Electronic Cigarettes: Neurological Effects on Murine Offspring and the Response of Neuronal Cells

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Certificate of Original Authorship

I, Tara Nguyen declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Science, Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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Chen, H., Li, G., Chan, Y. L., **Nguyen, T.**, van Reyk, D., Saad, S., Oliver, B. G. Modulation of neural regulators of energy homeostasis, and of inflammation, in the pups of mice exposed to e-cigarettes. *Neuroscience Letter*. 2018, Volume: 684, Pages 61-66, https://doi.org/10.1016/j.neulet.2018.07.001.

Chen, H., Li, G., Chan, Y. L., Chapman, D. G., Sukjamnong, S., **Nguyen, T.**, Annissa, T., McGrath, K. C., Sharma, P., Oliver, B. G. Maternal E-Cigarette Exposure in Mice Alters DNA Methylation and Lung Cytokine Expression in Offspring. *Am J Respir Cell Mol Biol*. 2018, 58(3):366-377.

Conference proceedings arising from PhD research

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Australian Society for Medical Research NSW meeting 2019

Nguyen, T.; Li, G.E.; Chen, H.; Cranfield, C.G.; McGrath, K.C.; Gorrie, C.A. Switching to electronic cigarette from tobacco cigarette during pregnancy mildly improves offspring neurological outcomes. In; AMSR NSW meeting 2019; Sydney; Australia, 2019.

Australasian Neuroscience Society 2018 Conference

Nguyen, T.; Li, G.E.; Chen, H.; Cranfield, C.G.; McGrath, K.C.; Gorrie, C.A. Exposure to e-cigarette aerosols results in neurological changes in both *in vivo* and *in vitro* models. In; Australasian Neuroscience Society 2018 Conference; Brisbane; Australia, 2018.

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Abbreviations

ADHD:	Attention deficit hyperactive disorder
ANOVA:	Analysis of variance
BBB:	Blood brain barrier
BDNF:	Brain derived neurotrophic factor
BV2:	Microglial cells
CA:	Cornu ammonis
CNS:	Central nervous system
COPD:	Chronic obstructive pulmonary disease
CpG:	Cytosine-phosphate-guanine
C_T:	Threshold cycle
DCF:	2' 7'-Dichlorofluorescein
Diff-SHSY5Y:	Differentiated SHSY5Y
DMSO:	Dimethyl sulfoxide
E-cigarettes:	Electronic cigarettes
E-liquids:	Electronic liquids
ECAC:	E-cigarette aerosol condensate
ENDS:	Electronic nicotine delivery systems
EPM:	Elevated plus maze
EU:	European Union

FBS:	Foetal bovine serum
FDA:	Food and Drug Administration
FITC:	Fluorescein isothiocyanate
GABA:	Gamma-aminobutyric acid
GAP43:	Growth associated protein 43
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
H_2O_2:	Hydrogen peroxide treatment
HBEC:	Human brain microvascular endothelial cells
IBA-1:	Ionised calcium-binding adaptor molecule -1
IL-:	Interleukin
iNOS:	Inducible nitric oxide synthase
nAChR:	Nicotinic acetyl-choline receptor
NEAA:	Non-essential amino acids
NGFR:	Nerve growth factor receptor
NNK:	4-Methylnitrosamino-1-(3-pyridyl)-1-butanone
NOR:	Novel object recognition
NOS2:	Nitric oxide synthase 2
NRT:	Nicotine replacement therapies
MCP-1:	Monocyte chemoattractant protein-1
MTT:	Methylthiazolydiphenyl-tetrazolium bromide

P1:	Postnatal day 1
P20:	Postnatal day 20
PBS:	Phosphate buffer with saline
PECAM-1:	Platelet endothelial cell adhesion molecule-1
PFA:	Paraformaldehyde
PMA:	Phorbol 12-myristate 13-acetate
ROI:	Region of interest
ROS:	Reactive oxygen species
SE:	Smoke exposure
SHSY5Y:	Neuroblastoma cells
TEER:	Trans-endothelial electrical resistance
TGA:	Therapeutics Goods Administration
THP-1:	Human monocyte leukaemia
TNF-α:	Tumour necrosis factor alpha
U87MG:	Glioblastoma
UK:	United Kingdom
US:	United States
VEGF-A:	Vascular endothelial growth factor-A
ZO-1:	Zonula occludens-1

Abstract

Electronic cigarettes (e-cigarettes) are battery-powered devices that convert an oily-flavoured liquid into an aerosol. E-cigarette liquids contain propylene glycol, glycerin, flavouring and varying concentrations of nicotine. Due to aggressive marketing, e-cigarettes are attractive to a number of vulnerable groups such as young people and pregnant women. It is perceived within these populations that e-cigarettes are a safer alternative to smoking tobacco cigarettes although there is limited evidence proving this.

In this thesis, Chapter 1 provides an extensive review on what is currently known about e-cigarettes within the literature. Chapter 2 describes a mouse pregnancy model of e-cigarette exposure and examines the offspring at three time-points; postnatal day 1 (right after birth), postnatal day 20 (right after weaning) and at week 13 (adulthood). Chapter 3 describes a pregnancy model of switching from tobacco cigarette to e-cigarette exposure during pregnancy. Behavioural assessments using the novel object recognition and the elevated plus maze tests were conducted in both Chapter 2 and 3 to determine changes to short-term memory, anxiety and exploration. In addition, epigenetic changes investigating DNA methylation and epigenetic gene expression on offspring brain were investigated. Finally, Chapter 4 investigated the effects of e-cigarette condensate on differentiated neuroblastoma cells (diff-SHSY5Y), microglial (BV2) cells and human brain endothelial cells (HBEC) in monoculture and in co-culture using a blood brain barrier (BBB) model.

The results showed that offspring from mothers exposed to e-cigarette aerosols with and without nicotine had significant changes to memory, anxiety, hyperactivity, DNA methylation and epigenetic gene expression compared to normal offspring. Continuous tobacco cigarette exposure showed significant effects on offspring behaviour and epigenetics, however, switching to e-cigarettes during pregnancy reduced some of these changes but not all to normal levels. In the cell culture experiments, e-cigarette exposure on diff-SHSY5Y, BV2 and HBEC showed reduced cell-

viability and an increase in oxidative stress in monoculture. In a co-culture model of the BBB, significant epigenetic gene changes were observed in diff-SHSY5Y cells after treatment with conditioned media from BV2 cells. All of these results are summarised in Chapter 5.

In summary, the *in vivo* experiments showed that neurological changes including behavioural and epigenetics occurred in the offspring after maternal e-cigarette exposure. The *in vitro* experiments showed that this may be due to a direct effect of e-cigarette constituents on neuronal cells, or through an indirect inflammatory response involving microglia. Overall, this study concluded that e-cigarettes are not safe to be used during pregnancy.