Effect of Limonene on Developing Human Brain Neurons

SaDazia Driffin and Omar Bagasra

South Carolina Center for Biotechnology, Claflin University, Orangeburg, South Carolina

Abstract

The objective of this experiment was to determine what effect limonene has on the developing brain neurons and how it relates to autism. The experiment design was to test limonene against fetal brain neurons to evaluate how neuroblastoma cell lines exposed to a minute concentration of limonene respond to the chemical. Limonene is a colorless liquid hydrocarbon classified as a cyclic terpene. It is used as a base solvent in cleaning supplies, perfumes, and cosmetic products (Gosselin, R.E. 2012). Autism Spectrum Disorder (ASD) is a heterogeneous developmental disorder that impacts most of the social interactions. ASD can be either genetically linked or environmentally linked or both. This study focuses on the environmental effect of ASD. This experiment is to analyze how this chemical can effect the fetal brain development when a pregnant mother is exposed to this chemical. In this experiment neurons were exposed to different concentrations of limonene. Morphological modifications where analyzed after staining with Hematoxylin and Eosin (H&E). There was a major adverse effects on both male and female cell lines (CRL-2267 and CRL-2266, cell lines, respectively). The higher the concentration of limonene the more morphological deformities were present. In conclusion, exposure to limonene impart serious adverse effects on fetal brain neurons. Therefore, it may be an environmentally linked cause of ASD.

Introduction

Autism spectrum disorder (ASD) is a condition related to brain development that impacts how a person perceives and socializes with others, causing problems in social interaction and communication. The cause or causes of autism are unknown. Autism involves very early changes in the development of a fetal brain that starts 8 week of gestation. This past several years increased evidence are being shown that chemical pollutants may affect brain development in ways that the risk of Autism is increased. Too little is known about how the timing or dose of exposure that influences risk; or biological mechanisms involved. This experiment will help to address further studies by examining the reaction of various concentrations of Limonene on fetal brain cells through various observations while recording rates of morphological deformities. It is also hypothesized that children with autism have lower levels of OXY and AVP. Therefore, fragrances that contain complex chemical mixtures may have different effects on the human neural development (Sealey et al. 2015).

Materials and Methods

Step 1: Culture of fetal brain cell lines CRL-2266 and CRL-2267

Step 2: Dilution of all synthetic chemicals in ethanol solution (concentration of 1 ng/ml)

Step 3: 1:10 Serial dilution using a 96-Well Plate exposing synthetic chemicals to fetal brain cell lines. In order to find an optimal dilution that doesn't kill cells completely, we choose 1:8000 dilution.

Step 4: Adding diluted chemicals to the cell lines in 4 8-well slides incubate at 37 degrees Celsius for 48 hours. (Final Concentration 0.125 ng/ml)

Step 5: Fix cells in 8 well slides with STF (Streck Tissue Fixative) To ensure cells will adhered to surface

Step 6: Observe any morphological changes by H&E staining

Results

Figure 1 A&B

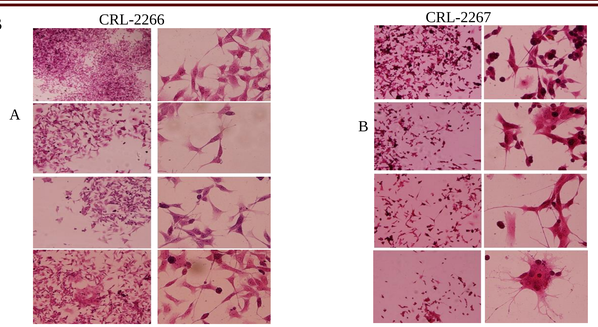
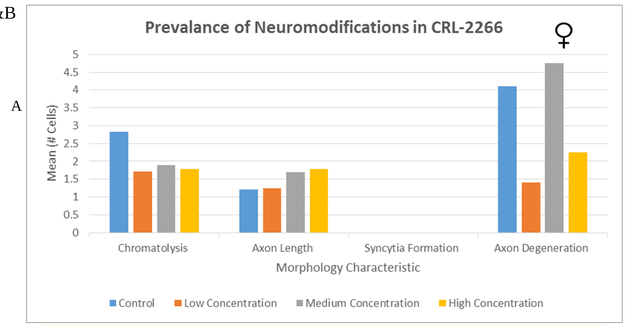


Figure 1A &B represents H&E staining of a particular cell line at 10x and 40x magnification. Cells were exposed to limonene at 1:10 concentrations. The control contained media only.

Figure 2 A&B

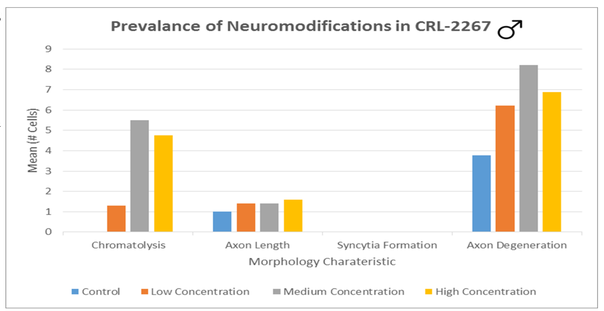


B

CRL- 2266	Chromatolysis	Axon Length	Syncytia Formation	Axonal Degeneration
Control	0.75	0.42	0	1.20
Low	0.75±0.76 P-Value: 0.0220	0.42±0.46 P-Value: 0.8138	0	1.20±1.35 P-Value: 0.0002
Medium	0.75±1.05 P-Value: 0.0817	0.42±0.48 P-Value: 0.0239	0	1.00±1.04 P-Value: 0.4122
High	0.75±1.48 P-Value: 0.1338	0.42±0.67 P-Value: 0.0355	0	13.25±1.16 P-Value: 0.1866

Figure 2 A&B represents the differences in by limonene exposure in the female cell line compared to the control fetal brain cells. The table shows P values, with significant differences (P<0.05)

Figure 3 A &B



B

CRL- 2267	Chromatolysis	Axon Length	Syncytia Formation	Axonal Degeneration
Control	0	0	0	0.97
Low	0.00±1.30 P-Value: 0.0001	0.00±0.52 P-Value: 0.0248	0	0.97±1.39 P-Value: 0.0005
Medium	0.00±1.05 P-Value: 0.0001	0.00±0.52 P-Value: 0.0248	0	0.97±1.20 P-Value: 0.0001
High	0.00±1.04 P-Value: 0.0001	0.00±0.52 P-Value: 0.0017	0	0.97±1.27 P-Value: 0.001

Figure 3 A&B represents the differences in by limonene exposure in the male cell line compared to the control fetal brain cells. The table shows P values, with significant differences (P<0.05)

Our morphologic analyses showed that exposure of developing neurons to 0.125, 0.062 and 0.032 ng/ml concentrations resulted in significant neuromodifications in both male and female neurons. Two observation were made as the concentration of limonene increased. High concentrations of limonene have been shown to have the highest degree of morphological deformities in CRL-2266. However, fewer cells were present in each chamber and more cells were found in clumps. It appears that male developing neurons were significantly more sensitive to limonene at all concentrations as compared to female developing neurons.

Conclusion

In conclusion, exposure to minuscule amounts of limonene (ranging from 0.125ng/ml to 0.032 ng/ml) resulted in significant damage to developing human brain. male and female fetal brain cell lines. Our analyses suggest that male develping brain neurons are significantly more sensitive to limonene than the female developing neurons and may explain the 1:5 male bias in ASD.

References

Sealey, L. A., Hughes, B. W., Pestaner, J. P., Steinemann, A., Pace, D. G., & Bagasra, O. 2015. Environmental factors may contribute to autism development and male bias: effects of fragrances on developing neurons. *Environmental Research, **142***, 731-738.

Gosselin, R.E., RRichards, Bryson J. "Safety and Absorption of Limonene**",** *Wellness Resource; All Health News.* October 12, 2012, January 15 2017.

Rosenberg, D. "Population Attributable Fractions for Three Perinatal Risk Factors for Autism Spectrum Disorders, 2002 and 2008 Autism and Developmental Disabilities' Monitoring Network", *National Center on Birth Defects and Developmental Disabilities;* U.S. Department of Health & Human Services. March 4, 2014, February 17, 2017

Acknowledgements

Findings reported in this publication were supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R25GM113740. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.