The Effects of Ethanol Exposure on the Development of *Caenorhabditis elegans*

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Summary

Prenatal exposure to ethanol results in a wide range of birth defects in humans classified as Fetal Alcohol Spectrum Disorders (FASD). As a result, the effect of ethanol on fetal development has been highly studied in many vertebrate and mammalian models. Recently, *Caenorhabditis elegans* has been introduced as an ideal model organism for studying the effects of ethanol on development. In this experiment, we exposed *C. elegans* to three different concentrations of ethanol. We hypothesized that increasing ethanol concentrations would result in fewer offspring that were smaller in size. Our results were inconclusive in the quantity of offspring produced, but we found that worms developing in the presence of ethanol were significantly smaller than those that were not. From these observations, we concluded that exposure to ethanol during development inhibited growth. Future experiments include developing a more precise experimental procedure and increasing the concentrations of ethanol used.

Introduction

Abnormal human fetal development has long been associated with women consuming alcohol (ethanol) during pregnancy. The resulting offspring frequently present what has come to be classified as Fetal Alcohol Spectrum Disorder (FASD) – colloquially known as Fetal Alcohol Syndrome (Davis, et al., 2008). FASD is characterized by physical and neurological abnormalities. These abnormalities result in observable behavioral symptoms, learning disorders, and irregular immune system function (Davis, et al., 2008).

To further understand the teratogenic effects alcohol may have on human development, studies on vertebrate models (mouse, rat, guinea pig, chick, sheep, pig-tail macaque) have been extensively conducted over the past several decades (Davis, et al., 2008). Evidence from these experiments has consistently demonstrated the role ethanol likely plays in interfering with neural crest cell survival and migration (Ahlgren, et al., 2002). During vertebrate embryonic development, neural crest cells migrate anteriorly along the developing head, ultimately differentiating into associated facial and neural tissues (Ahlgren, et al., 2002). Sonic hedgehog (Shh) signaling has been identified as a prominent pathway directing this neural crest cell migration and is therefore largely responsible for producing normal head, brain, and facial structures (Ahlgren, et al., 2002). In mouse models and humans with Shh mutations, extensive neural crest cell apoptosis has been observed, which result in phenotypic abnormalities coinciding with those demonstrated in FASD patients (Ahlgren, et al., 2002). Experimentally administering antibodies blocking Shh signaling in chick embryos also produces phenotypes observed in humans with FASD and mouse, chick, and quail embryos exposed to ethanol (Ahlgren, et al., 2002). When chick embryos are treated with ethanol, depressed levels of mRNAs involved in the Shh signaling cascades are detected (Ahlgren, et al., 2002). Administering exogenous Shh to the ethanol treated chick models revives normal gene activity and mRNA concentrations (Ahlgren, et al., 2002). Subsequently, interfering with Shh signaling appears to be the mechanism by which ethanol promotes neural crest cell apoptosis and abnormal embryonic development.

While data from these experiments has supported the observations of humans with FASD, vertebrate models make examining microbiological components difficult (Davis, et al., 2008; Wolf, et al., 2003). Recently, in order to further observe the teratogenic effects of ethanol on this level, invertebrate models such as *C. elegans* and *Drosophilia melanogaster* have been proposed as alternate models for experimentation (Davis, et al., 2008; Wolf, et al., 2003; Ahlgren, et al., 2002; Kwon, et al., 2003). These models have previously been used with success to investigate the microbiological components and pathways involved in drug and nicotine dependence, supporting their use in the investigation of alcohol's teratogenic effects (Davis, et al., 2008; Wolf, et al., 2003; Feng, et al., 2006; Kwon, et al., 2003; Dhawan, et al., 1999; Davies, et al., 2003; Davies, et al., 2004; Ahlgren, et al., 2002). Justified by these past successes, recent experiments have been conducted with ethanol and *C. elegans*. The results of these studies have provided insight into ethanol's physiological impact on *C. elegans*, many of which are postulated to have conserved mechanisms of action responsible for mammalian responses to ethanol (Davies, et al., 2003; Feng, et al., 2006; Kwon, et al., 2003). More specifically, the results of Kwon, et al. (2003) found that 230 genes in *C. elegans* were altered in their expression when the nematodes were treated with ethanol, many of which are known homologs to genes found in humans (Kwon, et al., 2003). Furthermore, Davis, et al. (2008) observed that ethanol inhibited normal *C. elegans* larval development, growth, onset of reproductive maturity, and negatively affected longevity and the ability to produce normal progeny in normal quantity (Davis, et al., 2008). Many of these results correlate with observations of phenotypes seen in humans with FASD. The potential of the *C. elegans* model in researching ethanol's impacts on development – including human development – has yet to be fully realized. With the *C. elegans* genome and normal embryonic developmental fates mapped in their entirety, this nematode appears to be an ideal model to study (Kwon, et al., 2003).

Our aims were to conduct an experiment using this model to test two hypotheses: (1) the quantity of progeny will negatively correlate with increasing alcohol concentrations and (2) the progeny will be smaller with increasing alcohol concentrations. Based on the results of Davis, et al. (2008) and Wolf, et al. (2003), we expected to observe evidence in support of our hypotheses as well as associated behavioral symptoms that increased in severity with exposure to increasing ethanol concentrations.

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Results

24 hours
The worms were observed 24 hours after being placed on the ethanol plates. Plate set-up is depicted in Table 1. The worms on the control plates had normal movement, two of the worms on control plate A (CPA) were gravid and one worm on control plate B (CPB) is obviously gravid. At 0.32%, the adult worms appeared to be moving very slowly on both plates and stopped frequently. None of them appeared to be gravid. On plate 3 (P3), at 0.16% ethanol, the worms had slightly inhibited movement, and two appeared swollen with eggs. On plate 4 (P4), at 0.16% ethanol, the worms appeared normal and none were gravid. At 0.08% ethanol, movement was normal in all worms. On plate 5 (P5), one appeared gravid and on plate 6 (P6), all three worms appeared gravid.

72 hours
The worms were observed 72 hours after being transferred to ethanol plates. The CPA and CPB had adult worms that exhibited normal movement (Table 2). P1, at 0.32% ethanol, had approximately 150 adult worms and over 400 worms that were half the size or less. The adult worms had abnormally slow movement while the small worms appeared to move normally. P2 showed a similar trend but there were also many eggs that had not hatched yet (Table 2). P3 was also similar to P1 and P2, but P4 differed in that the adult worms outnumbered the small worms (Table 2). P5, at 0.08% ethanol, had similar quantities of worms as P1-4, but the adult worms showed normal movement. P6 had fewer worms, and hundreds of unhatched eggs.

96 hours
The worms were observed after 96 hours. CPA and CPB contained mostly large adult worms with approximately half as many small worms (Table 3, Figure 1A). P1 and P2 (0.32% ethanol), showed hundreds of small worms and slightly less large worms than the control plates (Table 3, Figure 1C, 1D). The small worms were approximately five grid units long while the normal adults were about nine grid units long (Figure 2). The normal adult worms were estimated to be 0.5 grid units wide and the small worms were about 0.25 grid units wide (Figure 2). P3 and P4 (0.16% ethanol) had similar quantities of large adult worms as P1 and P2, but fewer smaller worms (Table 3, Figure 1E, 1F). P5 and P6 (0.08% ethanol) had similar quantities of large adult worms, fewer smaller worms than P1-4 but still more than the control plates (Table 3, Figure 1G, 1H).

Plate	Ethanol concentration
Control A	0
Control B	0
1	0.32%
2	0.32%
3	0.16%
4	0.16%
5	0.08%
6	0.08%

Table 1. The plates were created with specific ethanol concentrations absorbed into the agar.

Plate	Ethanol Concentration	Number of Adult Worms	Number of Small Worms	Eggs Visible
Control A	0	150	40	0
Control B	0	175	45	0
1	0.32%	150	400	0
2	0.32%	120	350	40
3	0.16%	140	300	35
4	0.16%	200	100	120
5	0.08%	120	400	5
6	0.08%	170	100	300

Table 2. Observations after 72 hours. The number of adult and small worms were counted as well as the number of eggs that had not hatched yet.

Plate	Ethanol Concentration	Number of Adult Worms	Number of Small Worms	Ratio of Adult/Small Worms
Control A	0	130	45	2.888888889
Control B	0	125	60	2.083333333
1	0.32%	100	475	0.210526316
2	0.32%	115	505	0.227722772
3	0.16%	95	350	0.271428571
4	0.16%	75	290	0.25862069
5	0.08%	120	210	0.571428571
6	0.08%	95	250	0.38

Table 3. Observations after 96 hours. More small worms were visible on ethanol plates than controls as shown by the ratio of adult/small worms.

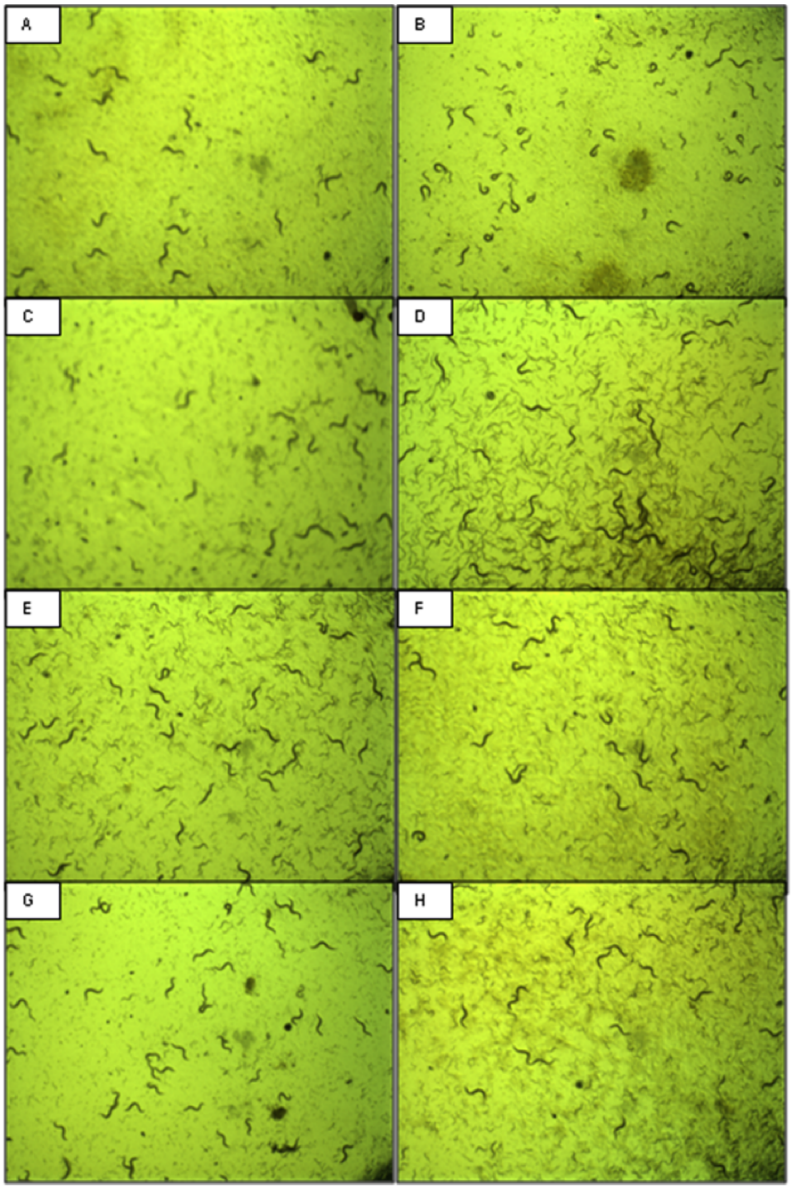

Figure 1. *C. elegans* photographs at 96 hours; 40x magnification. A) CPA; mostly adult worms were visible, few small worms. B) CPB; mostly adult worms visible, few small worms. C) P1; at 0.32%, many more small worms visible than in controls. D) P2; at 0.32%, abundance of small worms. E) P3; at 0.16%, more small worms than controls but less than P1 and P2. F) P4; at 0.16%, more small worms than controls but less than P1 and P2. G) P5; at 0.08%, worm quantities appear similar to control. H) P6; at 0.08%, slightly more small worms than control groups but less than P1-4.

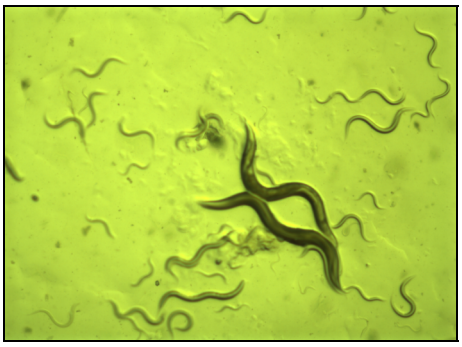

Figure 2. (P1) Normal adult worms were significantly larger than the small worms.

Discussion

Our results indicated that ethanol did have an effect on the development of *C. elegans*. After 24 hours exposure to ethanol, no first generation offspring were visible, but the parent worms on the 0.32% and 0.16% plates showed behavioral abnormalities; primarily slow, uncoordinated movement. The worms on the 0.08% plates exhibited normal movement, suggesting that this concentration of ethanol is not high enough to cause behavioral abnormalities.

The quantity of offspring produced did not appear to be affected by exposure to different concentrations of ethanol, which contradicts our original hypothesis. Recent studies suggested that the number of eggs laid and hatched is reduced in *C. elegans* that have been exposed to ethanol (Davis, et al., 2008). In our experiment, however, it was

found that the plates with the highest ethanol concentration (0.32%) had the most worms present. A possible explanation for this observation is that our methods were not meticulous enough to determine the exact stage each worm was at when it was transferred from a normal plate to a plate containing ethanol. The worms transferred to the 0.32% plates may have contained more eggs, or were at a slightly different stage than those put on the lower concentration and control plates. This could have altered the effect the ethanol had on the first generation offspring.

A significant difference in the size of the worms was observed between the ethanol plates and the control plates. The 0.32% plates had over seven times as many small worms as the control plates but only slightly less large worms. The large worms were assumed to be adults, but we did not have a conclusive method for differentiating between baby worms and adult worms with inhibited growth. As the concentration of ethanol decreased, however, the amount of worms and their sizes became increasingly similar to the control plates. The 0.08% concentration had a much smaller effect on the size and quantity than did 0.32% and 0.16%. These observations support our hypothesis that worm size would be decreased in response to ethanol exposure during development. Recent research has shown that *C. elegans* that have been exposed to ethanol during development appear to be shorter in length than normal organisms (Davis, et al., 2008). However, the decreased size of the worms on the ethanol plates could also have been caused by a shortage of food. There were significantly more worms present on all plates containing ethanol, especially the 0.32% concentration plates. This could be a result of a shortage of food and the worms may not have had the nutrients to develop to their normal adult size.

Another trend was noticed in the behavior of the different generations of worms. First generation offspring exposed to 0.32% and 0.16% concentrations had slow, uncoordinated movement, but the second generation offspring, while smaller, did not exhibit this behavior. This indicates they may have the ability to adapt to this environmental condition. *C. elegans* has been shown to adapt its response to stimuli in the presence of a drug, especially when exposed to that drug prior to the stimulus (Bettinger and McIntire, 2005). Studies have shown that ethanol causes a decrease in the rate of locomotion of *C. elegans*, which becomes more pronounced as the concentration increases (Davies and McIntire, 2004). A genetic screen for mutants with normal locomotion in the presence of ethanol showed multiple mutations of the *slo-1* allele. *C. elegans slo-1* loss-of-function mutants were found to be resistant to ethanol's intoxicating effects (Davies, et al. 2003). Inversely, *C. elegans slo-1* gain-of-function mutants presented behavior normally observed only in the presence of ethanol (Davies. et al., 2003). *slo -1* codes for the BK potassium channel, a calcium-dependent channel with many mammalian homologs (Davies and McIntire, 2004). Based on the observations of Davies, et al., it was concluded that BK potassium channels become hyper-activated by ethanol, producing diminished neural functionality. This model provides a possible mechanism for altered human behavior in the presence of ethanol.

In future experiments, the concentrations of ethanol should be increased to determine if there are more significant effects on the development of *C. elegans*. Recent studies have shown more extensive effects than were seen in our experiment (Davis, et al., 2008), suggesting an altered method may provide more conclusive results. Another future study could include transferring a second generation offspring organism to a normal plate and observing the development of its offspring. This could show whether exposure to ethanol has altered its ability to survive and reproduce in an ethanol-free environment, and whether the later generations would be affected by parental exposure to ethanol. The genetic effects ethanol has on *C. elegans* to cause abnormal development should also be further studied. *C. elegans* provides many advantages as a model organism, notably its completely mapped genome and short life cycle. The results of such an experiment could provide insight into the study of Fetal Alcohol Syndrome in humans.

Materials and Methods

Five N2 strain adult worms at L2 stage were placed in a standard 60mm agar plate to produce eggs. The adults were removed 24-hours later so only the eggs remained. This process was imperative to ensure that the worms were of the same age. At 48 hours the N2 worms were hatched, and three worms were each placed into a single plate. A total of eight plates were made, with two serving as a control. The remaining six plates contained three different concentrations of ethanol; two plates per ethanol concentration. 95% ethanol solution was diluted to achieve the desired concentrations, assuming the agar gel in the plates is essentially water and 1.0 g was equal to 1.0 mL of distilled water. The worms were exposed to 0.08%, 0.16%, and 0.32% concentrations of ethanol throughout development and stored in a 25°C incubator. 25°C was chosen as an ideal temperature with the intention of speeding up the rate of development in accordance to our timeline. The exposure of ethanol chronicled from the placement of adult N2 worms on to the cultured plates, to their offspring and subsequent generations. Observations were recorded 24, 72, and 96 hours after the worms had been transferred to the ethanol plates. A Leica GZ6E stereo zoom microscope with a 1X objective, 10X eye pieces and zoom factor of 0.63X to 4.0X was used to carry out observations. Size was determined through pictures obtained from a Diagnostic Instruments Spot Insight Color CCD microscope camera overlaid with a grid created in Microsoft Powerpoint. Approximate numbers of worms were recorded by estimating 1/10 of the plate.

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