronal maturation in a rat model of fetal alcohol syndrome. *Brain Res.* **1384**, 29-41, doi:10.1016/j.brainres.2011.01.116 (2011).
40. Popovic, M., Caballero-Bleda, M., & Guerri, C. Adult rat's offspring of alcoholic mothers are impaired on spatial learning and object recognition in the Can test. *Behav Brain Res.* **174**, 101-111, doi:10.1016/j.bbr.2006.07.012 (2006).
41. Guerri, C., & Sanchis, R. Alcohol and acetaldehyde in rat's milk following ethanol administration. *Life Sci.* **38**, 1543-1556 (1986).
42. Matta, S. G., & Elberger, A. J. Combined exposure to nicotine and ethanol throughout full gestation results in enhanced acquisition of nicotine self-administration in young adult rat offspring. *Psychopharmacology (Berl).* **193**, 199-213, doi:10.1007/s00213-007-0767-2 (2007).
43. Olney, J. W. Fetal alcohol syndrome at the cellular level. *Addict Biol.* **9**, 137-149; discussion 151, doi:10.1080/13556210410001717006 (2004).
44. Sulik, K. K. Genesis of alcohol-induced craniofacial dysmorphism. *Exp Biol Med (Maywood).* **230**, 366-375 (2005).
45. Lewis, S. M. *et al.* Modifying a displacement pump for oral gavage dosing of solution and suspension preparations to adult and neonatal mice. *Lab Anim (NY).* **39**, 149-154, doi:10.1038/laban0510-149 (2010).
46. Cebolla, A. M. *et al.* Effects of maternal alcohol consumption during breastfeeding on motor and cerebellar Purkinje cells behavior in mice. *Neurosci Lett.* **455**, 4-7, doi:10.1016/j.neulet.2009.03.034 (2009).
47. Becker, H. C., & Hale, R. L. Repeated episodes of ethanol withdrawal potentiate the severity of subsequent withdrawal seizures: an animal model of alcohol withdrawal "kindling". *Alcohol Clin. Exp. Res.* **17**, 94-98 (1993).
48. Galindo, R., & Valenzuela, C. F. Immature hippocampal neuronal networks do not develop tolerance to the excitatory actions of ethanol. *Alcohol.* **40**, 111-118, doi:10.1016/j.alcohol.2006.11.001 (2006).
49. Workman, A.D., Charvet, C.J., Clancy, B., Darlington, R.B., Finlay, B.L. Modeling transformations of neurodevelopmental sequences across mammalian species. *J Neurosci.* **33**, 7368-7383 (2013).
50. Becker, H. C., Diaz-Granados, J. L., & Weathersby, R. T. Repeated ethanol withdrawal experience increases the severity and duration of subsequent withdrawal seizures in mice. *Alcohol.* **14**, 319-326 (1997).
51. Ukita, K., Fukui, Y., & Shiota, K. Effects of prenatal alcohol exposure in mice: influence of an ADH inhibitor and a chronic inhalation study. *Reprod Toxicol.* **7**, 273-281 (1993).
52. Varma, P. K., & Persaud, T. V. Influence of pyrazole, an inhibitor of alcohol dehydrogenase on the prenatal toxicity of ethanol in the rat. *Res Commun Chem Pathol Pharmacol.* **26**, 65-73 (1979).

53. Balcombe, J. P., Barnard, N. D., & Sandusky, C. Laboratory routines cause animal stress. *Contemp Top Lab Anim Sci.* **43**, 42-51 (2004).
54. Arias, C., Molina, J. C., Mlewski, E. C., Pautassi, R. M., & Spear, N. Acute sensitivity and acute tolerance to ethanol in preweanling rats with or without prenatal experience with the drug. *Pharmacol Biochem Behav.* **89**, 608-622, doi:10.1016/j.pbb.2008.02.017 (2008).
55. Nizhnikov, M. E., Molina, J. C., Varlinskaya, E. I., & Spear, N. E. Prenatal ethanol exposure increases ethanol reinforcement in neonatal rats. *Alcohol Clin Exp Res*. **30**, 34-45, doi:10.1111/j.1530-0277.2006.00009.x (2006).

