UNIVERSITY OF CALIFORNIA
RIVERSIDE

Search for the Holy Grail of Smoking: Hidden Dangers of Electronic Nicotine Delivery Devices

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular, and Developmental Biology

by

Barbara Davis

December 2018

Dissertation Committee:
Dr. Prue Talbot, Chairperson
Dr. David Eastmond
Dr. Martin Garcia-Castro

Copyright by
Barbara Davis
2018

The Dissertation of Barbara Davis is approved:

____

____

____

Committee Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

Chapter 1: The text and figures in this chapter are a reprint of the materials as it appears in *Nicotine & Tobacco Research*, 2015;17:134–41. We are grateful for the support from the Tobacco-Related Disease Research Program of California (TRDRP), the TRDRP Cornelius Hopper Fellowship, the UCR Academic Senate, and the National Science Foundation Graduate Research Fellowship Program. We are also grateful to Dr. R. J. Talbot for his helpful suggestions on the manuscript.

Chapter 2: The text and figures in this chapter are a reprint of the materials as it appears in *Tobacco Control*, 2015;25:e67–8. We are grateful for the support from the National Institutes of Health and the National Science Foundation Graduate Research Fellowship Program.

Chapter 3: The text and figures of this chapter are a reprint of the material as it appears in *Tobacco Control* Published Online First: 13 March 2018. We are grateful for the support from the Tobacco-Related Disease Research Program of California (TRDRP). We would also like to thank Careen Khachatoorian for her help setting up the GC-MS analysis and Vivian To for her help with GC-MS analysis.

Chapter 4: We are grateful for the support from the Tobacco-Related Disease Research Program of California (TRDRP). We also thank Dr. Rachel Behar for her helpful comments and suggestions on the manuscript and Dr. Jun Li and Dr. Monique Williams their help with the statistical analyses

Chapter 5: We are grateful for the support from the Tobacco-Related Disease Research Program of California (TRDRP). We thank Vivian To for her help making the aerosol solutions.

Chapter 6: We are grateful for the support from the Tobacco-Related Disease Research Program of California (TRDRP). We would like to thank Dr. Songquin Pan from the UCR Proteomics Core Facility for his assistance with the MudPIT analysis. I would also like to thank Malcolm Tran for his assistance with bioinformatics analysis and Dr. Rachel Behar for her helpful comments.

Appendix A: The text and figures of this chapter are a reprint of the material as it appears in *Reprod Toxicol* 2012;34:529–37. We are grateful for the support from the Tobacco-Related Disease Research Program of California (TRDRP), the California Institute for Regenerative Medicine, the UCR Academic Senate, UCR Dean's Fellowship, and UCR STEM Pathways Award. We are also grateful to Dr. Evan Snyder (Stanford-Burnham Medical Institute, San Diego, CA) for providing the mNSC. We thank Monique Williams, R. Talbot, Kamlesh Asotra, and Rachel Behar for their comments on the manuscript, Laurie Graham for her assistance with equipment, Frank Monreal for help preparing Fig. 4, Crystal Hua for performing literature searching, and Jisso Kim and Michael Dang for assisting with the HPLC.

Appendix B: The text and figures of this chapter are a reprint of the material as it appears in *Toxicology In Vitro* 2014;28:198–208. We are grateful for the support from the Tobacco-Related Disease Research Program of California (TRDRP), the

California Institute for Regenerative Medicine, the TRDRP Cornelius Hopper Fellowship, UCR Dean's Fellowship, and UCR STEM Pathways Award. We thank Michael Dang and Jisoo Kim for assisting with the HPLC analysis, and Monique Williams for her comments on the manuscript. We would also like to thank the University of California, Riverside Analytical Chemical Instrumentation Facility and the Stem Cell Core for their assistance.

Appendix C: The text and figures of this chapter are a reprint of the material as it appears in *PLoS ONE* 2016;11:e0149251. We are grateful for the support from the National Institute of Health. We would like to thank An To, Ivana Villarreal, and Michael Dang for their assistance in the lab. We would also like to thank Esther Omaiye for her helpful suggestion on the manuscript and Laurie Graham for her help maintaining the smoking machine.

Appendix D: The text and figures of this chapter are a reprint of the material as it appears in *Tobacco Reg Sci* 2017;3:347–57. We are grateful for the support from the National Institute of Health, the FDA Center for Tobacco Products, and the National Science Foundation Graduate Research Fellowship Program.

This dissertation would not have been possible without the help and support of so many people. First, and foremost, I would like to thank my advisor, Dr. Prue Talbot, for giving me the opportunity to research in her lab as an undergraduate and for graciously accepting me into her lab as a graduate student. It is through her guidance that I have grown as a scientist and researcher. The things I have learned from my time in her lab are invaluable and will continue to help me as my scientific career progresses. I would also like to extend my deepest appreciation to my dissertation committee, Dr. David Eastmond and Dr. Martin Garcia-Castro for taking the time to participate on my committee, being available to help me when I needed advice, and for all of the constructive feedback and helpful tips given to me along my journey

I would also like to thank the National Science Foundation Graduate Research Fellowship Program, the Tobacco-Related Disease Research Program of California, the UCR Graduate Research Mentoring Program, and the UCR Cell, Molecular and Developmental Biology Program for providing the funding that led to the development of this research project.

I would also like to acknowledge the contributions of several other people for their invaluable help. I thank all of my co-authors and all of those collaborates who helped to make this work possible. I also thank Yu-huan Wang, Laurie Graham, Tara Pastucha, and Maggie Tello for their patience, assistance, and friendship over the years.

Lastly, I like to thank my amazing support system, without you none of this would have been possible. I am eternally grateful to Dr. Sabrina Lin for mentoring me in cell culture, without her training and expertise this dissertation would not have been possible. Moreover, I also thank her for her friendship and support. I am also extremely grateful to Dr. Matthew Collin, Dr. Devon Ehnes, Dr. Rachel Behar, Dr. Nikki Weng, Dr. Monique

Williams, and Careen Khachatoorian, without whom I would have never survived graduate school. You have all been my rocks and I can never thank you enough! Finally, I would like to thank my family, Frank, Elise and Evan, I cannot express how much your love and support have meant to me over these years. Thank you!

DEDICATION

This dissertation is dedicated to all the important people in my life that have supported me in so many ways. I cannot begin to express my gratitude to you all. Frank, I thank you for encouraging me to return to school; without your support I would not have been able to achieve the academic heights that I have. Evan and Elise, I thank you for your patience, understanding and for all the sacrifices you made to support my success, I know it wasn't and easy road, but I hope you understand that I did this to make our lives better. Devon, you have been such an amazing and supportive friend, your generous heart cannot be matched. Sabrina, not only were you my mentor and someone I have the utmost respect for and have strived to emulate, but you are an incredible person and loyal friend. Rachel, you have been my rock, without you I would not have survived the stresses of graduate school. Thank you all for putting up with my crazy, I know it wasn't easy and I love you all.

Lastly, I want to dedicate this dissertation to my grandmother, whose unconditional love, guidance, and support has helped me to become the person I am today. You saw in me something more than I ever though I could become. I have achieved successes I never thought I could, and it is because you believed in me, when no one else did, not even myself. Everything you have taught me has helped to mold me into the person you knew was deep inside, a better person than I thought I could be, a person I didn't even know could exist. My only regret is that I found her too late and that I could not share this moment with you. I love you and I miss you tremendously! Thank you!

ABSTRACT OF THE DISSERTATION

Search for the Holy Grail of Smoking:
Hidden Dangers of Electronic Nicotine Delivery Devices

by

Barbara Davis

Doctor of Philosophy, Graduate Program in Cell, Molecular, and Developmental Biology
University of California, Riverside, December 2018
Dr. Prue Talbot, Chairperson

For decades, smokers have searched for a safer alternative to cigarette smoking; to minimize smoking-related toxicant exposure and avoid illness. Recently, electronic nicotine delivery devices (ENDS), e-cigarettes and heat-not-burn systems, have emerged as a potential solution. The promise of reduced-risk advertised by ENDS has led to a surge in popularity, but the potential health hazards they present are just beginning to be elucidated. This dissertation sought to determine if the claims of harm-reduction touted were true for ENDS products. For e-cigarettes, quality control in refill fluid and Do-It-Yourself flavoring-product manufacturing was evaluated by testing the accuracy of nicotine concentration labeling and examining the presence of nicotine in flavoring products that are presumed nicotine-free. Analysis showed that a majority of refill fluids deviated by more than 10%, primarily in excess, of the label. Flavoring product testing showed the presence of nicotine in concentrations high enough to cause illness or death if accidentally ingested. IQOS, a novel heat-not-burn product, was also investigated. Testing of IQOS performance and toxicant emission from polymer-film filter

melting was conducted under two cleanliness conditions, in which the device was cleaned after every heatstick (C1) or after the 20th heatstick (C20), per the instruction manual. Performance evaluations showed IQOS is a well manufactured product. Comparisons of C1 and C20 aerosols showed that device cleanliness played a role in heat generation, leading to increased charring and polymer-film filter melting, emitting formaldehyde cyanohydrin, a deadly toxicant. Cytotoxicity testing comparing IQOS aerosols to 3R4F cigarette smoke showed that some aerosols had equivalent toxicities to smoke; and that A549, a cancer cell line, was the least sensitive to treatment. IQOS induced an epithelial-to-mesenchymal transition (EMT), the first step in cancer metastasis, in A549 cells. The EMT was characterized by decreased e-cadherin, increased vimentin, and increased cell motility. Proteomic evaluation of acute IQOS exposure showed that aerosols effected proteins involved in metabolism and respiratory diseases, and activated oxidative and inflammatory pathways. Device cleanliness affected protein expression. This investigation demonstrates that ENDS are not harm-free and emphasizes the need for further testing to accurately assess the risk these products pose.

TABLE OF CONTENTS

Introduction……………………………………………………………………………….... 1

References…………………………………………………………………………….. 26

Section I: Electronic Cigarettes………………………………………………………... 39

Chapter 1: Nicotine Concentrations in Electronic Cigarette Refill and Do-It-Yourself Fluids

Abstract…………………………………………………………………………………41

Introduction……………………………………………………………………………..42

Materials and Methods………………………………………………………………..43

Results……………………………………………………………………………….46

Discussion……………………………………………………………………………... 54

References…………………………………………………………………………….. 58

Chapter 2: Unexpected nicotine in Do-it-Yourself electronic cigarette flavourings

Research Letter…………………………………………………………………..…62

References…………………………………………………………………………….. 66

Section II: Heat-Not-Burn……………………………………………………………67

Chapter 3: iQOS: evidence of pyrolysis and release of a toxicant from plastic

Abstract……………………………………………………………………………… 69

Introduction……………………………………………………………………………..70

Materials and Methods……………………………………………………………….. 71

Results……………………………………………………………………………… 76

Discussion…………………………………………………………………………….. 87

References………………………………………………………………………….. 91

Chapter 4: Comparison of cytotoxicity of IQOS aerosols to smoke from Marlboro Red and 3R4F reference cigarettes

Abstract………………………………………………………………………………… 95

Introduction……………………………………………………………………………..96

Materials and Methods…………………………………………………………….. 98

Results………………………………………………………………..……………….101

Discussion…………………………………………………..………………………...114

References…………………………………………………..………………………..118

Chapter 5: IQOS aerosols promote an epithelial-to-mesenchymal transition in lung cancer cells

Abstract……………………………………………………………………………...………121

Introduction……………………………………………………………………...…………..122

Materials and Methods…………………………………………………………..……..123

Results……………………………………………………………………………..……….129

Discussion…………………………………………………………………………..……...137

References…………………………………………………………………………..……..140

Chapter 6: Proteomic analysis of normal human bronchial epithelial cells after IQOS aerosol exposure

Abstract……………………………………………………………………………..……145

Introduction…………………………………………………...……………………146

Materials and Methods……………………………………..……………………..147

Results…………………………………………………..…………………………152

Discussion…………………………………………………..……………………...160

References……………………………………………………………..…………..166

Conclusion…………………………………..…………..……………..………………..171

Appendix A: Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models………………………..………………………….…..…………………..175

Appendix B: Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids…………………………..…...………………………………..…..………………..185

Appendix C: Comparison of the Performance of Cartomizer Style Electronic Cigarettes from Major Tobacco and Independent Manufacturers…………..………………...……..197

Appendix D: Counterfeit Electronic Cigarette Products with Mislabeled Nicotine Concentrations……………………………………………..………………..……..212

LIST OF FIGURES

Figure I.1. Field of *Nicotiana rustica* (tobacco plants)..…………………………………..…..1

Figure I.2. Evolution of e-cigarettes…………………………………….…………….….. 15

Figure I.3. Collection of modern refill fluids……………………………….…………….….17

Figure I.4. IQOS heat-not-burn system……………………………...………………..…. 20

Figure 1.1. Examples of color variation among duplicate refill fluid pair……………….….. 53

Figure 2.1. Analysis of Tasty Puff flavorings..……………..……………………………….. 64

Figure 3.1. The I quit original smoking (iQOS) heat-not-burn system..………………..…...77

Figure 3.2. Internal view of the I quit original smoking holder. ………………………….. 79

Figure 3.3. Performance characteristics of the I quit original smoking heat-not-burn system..……………………………………………………………………………….….... 81

Figure 3.4. Charring of the tobacco plug and melting of the polymer-film filter.……..… 85

Figure 4.1. Representative concentration-response graphs for NHBE-nonsmoker treated with 30% WA…...……………………………………………………………………...…...102

Figure 4.2. LDH cytotoxicity data for IQOS and conventional cigarettes....……………...104

Figure 4.3. MTT cytotoxicity data for IQOS and conventional cigarettes…......………...107

Figure 4.4. NRU cytotoxicity data for IQOS and conventional cigarettes....…...………...110

Figure 4.5. Cell type treatment sensitivity hierarchy…………………........…………...113

Figure 5.1. Schematic of IQOS experimental timeline……………...……………........126

Figure 5.2. Cell count and morphology analysis…………………………...……………...131

Figure 5.3. Vimentin and E-cadherin expression analysis………………....…...……...133

Figure 5.4. Motility analysis……………………………………………...……………...135

Figure 6.1. Protein identification and differential protein expression in NHBE cells treated with IQOS aerosol..………………...….....……....153

Figure 6.2. Diseases and function annotations...………………………......…....……....156

Figure 6.3. IPA canonical pathway activation..……………………...…......……...…..…159

LIST OF TABLES

Table 1.1. Evaluated refill fluids. ……………………….…………………………….…...48

Table 1.2. Comparison of exact duplicate refill fluids. …….……………………….…...51

Table 3.1. Performance of iQOS heat-not-burn holders. ……………………….…... 74

INTRODUCITON

THE HISTORY OF SMOKING

Early tobacco use

While the exact dates are unknown, historians agree that tobacco had ritualistic uses as early as 3000 BC in its native North America [1]. There exist three natural varieties of tobacco, the first, *Nicotiana persica*, is native to Persia, and the other two, *Nicotiana rustica* and the most well-known *Nicotiana tabacum*, are native to North America [2]. It is thought that tobacco was first cultivated by humans in Mesoamerica

Figure I.1. Field of *Nicotiana rustica* (tobacco plants). Image downloaded from http://tns.thenews.com.pk/framework-mpowerment/#.W-eVRC2ZNTY

and South America where natives throughout the continent used it in shamanistic rituals and in medicinal applications. Ritualistically, tobacco was offered to the gods and used in religious ceremonies and was blown in the faces of warriors prior to battle and over the bodies of women prior to intercourse [3]. Medicinally, it was drunk, eaten and

chewed because of its analgesic properties. It was smeared over skin as a way to purge parasites and lice, spread over agricultural fields as an insecticide, and it was thought to cure many ailments because of its antiseptic properties [2].

Scholars believe that the practice of smoking arose from the practice of snuffing because snuff pipes are among the most common tobacco-related paraphernalia unearthed in archaeological digs [1]. Snuffing is the act of inhaling pulverized tobacco leaves into the nasal cavity. While this remained a popular method of tobacco consumption, by the time Christopher Columbus arrived in the Americas in 1492, smoking tobacco had already become the most popular method of tobacco use in both ritualistic and social applications. It was *Nicotiana tabacum* that was offered to Christopher Columbus by the natives, making him and his crew the first European smokers [1]. Tobacco was introduced in Spain around 1528, but was initially ill-received and touted as an evil and harmful practice [4]. Eventually, doctors began to recognize its "medicinal properties" and tobacco gained widespread acceptance [5].

The dawn of social tobacco use

As with most socially acceptable practices of the time, smoking didn't gain widespread popularity until it was accepted by royalty and used at court. Although tobacco was initially recognized only for medicinal purposes, and despite initial abhorrence, the English court began smoking during the reign Queen Elizabeth I and the practice was eventually adopted by the queen herself [6]. Tobacco was an expensive imported commodity that only affluent English society could afford, so the practice of smoking was initially limited to the wealthy. As the practice became more widespread,

the English began growing their own tobacco, opening smoking and snuffing to more people and solidifying it in mainstream society [7].

Tobacco made its way to other parts of Europe and Asia via Eurasian trade routes where it evolved specific cultural practices and medicinal applications in each country. It also spread to other parts of the world through colonization over the coming centuries, eventually gaining worldwide use and acceptance [8].

Early health concerns

Concern about health effects of tobacco has a long history. The earliest outspoken critic of the practice was English King James I, who condemned the practice in his 1604 polemic [9]. Shortly thereafter, the Roman Catholic Church threatened to excommunicate anyone who smoked in church [7,10]. By the 1700's, an association had been made between several cancers and tobacco use, and this observation was recapitulated from the late 1800's into the early 1920's, although this reiteration was routinely ignored. In the early 19th century, American naturalist and practitioner of botanical medicine, Gideon Lincecum, wrote that "this poisonous plant has been used a great deal as a medicine by the old school faculty, and thousands have been slain by it… it is a very dangerous article, and use it as you will, it always diminishes the vital energies in exact proportion to the quantity used - it may be slowly, but it is very sure" [11].

Despite the concerns of medical professionals, the advent of the automated cigarette-making machinery in the 1880's made it possible to mass-produce cigarettes at low cost, and smoking increased [7]. From the 1890's onwards, associations of tobacco use with cancers and vascular disease were regularly reported [12]. Before World War I,

lung cancer was classified as a rare disease that most physicians would never encounter during their career. Following the war, there was a rise in popularity of cigarette smoking, and concomitantly, an epidemic of lung cancer [13]. In 1912, American Dr. Isaac Adler was the first to advocate that lung cancer was connected with smoking [14], and in 1924, economist Irving Fisher wrote an anti-smoking article for Reader's Digest admonishing its use, calling it a poison and comparing it to opium [15]. Tobacco criticism continued in 1930, when German sociologist Fritz Lickint published a meta-analysis citing 167 other works to link tobacco use to lung cancer [12]. Lickint showed that people with lung cancer were likely to be smokers. He contended that tobacco use explained the fact that lung cancer struck men 4-5 times more often than women, who smoked much less than men, and he discussed the underlying contribution of smoking on liver and bladder cancer. Many more observational studies were published throughout the 1930's, and 1940's, but several were not in English and most of them were largely ignored [16]. However, in the 1950's, five case-control studies were published by researchers from the United States (US) and United Kingdom (UK) and these drew widespread attention [17] .These studies, which were among the first to use valid methodologies in which they excluded ex-smokers from their nonsmokers group, clearly demonstrated a close correlation between smoking and lung cancer, but received criticism for not showing causality.

The rise of public awareness to the dangers of tobacco use

Despite the early studies, the public largely ignored medical or scientific reports that correlated smoking to health concerns. However, in 1952 the very popular Reader's Digest published an article entitled "Cancer by the Carton" [18], in which they publicized

several studies that investigated the rise in incidence of the once-rare lung cancer and other cancers in response to the astronomical rise in tobacco consumption. Then, in 1953, scientists at the Sloan-Kettering Institute in New York City demonstrated experimentally that cigarette tar painted on the skin of mice caused several devastating cancers [19]. This work attracted extensive media coverage; the New York Times and Life Magazine both covered the study [18]. Then, in 1964, the United States Surgeon General published the first report on Smoking and Health, which determined, based on a massive review of 7,000 biomedical articles, that smoking was the cause of lung and pharyngeal cancer in men and women, and the primary cause of chronic bronchitis[20]. This report was one of the top-ranked news stories of the year, spending several days as headline news both in print and on television. This massive public awareness led to the Federal Cigarette Labeling and Advertising Act of 1965 and the Public Health Cigarette Smoking Act of 1969 [21].

The adverse health effects of tobacco

Over the last century, the list of health problems associated with cigarette smoking has become extensive. Health issues have been identified in Psychological, Reproductive, Immune, Oral, and Pulmonary/Cardiovascular organs, and smoking has been causatively associated with various types of cancers.

Psychological

Although surveys of smokers often report that cigarettes help relieve feelings of stress, studies demonstrated that measurable stress cues are higher in smokers than in nonsmokers, and smoking cessation leads to reduced stress [22]. Rather than as an aid

for mood control, nicotine dependency has been shown to lead to mood instability: in the daily mood patterns described by smokers, moods are normal during smoking, but worsen between cigarettes. Thus, the apparent relaxant effect of smoking only reflects the reversal of the tension and irritability that develop during nicotine depletion, meaning dependent smokers need constant nicotine to maintain mood control [23].

Reproductive

A number of studies have shown that tobacco use is a significant factor in miscarriages among pregnant smokers, and that it contributes to a number of other threats to the health of the fetus, including neural tube defects [24], and other developmental defects including cleft lip and palate. It has also been associated with preterm labor, ectopic pregnancy, stillbirth, and sudden infant death syndrome (refs). Multiple studies have demonstrated that environmental tobacco smoke also has adverse effects on developing fetuses [25]. Outside of birth defects, smoking has been shown to lead to infertility. Prolonged nicotine exposure can interfere with the body's ability to create estrogen, damaging ovaries and inhibiting folliculogenesis [26]. The degree of damage is dependent upon the amount and length of time a woman smokes, and can be irreversible [27,28]. Studies have shown that smokers are 60% more likely to be infertile than nonsmokers, and that smoking reduces the chances of successful in-vitro fertilization (IVF) by 34% and increases the risk of an IVF pregnancy miscarriage by 30%, and has also been associated with accelerated menopause [29,30].

In men, the incidence of impotence in smokers is approximately 85% higher than in nonsmokers [31]. Smoking is a key cause of erectile dysfunction as it promotes arterial

narrowing and damages the cells lining the inside of the arteries, leading to reduce penile blood flow [32]

Immune

Several correlative studies suggest that smoking can repress immunity. According to a retrospective study of 1,900 male cadets following the 1968 Hong Kong A2 influenza epidemic, heavy smokers (those who smoked more than 20 cigarettes per day) had a 21% increase in illnesses and required 20% more bed rest than nonsmokers, whereas light smokers (those who smoked less than 20 cigarettes per day) only experienced 10% increase and required 7% more bed rest [33]. A more comprehensive 1982 study of an outbreak of influenza in an Israeli military unit of 336 healthy young men revealed that out of the 168 smokers, 68.5% contracted influenza, as compared to 47.2% of nonsmokers. Symptoms were also more severe in smokers, 50.6% of them lost work days or required bed rest, compared to 30.1% of the nonsmokers [34].

Smoking is also linked to susceptibility to other infectious diseases, particularly of the lungs. Smoking more than 20 cigarettes a day increases the risk of contracting pneumonia by 2-4 times [35,36], and being a current smoker has been linked to a 4-fold increase in the risk of infection by Streptococcus pneumoniae [37]. Smokers are also susceptible for increased risk of other pulmonary and respiratory tract infections both through structural damage and through effects on the immune system. Studies have shown that smokers exhibit an increase in CD4+ cell production, which is attributable to nicotine [38]. CD4+ cells play an essential role in immunosuppressive function, thus and increase in these cells leads to decreased immunity [39]. Increase in CD4+ cell production have also been tentatively linked to increased HIV susceptibility [40].

Moreover, smoking increases the risk of Kaposi's sarcoma in people without HIV infection [41].

Oral

Smoking increases the risk for multiple oral diseases, some almost completely unique to tobacco users. The National Institutes of Health, through the National Cancer Institute, determined in 1998, that cigar smoking causes a variety of oral cancers [42], and that pipe smoking also involves significant health risks [43], particularly oral cancer. Dentists have reported that nearly half of the cases of periodontitis or inflammation around the teeth are attributed to current or former smoking, and up to 90% of patients that do not respond to traditional treatments for these illnesses are smokers. Smokeless tobacco is known to cause gingival recession and white mucosal lesions. As with smoking, up to 90% of periodontitis patients who are not helped by common modes of treatment use these products. Smokers have significantly greater loss of socket bone height than nonsmokers, and the trend can be extended to pipe smokers who have more bone loss than non-smokers [44].

Less severe oral issues associated with smoking include staining of teeth [45,46], halitosis [47], tooth loss [48], and leukoplakia, the adherent white plaques or patches on the mucous membranes of the oral cavity, including the tongue [49].

Pulmonary/Cardiovascular

Long-term exposure to compounds found in tobacco smoke (e.g., carbon monoxide and cyanide) are thought to be responsible for pulmonary damage and for loss of elasticity in the alveoli, leading to chronic bronchitis, emphysema and Chronic

Obstructive Pulmonary Disease (COPD), a permanent, incurable, often terminal reduction of pulmonary capacity characterized by shortness of breath and damage to the lungs [50,51]. Inhalation of tobacco smoke also causes several cardiac issues [52]. Tobacco use has been linked to Buerger's disease, in which the veins of the hands and feet exhibit acute inflammation and thrombosis of arteries [53]. It also leads to increase blood cholesterol levels: smokers tend to have higher levels of low-density lipoprotein (the "bad" cholesterol) compared to nonsmokers. Smokers also display higher levels of fibrinogen and increased platelet production (both involved in blood clotting) making the blood thicker and more likely to clot unnecessarily, increasing the risk of developing various forms of arteriosclerosis, heart disease, stroke, and peripheral vascular disease [54]. According to a collaborative international research study, smokers under the age of 40 are five times more likely to have a heart attack than nonsmokers [55].

Cancer

The major risk of tobacco usage includes many forms of cancer, primarily lung cancer [56], cancer of the larynx, head and neck [57], cancer of the esophagus [58], and cancers of the pancreas [59,60], stomach [61], kidney, and bladder [62]. Studies have also established a relationship between tobacco smoke, including secondhand smoke, and cervical cancer in women [63-65]. Lung cancer risk is greatly increased by smoking, causing the great majority of all lung cancer cases and increasing the risk of developing lung cancer with every year a person smokes and the number of cigarettes smoked daily [13,66,67].

THE INTRODUCTION OF HARM REDUCTION PRODUCTS

The concept of tobacco harm reduction was established in 1976 when British Psychologist Michael Russell wrote: "People smoke for nicotine but they die from the tar" [68] and suggested that the ratio of tar to nicotine could be the key to safer smoking. Since then, despite Russell's conflict of interest and collaboration with British American Tobacco, it has been widely accepted that the harm from smoking is derived almost exclusively from toxins released through the combustion of tobacco [69], leading to the development of non-combustible, pure nicotine tobacco products believed to be considerably less harmful [70-72].

In 2008, the American Association of Public Health Physicians became the first medical organization in the US to officially endorse tobacco harm reduction as a viable strategy to reduce the death toll related to cigarette smoking [69]. However, while the US accepted harm reduction products, many other parts of the world have not [73]. Debates on tobacco harm reduction tend to differ geographically due to varying legal and moral status of tobacco, as well as the different types of tobacco products and use in different cultures around the world [74]. Anti-smoking advocacy efforts and widespread popularization of the negative health effects of smoking over the past few decades have led to restrictions in the sale and use of regular tobacco products and a rise in sales of harm reduction products [75]. However, the European Union (EU), which allows traditional tobacco products, prohibits tobacco harm reduction in many EU countries because of the lack of peer-reviewed research data to support evidence-based policy making for the products [76].

Light cigarettes

Light cigarettes are a form of ventilated cigarette that purportedly causes the smoker to inhale lower levels of tar, nicotine, and other chemicals. The idea for light cigarettes came from the filters that were incorporated into cigarettes in the 1950's in response to the intensifying link between cancer and smoking [77]; however, through 50 years of repeated designs, cigarette manufactures have had trouble producing substantiated evidence that they do indeed reduce harm [78]. The belief that these innovations were harm reducing lead to an explosion in sales and they were the most popular tobacco product by the 1960's [79]. By the 1970's, "low-tar" cigarettes, which contained a measurably lower level of tar, became available. In 1976, investigators at the American Cancer Society published research concluding that light cigarettes were safer [80], writing that the "total death rates, death rates from coronary heart disease, and death rates from lung cancer were somewhat lower for those who smoked 'low' tar-nicotine cigarettes than for those who smoked 'high' tar-nicotine cigarettes." However, modern scientific evidence suggests that switching from regular to light or low-tar cigarettes does not reduce the health risks of smoking or lowers the smoker's exposure to the nicotine, tar, and carcinogens that are present in cigarette smoke [81]. This lack of reduction is due to nonlinear relationship between cigarette consumption and exposure to cigarette smoke constituents via compensatory smoking; the adjustments made to smoking patterns (i.e. inhale more deeply) by low-tar cigarette users to maintain a desired level of nicotine thus "compensating" for the reduction [82-84]. This adjustment not only increase nicotine to levels equivalent to regular-tar cigarettes but to other cigarette constituents as well, leading to no differences in cancer risk [82]. The World Health Organization subsequently recommend that misleading terms, such as "light" and

“mild”, should be removed from tobacco product advertising, packaging, and labeling [85].

Alternative nicotine delivery devices/methods (Smokeless Tobacco)

Non-electronic alternative nicotine delivery devices refer to methods of tobacco consumption that do not require the burning of tobacco, as do cigarettes, pipes, and cigars [86]. The term for this is smokeless tobacco, a broad term referring to a number of different types of tobacco products used orally or nasally. These include chewing tobaccos, dry snuff, moist snuff, and Swedish-style snus, as well as various cultural types of smokeless tobacco and newer dissolvable tobacco products [87]. Because the blanket term smokeless tobacco covers such a wide scope of products, explaining epidemiological associations between smokeless tobacco use and health becomes complicated, which had led to miscommunications in terms of the hazardous of smokeless products [88].

Snus

Scandinavian snus is a moist form of smokeless tobacco, which is usually placed under the upper lip and is not smoked or swallowed. Because of the cultural importance of snus in Sweden, snus is the only “harm-reduction” product that is legal in the EU [89]. Thus, in an effort to ease these restrictions, proposed legislation by the EU posits that Sweden, whose cigarette consumption is lower than the rest of the Union, appears to avoid smoking in favor of snus, and hence has the lowest rates of tobacco related disease in Europe [90]. However, a 2014 report commissioned by Public Health England on tobacco harm reduction said snus has a risk profile which includes possible increases

in esophageal and pancreatic cancer, and myocardial infarction, but not COPD or lung cancer, suggesting that the risk for snus tobacco is not less than cigarettes, but merely different [91]. Several subsequent peer reviewed studies have upheld this supposition [92-95].

Chewing Tobacco

One of the oldest methods of tobacco consumption, chewing tobacco is a type of smokeless tobacco product consumed by placing a portion of the tobacco between the cheek and gum or upper lip teeth then manually crushing the toabcco with the teeth to release flavor and nicotine. The resulting unwanted juices are then expectorated [96]. Chewing tobacco has been known to cause cancer, primarily of the mouth and throat [97]. As with other types of smokeless tobacco, chewing tobacco has been suggested to be used in smoking cessation programs because it partly reduces the exposure of smokers to carcinogens and the risk for cancer, but these claims have been challenged [98].

Snuff

Snuff is a smokeless tobacco made from ground or pulverized tobacco leaves. It is inhaled or "snuffed" into the nasal cavity, delivering a swift hit of nicotine and often a flavored scent. Traditionally, it is placed onto the back of the hand, pinched between thumb and index finger, or held by a "snuffing" device, then sniffed or inhaled lightly [99]. Studies relating dry snuff to cancers in the nasal mucosa are rare and not conclusive, but there are studies that show that long-term snuff users develop a form of chronic rhinitis [100].

Electronic nicotine delivery devices

Electronic cigarettes

Electronic cigarettes or e-cigarettes/e-cigs/vape were first developed in China in 2003, by a Chinese pharmacist, Hon Lik, who was motivated to create an ignition free electronic atomizing device that would mimic a cigarette. He was influenced by the death of his father who was a cigarette smoker and had succumbed to lung cancer. Lik was conducting medical research at a company called Golden Dragon Holdings that subsequently changed its name to Ruyan and became the first company to produce and sell e-cigarettes. In 2007, Ruyan received their international patent and began introducing their product to US and European markets, where sales of e-cigarettes began to boom [101-104].

E-cigarettes are electronic devices that are comprised of a battery, atomizer, and typically a cartridge or storage tank that houses fluid [105-107]; however, some current models do not use any storage vessel but rather the fluid is directly dripped onto the atomizer. The fluid solution is comprised of a solvent, propylene glycol, glycerin, or a combination of the two, and flavor chemicals [108,109]. Some fluids are nicotine-free but many contain nicotine at varying concentrations. E-cigarette aerosol is produced by drawing air through the device or by pushing a power button, which activates the battery and heats the atomizer, aerosolizing the fluid.

Since the inception of the first e-cigarette, the device has continued to evolve as its popularity has grown, becoming ever more user friendly and/or customizable. The first iteration of e-cigarettes, cig-a-likes, were made to resemble a conventional cigarette and the three basic components, battery, atomizer, cartridge, were separate entities. Quickly, the atomizer and cartridge were combined to form the “cartomizer” unit,

simplifying the design [105-107]. The next evolution was the pen style, which offered a larger more powerful battery, followed by the tank model, which employed a larger vessel for holding more fluid. This was subsequently followed by another upgrade to the battery [110] called the box-mod, this model allowed for customizing by the user, including making changes to voltage/ wattage, this was followed by the advent of the drip system, doing away with any tank/vessel and dripping the fluid directly onto the atomizing coils [111] [112]. The latest in e- cigarette evolution is the pod style, e.g. JUUL, a sleek, engineered product that resembles a USB flash-drive and utilizes single-use fluid-filled pod [113].

Figure I.2. Evolution of e-cigarettes. (from left to right) Cig-a-like models, vape pen, tank model, box-mod, pod models (JUUL). Image has been modified from the original version. Image downloaded from http://www.ansrmn.org/ecigs

Over time the device has not been the only component to evolve, the fluid used to generate aerosols has also. Early e-cigarette fluids came in pre-fill cartridges but with

the growing popularity of refillable models, the refill fluid industry was born. Refill fluids had the same basic composition of pre-fill fluids but were sold in drip-top bottles. This allowed the user to control the amount of solution dispensed as they manually added or refiled the solution of their choice. This option was also more cost effective and allowed users to compound their own fluids at home by purchasing the individual ingredients. Also, many companies allowed their users to customize every aspect of the fluid from solvent makeup, flavor choices, and nicotine concentration. However, initially, in an effort to emulate conventional cigarette use, fluids were nicotine containing and had rudimentary flavor profiles, primarily of the tobacco variety. Over time and as “vaping” culture grew among young users, the flavor profiles became ever more complex, with flavors ranging from strawberry to chocolate covered bacon. By 2014, a study by Zhu et al. [114] found that there were over 7000 different flavors of fluids. Currently, refill fluids flavors have become even more elaborate and have names that give no indication of the flavor profile at all, such as Unicorn Puke and The Mystery Flavor [115]. Another shift in fluid evolution was the decrease or complete lack of nicotine in fluids. As some e-cigarette users were previously nonsmokers, there was an interest in using the device nicotine free. Also, e-cigarettes are an attractive alternative to cigarettes for some expectant mothers, who have become one of the fastest growing user bases. Many of these women, believing e-cigarettes were harm reduction, sought to use the device as an alternative method to satisfy their nicotine needs without bringing harm to their babies or as a method of smoking cessation since refill fluids allowed them to step-down their nicotine intake gradually [116-118].

Figure I.3. Collection of modern refill fluids. Shows colorful labeling without indication of flavor profile. Image downloaded from http://www.bu.edu/research/articles/behind-the-vapor/

Since their inception, electronic cigarettes have been marketed as a smoking cessation device, delivering nicotine without the dangerous chemicals wrought in tobacco combustion [71]. Currently, there is no consensus on whether e-cigarette use aids in cigarette smoking cessation, there is data to support both sides; however, there is growing scientific evidence that they are not harm-free. New research over the last 2 years has found evidence that the heating of these unstable fluids can induce toxicity, inflammation and oxidative stress in the mothers and can accumulate in the developing fetus, affecting intrauterine development and causing respiratory and neurological disorders [119].

Their novelty means that regulation of e-cigarettes varies globally. The Institute for Global Tobacco Control (IGTC) has identified 68 countries that have laws regulating e-cigarettes, as of November 2016 [120]. Types of regulation range from complete outright prohibition on the sale and marketing of e-cigarettes (Brazil, Singapore) to prohibition on their use in enclosed public places (United States) to a simple minimum age of purchase regulation (many countries). There also exist restrictions on whether vendors are permitted to sell nicotine-containing or nicotine-free solution cartridges. The World Health Organization acknowledged that e-cigarettes may play a role in harm reduction strategies, but in light of newly published and imminent studies, should be regulated to minimize any potential risks [121].

Heated tobacco products

In 1988, R.J. Reynolds released Premier, a smokeless tobacco product that mimicked the appearance of a conventional cigarette but functioned by igniting a combustible fuel element of a carbonaceous material that would heat (without burning) tobacco saturated in glycerin to produce an aerosol [122]. In 1989, Premier was pulled from the market only to reappear in the mid 1990's as the Eclipse [123], subsequently the Eclipse was redesigned as the Revo, only to be pulled from commercial markets in 2015 [124].

In 1998, PMI launched the Accord, the predecessor to the IQOS. In 2008, the Accord we redesigned and reintroduced as the Heatbar. Accord, Heatbar and IQOS function similarly and were the first electronic heated tobacco products, employing a battery powered heating unit/holder outfitted with a flange to heat a rolled, reconstituted tobacco sheet impregnated with glycerin (called a cast-leaf), housed within a cigarette-

like heatstick that generates a nicotine containing aerosol when heated [125,126]. Prior to the IQOS, earlier iterations of heat-not-burn products were not well received, and all were discontinued.

The IQOS heat-not-burn system was successfully launched in 2014 to test markets in Japan and Italy [127]. However, recently PMI observed a 16% drop in sales in Asia [128], and although sales in Italy are still growing, they have received criticism because IQOS was marketed as a smoking cessation aid, but in the 4 years they have been sold, there has not been any significant decrease in conventional cigarette use, but rather a substantial increase in IQOS use [129].

IQOS is currently commercially available in 43 countries but is not available for purchase in the US [130,131]. In December 2016, PMI filed an application with the US the Food and Drug Administration (FDA) to market IQOS as a modified risk tobacco product (MRTP) [129,132,133]. In January 2018, the FDA held a meeting of the Tobacco Products Scientific Advisory Committee (TPSAC) during which they acknowledge that IQOS studies showed a significant reduction in exposure to harmful or potentially harmful chemicals (HPHC); however, they did not feel this reduction was sufficient "to have a measurable and substantial reduction in morbidity and/or mortality", and thus voted to deny their application to market IQOS as a MRTP and continued the ban IQOS sales in the US [132,133].

Figure I.4. IQOS heat-not-burn system. Shows a carton of Marlboro iQOS heatsticks, a pack of heatsticks, three individual heatsticks, two IQOS chargers and Holders. The black? charger is shown with the lid closed, the black? blue Holder is shown with a heatstick inserted, and the white Holder is placed inside the white charger (lid open).

Concerns surrounding "safe" nicotine and tobacco products

Smoking has become socially unacceptable over recent years in many countries, a result of a repeatedly published scientific evidence and public awareness campaigns, but this has led to a rise in the use of "harm-reduction" products, and thus a host of new public health concerns [88,134]. As discussed in previous sections, research has shown that most harm reduction products (light cigarettes and chewing tobacco/snus) parallel the health problems of cigarettes, leading to other types of orofacial cancers, dental issues, and other health concerns [42,92-94,97,98]. The newest harm-reduction products, e-cigarettes debuted to rave reviews for their potential as a smoking cessation device and complete lack of tobacco, which has been publicly accepted as the causative culprit for the aforementioned health problems. Public Health Officials are concerned that with the popularization of e-cigarettes, there will be a renormalization of smoking, for example through their use in locations where conventional cigarettes are prohibited, ultimately leading to an unintended increase in use of conventional tobacco products by nonsmokers. In fact, this been demonstrated to occur with e-cigarettes [135-137], and has already begun to occur with the IQOS heat-not-burn system. In just 4 years since its debut, polls indicated that in Italy 51% of IQOS consumers were previously nonsmokers, introducing a substantial new population to addictive nicotine levels on par with conventional cigarettes [138].

More pressing than social concerns are the latest publications that suggest heating of refill fluids can have physical ramifications in the body of the user. Nicotine delivery has increased with e-cigarette evolutions [139], and it has been suggested that repeated use of larger battery/ more powerful devices can result in continual venous blood levels of nicotine equal to those of cigarette smokers [140]. Although nicotine has

been touted as a safe alternative to tobacco, various studies have demonstrated that nicotine, independent of other chemical found in cigarettes, affects cell proliferation, increases oxidative stress, apoptosis, and promotes DNA mutation by various mechanisms, leading to cancer [141]. It can also affect tumor proliferation and metastasis and can cause resistance to chemo- and radio-therapeutic agents [142]. Studies in which animals were exposed to vaporized refill fluids provided evidence that vapor inhaled from e-cigarettes promotes an inflammatory response in the lungs (refs). This response is more pronounced in chemically flavored refill fluids, compared to unflavored ones, suggesting that prolonged vaping can cause irreversible damage to the lung tissue and can increase pulmonary susceptibility to viral infection [143]. Moreover, dental studies have shown that people who vape exhibit increased levels of pro-inflammatory cytokines, leading to increased peri-implant complications. Cell studies have mirrored *in vivo* studies, demonstrating decreased cell viability and increased inflammation [144-146].

Another concern with electronic cigarettes is that they are a common alternative choice for pregnant smokers. Despite e-cigarette's lack of extraneous chemicals that are commonly found in tobacco, multiple animal studies have demonstrated that nicotine is a developmental neurotoxin [147-151] and is responsible for many of the health consequences associated with *in utero* tobacco smoke exposure, including heart and craniofacial defects [152,153]. Recent studies have demonstrated that exposing embryos to aerosols caused developmental defects including heart deformities [154] and some craniofacial defects [155]. Animal studies have also been done to determine whether nicotine-free vape solutions were safe. In these studies, embryos (e.g. mouse and monkey) exposed to the nicotine-free e-cigarette solutions produced the same

results, indicating that either the solution components alone, or the heating of these components is teratogenic [156].

While most of the data currently available on electronic nicotine delivery systems is deals with electronic cigarettes (due to the length of time of their commercial availability), more data are becoming available for the IQOS system, some of these data challenge the claim of harm reduction. The new data, pioneered to a great extent by researchers from the University of California San Francisco, contradicts PMI's internal data that touts its safety and efficacy in smoking cessation.

The Gotts research group at the University of California, San Francisco, (UCSF) conducted an independent review of PMI's internal preclinical trial data revealing a different story than the one they flaunt publicly. Their own internal data suggested that the usage of the IQOS system presented a new tobacco related complication: hepatotoxicity [157]. Preclinical studies demonstrated that after only 5 days of use in human subjects, plasma bilirubin was elevated by 8.8% compared with 0% elevation in conventional cigarette smokers, suggesting cholestatic liver injury. Ninety-day studies in male and female Sprague-Dawley rats also showed that rats exposed to IQOS aerosols had increased liver weights, a symptom of hepatocellular hypertrophy. Measures of blood levels of alanine aminotransferase, an enzyme released into the blood by hepatocytes during hepatocellular injury, were significantly higher with IQOS than with conventional cigarettes in female animals. Moreover, livers of IQOS rats exhibited hepatocellular vacuolization, a sign of acute liver injury and an effect not seen in cigarette-exposed animals. Aside from liberally interpreting data to fit a desirable outcome, former employees of PMI have criticized their research methods. In an interview with Reuters, former IQOS preclinical trial coordinator Tamara Koval stated

that when she pointed out irregularities in the study, she was simply excluded from meetings. Interviews with principal investigators revealed issues with basic scientific technique. Regrettably, this is not the first time PMI's internal research has been brought into question. A former FDA commissioner was quoted as saying "Taken as a whole, it's clear they do not have the sophistication to carry out adequate and well-controlled clinical trials," which highlights the need for external research to authenticate PMI's claims [158].

In addition to this analysis, Moazed et al. also reviewed PMI's data for anomalies with pulmonary health and found evidence that IQOS usage is associated with significant pulmonary and immunomodulatory toxicities [159], and that there was no detectable differences between conventional cigarette smokers and those who switched to IQOS, challenging PMI's claim that their device is less harmful than conventional cigarettes. Moazed et al. also noted that PMI did not conduct studies to examine the impact of dual use (both IQOS and cigarettes) or secondhand aerosol exposure, highlighting the inadequacy and bias of their studies.

A second research group at UCSF conducted independent research to assess the effect of IQOS aerosols on vascular endothelial function and found that exposure to these aerosols produces vascular damage on par with conventional cigarettes, and effects were observed at a lower dose, demonstrating that IQOS products will expose users to similar cardiovascular risks [160]. These results were true for IQOS systems from three different countries (each of which have different tobacco regulations), suggesting that this problem is related to a core aspect of this device.

PURPOSE OF THE DISSERTATION

Since the popularization of tobacco products in Europe, humans have been entangled in a cyclical back-and-forth where the tobacco industry debuts a new, popular product, medical and research professionals point out their danger, and the tobacco industry develops a new “safer” product that is subsequently disavowed. Increasing data are available to demonstrate that electronic cigarettes are not harm-free. The novelty of the IQOS system combined with independent reevaluation of PMI’s preclinical data demonstrate that this product can lead to physiological changes and suggest that history is about to repeat itself.

The research presented here aims to expand upon existing research that demonstrates that the electronic nicotine delivery devices, e-cigarettes and particularly the new IQOS system are not harm free products. This study addresses both electronic cigarettes and IQOS products and provides new data showing that: (1) nicotine concentration labeling on electronic cigarette refill products is often inaccurate and misleading; (2) refill fluids and Do-it-Yourself (DIY) products require stricter quality control guidelines/oversight due to presence of nicotine in DIY electronic cigarette flavorings; (3) performance of IQOS heat-not-burn devices is affected by cleanliness, and use leads to heat generation and polymer-film filter melting, as well as emission of formaldehyde cyanohydrin from heated polymer-film filters; (4) IQOS aerosols have equivalent cytotoxicity to 3R4F reference cigarettes under certain conditions of testing; (5) IQOS exposure induced an epithelial-to-mesenchymal transition in primary lung carcinoma cells in vitro; and (6) the proteome of normal human bronchial epithelial cells from a nonsmoker is altered by exposure to IQOS aerosol. .

References

1 Musk AW, De Klerk NH. History of tobacco and health. *Respirology* 2003;8:286–90.

2 Gately I. *Tobacco*. 1st ed. Grove Press 2001.

3 Wilbert J. *Tobacco and Shamanism in South America*. Yale University Press 1993.

4 Fairholt FW. *Tobacco: Its history and Associations*. Chapman and Hall 1859.

5 Fairholt FW. *Tobacco: Its History and Associations*. Chapman and Hall 1859.

6 Gershon L. A Brief History of Tobacco in America. 2016.

7 Gilman SL, Zhou X, Robicsek F. *Smoke*. Reaktion Books 2004.

8 Romaniello M. Transregional Trade in Early Modern Eurasia. *Oxford Research Encyclopedias* Published Online First: 2017. doi:10.1093/acrefore/9780190277727.013.296

9 Milne I. A counterblaste to tobacco: King James's anti-smoking tract of 1616. *Journal of the Royal College of Physicians of Edinburgh* 2011;41:89–9. doi:10.4997/JRCPE.2011.118

10 Radke PW, Schunkert H. Public smoking ban: Europe on the move. *European Heart Journal* 2006;27:2385–6. doi:10.1093/eurheartj/ehl266

11 Lincecum G. Nicotiana tabacum. w3.biosci.utexas.edu. http://w3.biosci.utexas.edu/prc/lincecum/pages/Nicotiana_tabacum-notes.html (accessed 8 Nov2018).

12 Proctor RN. *International Journal of Epidemiology* 2001;30:31–4. doi:10.1093/ije/30.1.31

13 Spiro SG, Silvestri GA. One Hundred Years of Lung Cancer. *Am J Respir Crit Care Med* 2005;172:523–9. doi:10.1164/rccm.200504-531oe

14 Ruegg TA. Historical Perspectives of the Causation of Lung Cancer. *Global Qualitative Nursing Research* 2015;2:233339361558597. doi:10.1177/2333393615585972

15 Fisher I. Does Tobacco Injure the Human Body? 1924.

16 Doll R. Uncovering the effects of smoking: historical perspective. *Statistical Methods in Medical Research* 1998;7:87–117. doi:10.1191/096228098668199908

17 Michaels D. *Doubt Is Their Product: How Industry's Assault on Science Threatens Your Health*. Oxford University Press 2008.

18 Oreskes N, Conway EM. *Merchants of doubt*. Bloomsbury 2012.

19 Graham EA, Croninger AB, Wynder EL. Experimental production of carcinoma with cigarette tar.III. Occurrence of cancer after prolonged latent period following application of tar. *Cancer* 1957;10:431–6. doi:10.1002/1097-0142(195705/06)10:3<431::aid-cncr2820100303>3.0.co;2-q

20 WELLMANN KF. [SMOKING AND HEALTH. ON THE REPORT OF THE ADVISORY COMMITTEE TO THE SURGEON GENERAL OF THE PUBLIC HEALTH SERVICE]. *Dtsch Med Wochenschr* 1964;89:1085–6.

21 Warner KE. *American Journal of Public Health* 2014;104:5–8. doi:10.2105/ajph.2013.301722

22 Parrott AC. Does cigarette smoking cause stress? *American Psychologist* 1999;54:817–20. doi:10.1037/0003-066x.54.10.817

23 Cohen S, Lichtenstein E. Perceived stress, quitting smoking, and smoking relapse. *Health Psychology* 1990;9:466–78. doi:10.1037/0278-6133.9.4.466

24 Wang M, Wang Z-P, Gong R, *et al.* Maternal smoking during pregnancy and neural tube defects in offspring: a meta-analysis. *Childs Nerv Syst* 2013;30:83–9. doi:10.1007/s00381-013-2194-5

25 Ward C, Lewis S, Coleman T. Prevalence of maternal smoking and environmental tobacco smoke exposure during pregnancy and impact on birth weight: retrospective study using Millennium Cohort. *BMC Public Health* 2007;7. doi:10.1186/1471-2458-7-81

26 Dechanet C, reproduction TAH, 2010. Effects of cigarette smoking on reproduction. *academicoupcom*

27 Fredricsson B, Gilwam H. Smoking and reproduction: Short and long term effects and benefits of smoking cessation. *Acta Obstetricia et Gynecologica Scandinavica* 1992;71:580–92. doi:10.3109/00016349209006225

28 Sapra KJ, Barr DB, Tobacco JMN, *et al.* Time-to-Pregnancy Associated With Couples' Use of Tobacco Products. *academicoupcom*

29 Hyland A, Piazza K, Hovey KM, *et al.* Associations between lifetime tobacco exposure with infertility and age at natural menopause: the Women's Health Initiative Observational Study. *Tobacco Control* 2015;25:706–14. doi:10.1136/tobaccocontrol-2015-052510

30 Soares SR, and MMCOIO, 2008. Cigarette smoking and reproductive function. *journalslwwcom*

31 Harlev A, Agarwal A, of SGTWJ, *et al.* Smoking and male infertility: an evidence-based review. *synapsekoreamedorg*

32 Qiao Z-D, Dai J-B, Wang Z-X. The hazardous effects of tobacco smoking on male fertility. *Asian Journal of Andrology* 2015;17:954. doi:10.4103/1008-682x.150847

33 FINKLEA JF, SANDIFER SH, SMITH DD. CIGARETTE SMOKING AND EPIDEMIC INFLUENZA12. *American Journal of Epidemiology* 1969;90:390–9. doi:10.1093/oxfordjournals.aje.a121084

34 Kark JD, Lebiush M, Rannon L. Cigarette smoking as a risk factor for epidemic a(h1n1) influenza in young men. *New England Journal of Medicine* 1982;307:1042–6. doi:10.1056/NEJM198210213071702

35 Davies PDO, Yew WW, Ganguly D, *et al.* Smoking and tuberculosis: the epidemiological association and immunopathogenesis. *Trans R Soc Trop Med Hyg* 2006;100:291–8. doi:10.1016/j.trstmh.2005.06.034

36 Jha P, Jacob B, Gajalakshmi V, *et al.* A nationally representative case-control study of smoking and death in India. *New England Journal of Medicine* 2008;358:1137–47. doi:10.1056/NEJMsa0707719

37 Nuorti JP, Butler JC, Farley MM, *et al.* Cigarette smoking and invasive pneumococcal disease. Active Bacterial Core Surveillance Team. *New England Journal of Medicine* 2000;342:681–9. doi:10.1056/NEJM200003093421002

38 Nordman JC, Muldoon P, Clark S, *et al.* The α4 nicotinic receptor promotes CD4+ T-cell proliferation and a helper T-cell immune response. *Mol Pharmacol* 2014;85:50–61. doi:10.1124/mol.113.088484

39 Wang D-W, Zhou R-B, Yao Y-M, *et al.* Stimulation of α7 nicotinic acetylcholine receptor by nicotine increases suppressive capacity of naturally occurring CD4+CD25+ regulatory T cells in mice in vitro. *J Pharmacol Exp Ther* 2010;335:553–61. doi:10.1124/jpet.110.169961

40 Arcavi L, Benowitz NL. Cigarette Smoking and Infection. *Archives of Internal Medicine* 2004;164:2206. doi:10.1001/archinte.164.20.2206

41 Goedert JJ. Risk Factors for Classical Kaposi's Sarcoma. *CancerSpectrum Knowledge Environment* 2002;94:1712–8. doi:10.1093/jnci/94.22.1712

42 Chang CM, Corey CG, Rostron BL, *et al.* Systematic review of cigar smoking and all cause and smoking related mortality. *BMC Public Health* 2015;15:390. doi:10.1186/s12889-015-1617-5

43 Henley SJ, Thun MJ, Chao A, *et al.* Association Between Exclusive Pipe Smoking and Mortality From Cancer and Other Diseases. *JNCI Journal of the National Cancer Institute* 2004;96:853–61. doi:10.1093/jnci/djh144

44 Johnson GK, Education NSJOD, 2001. Impact of tobacco use on periodontal status. *Am Dental Educ Assoc*

45 Reibel J. Tobacco and oral diseases. Update on the evidence, with recommendations. *Med Princ Pract* 2003;12 Suppl 1:22–32. doi:10.1159/000069845

46 Ness L, Rosekrans D de L, Welford JF. An epidemiologic study of factors affecting extrinsic staining of teeth in an English population. *Community Dent Oral Epidemiol* 1977;5:55–60.

47 Zalewska A, Zatoński M, Jabłonka-Strom A, *et al.* Halitosis--a common medical and social problem. A review on pathology, diagnosis and treatment. *Acta Gastroenterol Belg* 2012;75:300–9.

48 Qureshi FH, Hamid S, Khan SM, *et al.* Effect of tobacco use on tooth loss among patients visiting the out-patient dental department of a tertiary care hospital in Pakistan. *J Pak Med Assoc* 2018;68:841–7.

49 Mecklenburg RE. *Tobacco effects in the mouth*. 2004.

50 Devereux G. Definition, epidemiology, and risk factors. *BMJ* 2006;332:1142–4. doi:10.1136/bmj.332.7550.1142

51 Facchinetti F, Amadei F, Geppetti P, *et al.* Alpha,beta-unsaturated aldehydes in cigarette smoke release inflammatory mediators from human macrophages. *Am J Respir Cell Mol Biol* 2007;37:617–23. doi:10.1165/rcmb.2007-0130OC

52 Glantz SA. Passive Smoking and Heart Disease. *JAMA* 1995;273:1047. doi:10.1001/jama.1995.03520370089043

53 Weisman MH. Lung Disease in Rheumatic Diseases. *Rheumatic Disease Clinics of North America* 2015;41:xi. doi:10.1016/j.rdc.2015.03.002

54 Shah RS, Cole JW. Smoking and stroke: the more you smoke the more you stroke. *Expert Review of Cardiovascular Therapy* 2010;8:917–32. doi:10.1586/erc.10.56

55 Mahonen MS. Current smoking and the risk of non-fatal myocardial infarction in the WHO MONICA Project populations. *Tobacco Control* 2004;13:244–50. doi:10.1136/tc.2003.003269

56 Thun MJ, Day-Lally CA, Calle EE, *et al.* Excess mortality among cigarette smokers: changes in a 20-year interval. *American Journal of Public Health* 1995;85:1223–30. doi:10.2105/AJPH.85.9.1223

57 Law MR, Morris JK, Wald NJ. Environmental tobacco smoke exposure and ischaemic heart disease: an evaluation of the evidence. *BMJ* 1997;315:973–80. doi:10.1136/bmj.315.7114.973

58 Yuan J-M, Knezevich AD, Wang R, *et al.* Urinary levels of the tobacco-specific carcinogen N'-nitrosonornicotine and its glucuronide are strongly associated with esophageal cancer risk in smokers. *Carcinogenesis* 2011;32:1366–71. doi:10.1093/carcin/bgr125

59 Midha S, Chawla S, Garg PK. Modifiable and non-modifiable risk factors for pancreatic cancer: A review. *Cancer Lett* 2016;381:269–77. doi:10.1016/j.canlet.2016.07.022

60 Early D. Smoking is bad for your colon. *Gastrointestinal Endoscopy* 2010;71:1241–3. doi:10.1016/j.gie.2010.01.032

61 Chao A, Thun MJ, Henley SJ, *et al.* Cigarette smoking, use of other tobacco products and stomach cancer mortality in US adults: The Cancer Prevention Study II. *Wiley Online Library*

62 Scélo G, Urology PBNR, 2007. The epidemiology of bladder and kidney cancer. *naturecom*

63 Tay S-K, Tay K-J. Passive cigarette smoking is a risk factor in cervical neoplasia. *Gynecol Oncol* 2004;93:116–20. doi:10.1016/j.ygyno.2003.12.032

64 Trimble CL, Genkinger JM, Burke AE, *et al.* Active and passive cigarette smoking and the risk of cervical neoplasia. *Obstet Gynecol* 2005;105:174–81. doi:10.1097/01.AOG.0000148268.43584.03

65 Wu M-T, Lee L-H, Ho C-K, *et al.* Lifetime exposure to environmental tobacco smoke and cervical intraepithelial neoplasms among nonsmoking Taiwanese women. *Archives of Environmental Health* 2003;58:353–9.

66 Rachtan J. Smoking, passive smoking and lung cancer cell types among women in Poland. *Lung Cancer* 2002;35:129–36. doi:10.1016/S0169-5002(01)00330-0

67 Smith RA, Andrews KS, Cancer DBCA, *et al.* Cancer screening in the United States, 2018: A review of current American Cancer Society guidelines and current issues in cancer screening. *Wiley Online Library*

68 Russell MA. Low-tar medium-nicotine cigarettes: a new approach to safer smoking. *BMJ* 1976;1:1430–3. doi:10.1136/bmj.1.6023.1430

69 Rodu B. The scientific foundation for tobacco harm reduction, 2006-2011. *Harm Reduct J* 2011;8:19. doi:10.1186/1477-7517-8-19

70 Nitzkin J. The Case in Favor of E-Cigarettes for Tobacco Harm Reduction. *International Journal of Environmental Research and Public Health* 2014;11:6459–71. doi:10.3390/ijerph110606459

71 Fagerström KO, Bridgman K. Tobacco harm reduction: the need for new products that can compete with cigarettes. *Addict Behav* 2014;39:507–11. doi:10.1016/j.addbeh.2013.11.002

72 Rodu B. *Tobacco harm-reduction strategies*. Unitec House, 2 Albert Place, London N3 1QB, UK: : Future Medicine Ltd 2013. doi:10.2217/ebo.13.3

73 Cox S, Dawkins L. Global and local perspectives on tobacco harm reduction: what are the issues and where do we go from here? *Harm Reduct J* 2018;15:569. doi:10.1186/s12954-018-0239-5

74 Mukherjea A, Morgan PA, Snowden LR, *et al.* Social and cultural influences on tobacco-related health disparities among South Asians in the USA. *Tobacco Control* 2012;21:422–8. doi:10.1136/tc.2010.042309

75 Martin EG, Warner KE, Lantz PM. Tobacco harm reduction: what do the experts think? *Tobacco Control* 2004;13:123–8.

76 Bates C. European Union policy on smokeless tobacco: a statement in favour of evidence based regulation for public health. *Tobacco Control* 2003;12:360–7. doi:10.1136/tc.12.4.360

77 Harris B. The intractable cigarette 'filter problem'. *Tobacco Control* 2011;20 Suppl 1:i10–6. doi:10.1136/tc.2010.040113

78 Rigotti NA, Tindle HA. The fallacy of “light” cigarettes. *BMJ* 2004;328:E278–9. doi:10.1136/bmj.328.7440.E278

79 Lancet MRT, 1974. Realistic goals for smoking and health A case for safer smoking. *Elsevier*

80 Hammond EC, Garfinkel L, Seidman H, *et al.* “Tar” and nicotine content of cigarette smoke in relation to death rates. *Environmental Research* 1976;12:263–74.

81 Benowitz NL, Jacob P, Bernert JT, *et al.* Carcinogen exposure during short-term switching from regular to “light” cigarettes. *Cancer Epidemiology Biomarkers & Prevention* 2005;14:1376–83. doi:10.1158/1055-9965.EPI-04-0667

82 Services USDOHAH. The Health Consequences of Smoking - 50 Years of Progress: A Report of the Surgeon General. 2014;:1–1081.

83 Kozlowski LT, method JPTFCT, 1996. Compensation for nicotine by smokers of lower yield cigarettes. In: *Smoking and Tobacco Control Monograph No.* cancercontrol.cancer.gov

84 Frost C, Fullerton FM, Stephen AM, *et al.* The tar reduction study: randomised trial of the effect of cigarette tar yield reduction on compensatory smoking. *Thoraxbmjcom*.doi:10.1136/thx.50.10.1038

85 World Health Organization. *WHO report on the global tobacco epidemic, 2013: enforcing bans on tobacco advertising, promotion and sponsorship*. 2013.

86 Kunze U, Klinische RSW, 1998. Alternative nicotine delivery systems (ANDS)--public health-aspects. *europepmcorg*

87 Stanisce L, Levin K, Ahmad N, *et al.* Reviewing smokeless tobacco epidemiology, carcinogenesis, and cessation strategy for otolaryngologists. *Laryngoscope* 2018;128:2067–71. doi:10.1002/lary.27104

88 Savitz DA, Meyer RE, Tanzer JM, *et al.* Public health implications of smokeless tobacco use as a harm reduction strategy. *American Journal of Public Health* 2006;96:1934–9. doi:10.2105/AJPH.2005.075499

89 Lancet NGT, 2005. Mixed feelings on snus. *thelancetcom*

90 Lund I, Scheffels J. Perceptions of the relative harmfulness of snus among Norwegian general practitioners and their effect on the tendency to recommend snus in smoking cessation. *Nicotine Tob Res* 2012;14:169–75. doi:10.1093/ntr/ntr159

91 Britton J, England IBPH, 2014. *Electronic cigarettes: A report commissioned by Public Health England*.

92 Byhamre ML, Gustafsson PE, Jansson J-H, *et al.* Snus use during the life-course and risk of the metabolic syndrome and its components. *Scand J Public Health* 2017;45:733–40. doi:10.1177/1403494817706631

93 Yang F, Pedersen NL, Ye W, *et al.* Moist smokeless tobacco (Snus) use and risk of Parkinson's disease. *International Journal of Epidemiology* 2017;46:872–80. doi:10.1093/ije/dyw294

94 Araghi M, Galanti MR, Lundberg M, *et al.* Smokeless tobacco (snus) use and colorectal cancer incidence and survival: Results from nine pooled cohorts. *Scand J Public Health* 2017;45:741–8. doi:10.1177/1403494817714191

95 Araghi M, Rosaria Galanti M, Lundberg M, *et al.* Use of moist oral snuff (snus) and pancreatic cancer: Pooled analysis of nine prospective observational studies. *Int J Cancer* 2017;141:687–93. doi:10.1002/ijc.30773

96 Christen AG, Glover ED. History of Smokeless Tobacco Use in the United States. *Health Education* 2013;18:6–13. doi:10.1080/00970050.1987.10614497

97 Moore GE, American LBJOT, 1953. Intraoral cancer and the use of chewing tobacco. *Wiley Online Library*

98 World Health Organization… - … some tobacco-specific …, 2007. Smokeless tobacco and some tobacco-specific N-Nitrosamines. *cabdirectorg*

99 Blyth H. A history of snuff and tobacco. *Notes and Queries* 1875;s5-IV:67–7. doi:10.1093/nq/s5-IV.82.67b

100 Khan SM, Nasreen S, Zai S. Naswar (snuff) dipping and oral cancer in north-west Pakistan. In: *Tobacco: The Growing Epidemic*. London: : Springer London 2000. 104–4. doi:10.1007/978-1-4471-0769-9_38

101 Sleight VJ. A Brief History of the Electronic Cigarette. *Journal of Lung, Pulmonary & Respiratory Research* 2016;3. doi:10.15406/jlprr.2016.03.00097

102 Ayers JW, Ribisl KM, Brownstein JS. Tracking the Rise in Popularity of Electronic Nicotine Delivery Systems (Electronic Cigarettes) Using Search Query Surveillance. *AMEPRE* 2011;40:448–53. doi:10.1016/j.amepre.2010.12.007

103 Etter J-F, Bullen C. Electronic cigarette: users profile, utilization, satisfaction and perceived efficacy. *Addiction* 2011;106:2017–28. doi:10.1111/j.1360-0443.2011.03505.x

104 McQueen A, Tower S, Sumner W. Interviews with “vapers”: implications for future research with electronic cigarettes. *Nicotine Tob Res* 2011;13:860–7. doi:10.1093/ntr/ntr088

105 Trtchounian A, Talbot P. Electronic nicotine delivery systems: is there a need for regulation? *Tobacco Control* 2011;20:47–52. doi:10.1136/tc.2010.037259

106 Bahl V, Lin S, Xu N, *et al.* Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models. *Reprod Toxicol* 2012;34:529–37. doi:10.1016/j.reprotox.2012.08.001

107 Williams M, Talbot P. Variability among electronic cigarettes in the pressure drop, airflow rate, and aerosol production. *Nicotine Tob Res* 2011;13:1276–83. doi:10.1093/ntr/ntr164

108 National Academies of Sciences, Engineering, and Medicine, Health and Medicine Division, Board on Population Health and Public Health Practice, *et al.* Public Health Consequences of E-Cigarettes. Published Online First: 23 January 2018. doi:10.17226/24952

109 Hahn J, Monakhova YB, Hengen J, *et al.* Electronic cigarettes: overview of chemical composition and exposure estimation. 2014;12:607–12. doi:10.1186/s12971-014-0023-6

110 Cantrell J, Huang J, Greenberg M, *et al.* History and Current Trends in the Electronic Nicotine Delivery Systems Retail Marketplace in the United States: 2010-2016. *Nicotine Tob Res* 2018;25:e16. doi:10.1093/ntr/nty214

111 Geiss O, Bianchi I, Barrero-Moreno J. Correlation of volatile carbonyl yields emitted by e-cigarettes with the temperature of the heating coil and the perceived sensorial quality of the generated vapours. *Int J Hyg Environ Health* 2016;219:268–77. doi:10.1016/j.ijheh.2016.01.004

112 Wagener TL, Floyd EL, Stepanov I, *et al.* Have combustible cigarettes met their match? The nicotine delivery profiles and harmful constituent exposures of second-generation and third-generation electronic cigarette users. *Tobacco Control* 2017;26:e23–8. doi:10.1136/tobaccocontrol-2016-053041

113 Willett JG, Bennett M, Hair EC, *et al.* Recognition, use and perceptions of JUUL among youth and young adults. *Tobacco Control* Published Online First: 18 April 2018. doi:10.1136/tobaccocontrol-2018-054273

114 Zhu S-H, Sun JY, Bonnevie E, *et al.* Four hundred and sixty brands of e-cigarettes and counting: implications for product regulation. *Tobacco Control* 2014;23 Suppl 3:iii3–9. doi:10.1136/tobaccocontrol-2014-051670

115 Hsu G, Sun JY, Zhu S-H. Evolution of Electronic Cigarette Brands From 2013-2014 to 2016-2017: Analysis of Brand Websites. *J Med Internet Res* 2018;20:e80. doi:10.2196/jmir.8550

116 Baeza-Loya S, Viswanath H, Carter A, *et al.* Perceptions about e-cigarette safety may lead to e-smoking during pregnancy. *Bull Menninger Clin* 2014;78:243–52. doi:10.1521/bumc.2014.78.3.243

117 Farquhar B, Mark K, Terplan M, *et al.* Demystifying electronic cigarette use in pregnancy. *Journal of Addiction Medicine* 2015;9:157–8. doi:10.1097/ADM.0000000000000100

118 Mark KS, Farquhar B, Chisolm MS, *et al.* Knowledge, Attitudes, and Practice of Electronic Cigarette Use Among Pregnant Women. *Journal of Addiction Medicine* Published Online First: 13 May 2015. doi:10.1097/ADM.0000000000000128

119 Whittington JR, Simmons PM, Phillips AM, *et al.* The Use of Electronic Cigarettes in Pregnancy: A Review of the Literature. *Obstet Gynecol Surv* 2018;73:544–9. doi:10.1097/OGX.0000000000000595

120 Feldman EA. Regulating E-Cigarettes: Why Policies Diverge. *SSRN Electronic Journal* Published Online First: 2018. doi:10.2139/ssrn.3236410

121 Chang AY, Barry M. The Global Health Implications of e-Cigarettes. *JAMA* 2015;314:663–4. doi:10.1001/jama.2015.8676

122 R. J. Reynolds Tobacco Company, editor. United States Patent Application Publication Pub.No.: US2017/0164654 A1. 2017;:A24F47/00–22.

123 Anderson SJ, Ling PM. “And they told two friends...and so on”: RJ Reynolds' viral marketing of Eclipse and its potential to mislead the public. *Tobacco Control* 2008;17:222–9. doi:10.1136/tc.2007.024273

124 Reynolds ends Revo test market in Wisconsin. Winston-Salem Journal. https://www.journalnow.com/business/business_news/local/reynolds-ends-revo-test-market-in-wisconsin/article_e23495d0-353e-11e5-98d3-cbaf921dc91c.html (accessed 8 Nov2018).

125 Elias J, Dutra LM, St Helen G, *et al.* Revolution or redux? Assessing IQOS through a precursor product. *Tobacco Control* 2018;27:s102–10. doi:10.1136/tobaccocontrol-2018-054327

126 Smith MR, Clark B, Lüdicke F, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 1: Description of the system and the scientific assessment program. *Regul Toxicol Pharmacol* 2016;81:S17–S26. doi:10.1016/j.yrtph.2016.07.006

127 Hair EC, Bennett M, Sheen E, *et al.* Examining perceptions about IQOS heated tobacco product: consumer studies in Japan and Switzerland. *Tobacco Control* Published Online First: 15 May 2018. doi:10.1136/tobaccocontrol-2018-054322

128 LaVito A. Philip Morris has worst day in a decade as iQOS growth in Japan “plateaus.” 2018.

129 St Helen G, Jacob P III, Nardone N, *et al.* IQOS: examination of Philip Morris International’s claim of reduced exposure. *Tobacco Control* 2018;:tobaccocontrol–2018–054321–7. doi:10.1136/tobaccocontrol-2018-054321

130 Phillip Morris International. IQOS. IQOS. https://www.pmi.com/smoke-free-products/iqos-our-tobacco-heating-system (accessed 8 Oct2017).

131 Phillip Morris International. What is IQOS? IQOS. https://www.pmi.com/faq-section/faq/what-is-iqos (accessed 27 Jul2018).

132 Food and Drug Administration. Meeting of the Tobacco Products Scientific Advisory Committee January 24-25, 2018

133 Food and Drug Administration. Meeting of the Tobacco Products Scientific Advisory Committee January 24-25, 2018. 2018. 1–262.

134 Franck C, Filion KB, Kimmelman J, *et al.* Ethical considerations of e-cigarette use for tobacco harm reduction. *Respiratory Research* 2016;17:53. doi:10.1186/s12931-016-0370-3

135 Etter J-F. Gateway effects and electronic cigarettes. *Addiction* 2018;113:1776–83. doi:10.1111/add.13924

136 Chatterjee K, Alzghoul B, Innabi A, *et al.* Is vaping a gateway to smoking: a review of the longitudinal studies. *Int J Adolesc Med Health* 2016;30:1. doi:10.1515/ijamh-2016-0033

137 Modesto-Lowe V, Alvarado C. E-cigs . . . Are They Cool? Talking to Teens About E-Cigarettes. *Clin Pediatr (Phila)* 2017;56:947–52. doi:10.1177/0009922817705188

138 Liu X, Lugo A, Spizzichino L, *et al.* Heat-Not-Burn Tobacco Products Are Getting Hot in Italy. *J Epidemiol* 2018;28:274–5. doi:10.2188/jea.JE20180040

139 Talih S, Balhas Z, Eissenberg T, *et al.* Effects of User Puff Topography, Device Voltage, and Liquid Nicotine Concentration on Electronic Cigarette Nicotine Yield: Measurements and Model Predictions. *Nicotine Tob Res* 2015;17:150–7. doi:10.1093/ntr/ntu174

140 Ramôa CP, Hiler MM, Spindle TR, *et al.* Electronic cigarette nicotine delivery can exceed that of combustible cigarettes: a preliminary report. *Tobacco Control* Published Online First: 31 August 2015. doi:10.1136/tobaccocontrol-2015-052447

141 Chaturvedi P, Mishra A, Datta S, *et al.* Harmful effects of nicotine. *Indian Journal of Medical and Paediatric Oncology* 2015;36:24. doi:10.4103/0971-5851.151771

142 Petros WP, Younis IR, Ford JN, *et al.* Effects of tobacco smoking and nicotine on cancer treatment. *Pharmacotherapy* 2012;32:920–31. doi:10.1002/j.1875-9114.2012.01117

143 Wu Q, Jiang D, Minor M, *et al.* Electronic cigarette liquid increases inflammation and virus infection in primary human airway epithelial cells. *PLoS ONE* 2014;9:e108342. doi:10.1371/journal.pone.0108342

144 Javed F, Abduljabbar T, Vohra F, *et al.* Comparison of Periodontal Parameters and Self-Perceived Oral Symptoms Among Cigarette Smokers, Individuals Vaping Electronic Cigarettes, and Never-Smokers. *J Periodontol* 2017;88:1059–65. doi:10.1902/jop.2017.170197

145 ArRejaie AS, Al-Aali KA, Alrabiah M, *et al.* Proinflammatory cytokine levels and peri-implant parameters among cigarette smokers, individuals vaping electronic cigarettes, and non-smokers. *J Periodontol* 2018;26:e8. doi:10.1002/JPER.18-0045

146 Bahl V, Johnson K, Phandthong R, *et al.* Thirdhand Cigarette Smoke Causes Stress-Induced Mitochondrial Hyperfusion and Alters the Transcriptional Profile of Stem Cells. *Toxicol Sci* 2016;:kfw102–15. doi:10.1093/toxsci/kfw102

147 Cholanian M, Wealing J, Levine RB, *et al.* Developmental nicotine exposure alters potassium currents in hypoglossal motoneurons of neonatal rat. *J Neurophysiol* 2017;117:1544–52. doi:10.1152/jn.00774.2016

148 Slotkin TA. Developmental Effects of Nicotine. In: *Effects of Nicotine on Biological Systems II*. Basel: : Birkhäuser Basel 1995. 111–7. doi:10.1007/978-3-0348-7445-8_14

149 Levin ED, Slotkin TA. Developmental Neurotoxicity of Nicotine. In: *Handbook of Developmental Neurotoxicology*. Elsevier 1998. 587–615. doi:10.1016/B978-012648860-9.50043-1

150 Slotkin TA. If nicotine is a developmental neurotoxicant in animal studies, dare we recommend nicotine replacement therapy in pregnant women and adolescents? *Neurotoxicol Teratol* 2008;30:1–19. doi:10.1016/j.ntt.2007.09.002

151 Kutlu MG, Holliday E, Gould TJ. Genetic, Developmental, and Receptor-Level Influences on Nicotine Withdrawal-Associated Deficits in Learning. In: *Negative Affective States and Cognitive Impairments in Nicotine Dependence*. Elsevier 2017. 53–70. doi:10.1016/B978-0-12-802574-1.00004-1

152 Zhang D, Cui H, Zhang L, *et al.* Is maternal smoking during pregnancy associated with an increased risk of congenital heart defects among offspring? A systematic review and meta-analysis of observational studies. *J Matern Fetal Neonatal Med* 2017;30:645–57. doi:10.1080/14767058.2016.1183640

153 Sabbagh HJ, Hassan MHA, Innes NPT, *et al.* Passive smoking in the etiology of non-syndromic orofacial clefts: a systematic review and meta-analysis. *PLoS ONE* 2015;10:e0116963. doi:10.1371/journal.pone.0116963

154 Palpant NJ, Hofsteen P, Pabon L, *et al.* Cardiac Development in Zebrafish and Human Embryonic Stem Cells Is Inhibited by Exposure to Tobacco Cigarettes and E-Cigarettes. *PLoS ONE* 2015;10:e0126259–19. doi:10.1371/journal.pone.0126259

155 Kennedy AE, Kandalam S, Olivares-Navarrete R, *et al.* E-cigarette aerosol exposure can cause craniofacial defects in Xenopus laevis embryos and mammalian neural crest cells. *PLoS ONE* 2017;12:e0185729. doi:10.1371/journal.pone.0185729

156 Li G, Saad S, Oliver BG, *et al.* Heat or Burn? Impacts of Intrauterine Tobacco Smoke and E-Cigarette Vapor Exposure on the Offspring's Health Outcome. *Toxics* 2018;6:43. doi:10.3390/toxics6030043

157 Chun L, Moazed F, Matthay M, *et al.* Possible hepatotoxicity of IQOS. *Tobacco Control* 2018;27:s39–s40. doi:10.1136/tobaccocontrol-2018-054320

158 Lasseter T, BANSAL P, WILSON T, *et al.* Scientists describe problems in Philip Morris e-cigarette experiments. 2018.

159 Moazed F, Chun L, Matthay MA, *et al.* Assessment of industry data on pulmonary and immunosuppressive effects of IQOS. *Tobacco Control* 2018;27:s20–5. doi:10.1136/tobaccocontrol-2018-054296

160 Nabavizadeh P, Liu J, Havel CM, *et al.* Vascular endothelial function is impaired by aerosol from a single IQOS HeatStick to the same extent as by cigarette smoke. *Tobacco Control* 2018;:tobaccocontrol–2018–054325–7. doi:10.1136/tobaccocontrol-2018-054325

SECTION I: ELECTRONIC CIGARETTES

CHAPTER 1

Nicotine Concentrations in Electronic Cigarette Refill and Do- It-Yourself Fluids

Barbara Davis, Michael Dang, Jisoo Kim, Prue Talbot

Nicotine Tob Res 2015;17:134–41. doi:10.1093/ntr/ntu080

ABSTRACT

Introduction: To evaluate the accuracy of nicotine concentration labeling on electronic cigarette refill products.

Methods: The nicotine concentration of 71 electronic cigarette refill fluid products and one related DIY (do-it-yourself) product was quantified using high performance liquid chromatography. Quantified data were compared to manufacturers labeled concentrations. Duplicate refill fluid products purchased at different times were evaluated by visual comparison of fluid coloration and quantified nicotine concentration.

Results: 35 of the 54 nicotine containing fluids had quantified nicotine concentrations that deviated by more than ±10% from the manufacturer labels with 46 of 50 being in excess of labeled values. Refill fluids labeled as zero nicotine had no detectable nicotine. Of five products that were unlabeled for nicotine concentration, three contained no detectable nicotine, while the remaining two contained nicotine in excess of 100mg/mL and may have been intended for do-it-yourself use. 16 of the 18 duplicate bottles of refill fluid varied greatly in their nicotine concentrations. One of five companies showed significant improvement in labeling accuracy in the most recently purchased products. Of the 23 total duplicate pairs, 15 of 23 varied in coloration from their mates.

Conclusions: Nicotine concentration labeling on electronic cigarette refill products was often inaccurate but showed improvement recently in products from one company.
To ensure the safety of refill fluids and DIY products, it is necessary to establish quality control guidelines for the manufacturing and labeling and to monitor products longitudinally.

INTRODUCTION

E-cigarettes (EC) are comprised of a battery that heats an atomizer, which aerosolizes a fluid that generally contains nicotine, a humectant(s), and flavorings [1,2]. EC cartridges, cartomizers, and tanks, which hold the fluid, can be refilled from drip bottles of refill fluid that are readily available over the Internet, in EC retail shops, and in malls. While EC per se are generally marketed in a limited number of flavors, refill fluids are available, often from third party vendors, in hundreds of different flavors. Thus, they expand the flavor options and offer EC users a more cost-effective option by enabling EC cartridges to be reused. However, some refill fluids were cytotoxic when tested in vitro with different cell types, and cytotoxicity of several products was attributed to flavorings [3,4].

Because nicotine contained in these fluids is both addictive and toxic (a dose of 6.5–13 mg/kg is fatal to adult humans) [5,6], it is important that the concentrations of nicotine on refill fluid and DIY product bottles be accurate. However, there are currently no federal regulations on the manufacturing of these products. Recently, the EC industry has begun some self-regulation with the creation of the American E-liquid Manufacturing Standards Association (AEMSA), which was formed to certify nicotine concentrations in EC products. According to the revised AEMSA guidelines (posted on the AEMSA website in February, 2014), nicotine concentrations should be ±10% of the label [7].

Previous studies have examined nicotine concentrations in a limited number of refill fluid products [8-10], and in some cases found significant discrepancies between what was on the bottle and what was measured [8,10]. The purpose of the current study was to quantify nicotine concentrations in a broad spectrum of refill fluids from different American manufacturers, to compare measured nicotine concentrations to those

provided by the manufacturer, and to determine if duplicate bottles of the same product purchased at different times had similar concentrations of nicotine. This study is the first to investigate nicotine concentrations in a broad range of American-made refill fluid products in longitudinal samples.

MATERIALS & METHODS:

Products tested

71 EC refill fluids and one DIY (do-it-yourself) product were purchased from five different manufacturers using the Internet or a local vendor and inventory numbers were assigned to each sample at the time of receipt (Table 1.1). Purchases were made on four different dates (inventory #s 1-41 April 2011; #s 49-68 summer 2011; #s 70-92 February 2012, and #s 93-96 May 2012). Johnson Creek (Johnson Creek, WI) and Red Oak (a subsidiary of Johnson Creek) are two major manufacturers of EC refill fluid products, as shown by Google Trends, with sales in 101 countries. Recently, Johnson Creek partnered with Blu Cig, one the most popular electronic cigarettes [11], thus increasing the potential distribution of their products. Freedom Smoke USA (Tucson, AZ) and V2 Cigs (Miami Beach, FL) were selected because the companies have consistently gained in popularity since their introduction to the refill fluid market in 2010, with their popularity still rising according to Google Trends. Global Smoke (Los Angeles, CA) was selected because it is marketed and readily available in shopping malls in our area. Lastly, e-cigexpress.com (Orlando, FL) was selected because, at the time of purchase, they were one of the few Internet vendors that sold flavorless nicotine in a propylene glycol base.

Of the 71 refill fluids/1 DIY products that were evaluated, 25 were purchased from Johnson Creek, and 20 of these were obtained from two sample kits. Kits were purchased at two different times, and 9 of the 10 products in each kit were exact duplicates (i.e., from the same manufacturer with the same label information). Of the additional five Johnson Creek refill fluids, two were Tennessee Cured (#s 31, 51) with labeled nicotine content that differed from the refill fluids in the sample kits, and three were J.C. Original (#s 34, 50, 88) of which two (#s50 and 88) were labeled with the same nicotine content as those in the sample kit and one (#34) was labeled at a lower concentration. 16 refill fluids were from Red Oak, and 14 of these were also obtained in two sample kits. The Red Oak kits each contained seven refill fluids, and all seven were duplicate flavors. Two additional Red Oak Marcado (#s 49, 70) refill fluids were purchased to further evaluate duplicates. For both Johnson Creek and Red Oak, all refill fluids contained within the kits had a different labeled humectant composition than individually purchased bottles. 24 refill fluids were purchased from Freedom Smoke-USA. Two (#24, #55) of the 24 products were unflavored nicotine in 100% propylene glycol. At the time of purchase, neither the labels nor website indicated the nicotine concentrations of these products or that they were concentrates to be used in diluted form. Therefore, these two products were also considered refill fluids, not DIY products. Four of the flavors (eight refill bottles) were purchased as exact duplicates. Two refill fluids were purchased from Global Smoke, four were from V2 Cigs, and the one DIY product was from e-cigexpress.com. All products were stored in the dark at 4°C, and all experiments were performed within three months of purchase.

Establishment of Nicotine Calibration Curve and Method Validation:

Our HPLC method for quantifying nicotine in refill fluids/ DIY products was adapted from Trehy et al (2011) which was shown previously to work well with EC refill fluids. Nicotine (≥99% purity) purchased from Sigma-Aldrich (St. Louis, MO) was used to establish a calibration curve. A stock solution of nicotine (10mg/mL) was prepared in non-buffered mobile phase consisting of 77% water and 23% acetonitrile. Serial dilutions (1 to 1500 µg/mL) were made, and the linear portion of instrumental response was determined. Three samples of 5 doses that spanned the linear range (100 µg to 1000µg/ml) were used to create a calibration curve. The accuracy and precision of the calibration curve was validated by injecting four samples of nicotine, prepared as described above, at two concentrations (500ug/ml and 637ug/ml) and determining the percent error at each concentration. For each concentration, the error was < 1% (0.408% for 500ug/ml and 0.843% for 637ug/ml). The calibration curve was periodically validated to ensure that no changes or drift were present.

HPLC analysis of nicotine concentrations:

HPLC grade chemicals (triethylamine, water, and acetonitrile) and phosphoric acid (85%) were purchased from Fischer Scientific (Fair Lawn, NJ). Sodium hydroxide was purchased from EM Scientific (Gibbstown, NJ). Samples were analyzed using a Hewlett Packard Series 1100 HPLC, consisting of a quaternary pump, degasser, column thermostat and manual injector. A 200 x 4.6 mm Thermo Scientific Hypersil ODS C18 column with a particle size of 5 µm was used at 35°C with a flow rate of 0.8 mL/min. The diode array detector signal was set to 260 nm with a bandwidth of 40 nm with a reference signal of 380 nm and bandwidth of 10 nm. An isocratic method was used with a buffered mobile phase consisting of 76.9% water, 23% acetonitrile, and 0.1%

triethylamine. The pH of the mobile phase was adjusted daily to 7.6 using phosphoric acid and sodium hydroxide. Since no extraction procedure was necessary, 5% stock solutions of each fluid were prepared in a non-buffered mobile phase. Care was taken to accurately pipette the fluids so as not to introduce air bubbles. The stock solutions were diluted down to the injection concentration of 0.5% by the further addition of non-buffered mobile phase. The injection volume for all samples was 5 µl. The limit of quantification for nicotine was 10 µg/mL with a limit of detection of 50 ng/ml. Each sample was injected and analyzed 5 times. The values reported in Tables 1 and 2 are the means and standard deviations of the 5 runs.

RESULTS

Table 1.1 shows the results, organized by ascending inventory number, for the 72 products that were evaluated in this study. For each product, the date of purchase, inventory number, flavor, humectant, color, manufacturers' nicotine concentration, quantified nicotine concentration, percent difference in nicotine concentrations, and whether the fluid was within ±10% of the concentration on the label. A broad range of flavors was included. In most cases, humectants were named on the label, and humectants varied among manufacturers and varied sometimes within a manufacturer. The color of the refill fluids also varied among products and ranged from clear to dark brown. Similarly flavored fluids produced by the same manufacturer sometimes varied in color. For example, Freedom Smoke Caramel (#26) was clear, while Freedom Smoke Caramel (#27) was orange-yellow.

Manufacturer labeled nicotine concentrations were compared to the HPLC quantified nicotine concentrations, and the percent differences were calculated (Table

1.1). Of the products tested, the 10 refill fluids that were labeled zero nicotine (#s 20, 21, 23, 26, 30, 36, 38, 41, 90 and 92) contained no detectable nicotine. Eight of 72 samples had nicotine concentrations below labeled values. Five of these eight were from Johnson Creek (#s 15, 82, 85, 86, 88) and three were from Freedom Smoke-USA (#s 19, 29, 37). Of the remaining 54 fluids, 46 had nicotine concentrations that were higher than the labeled amount. Five bottles had no labeled nicotine concentration; three contained no detectable nicotine (#s 25, 56, 87) and two (#s 24, 55) had nicotine concentrations in excess of 100mg/mL. Three of the four Red Oak Marcado samples (#s 3, 49, 73) were not analyzable using this HPLC method. Of the analyzable nicotine containing fluids, only 19 had concentrations within ±10% of the labeled concentration, which is the nicotine tolerance level set by AEMSA in their recently revised standard [7], as well as a standard that is acceptable for nicotine patches [12].

Table 1.1 Evaluated Refill Fluids

#	Manufacturer	Flavor	Hum	Color	[M]*	[Q] (mg/mL)	% Diff From [M]	AEMSA Standard
1	Red Oak	Domestic	G, V	Brown	18	22.8±0.93	+26.4	No
2	Red Oak	Island	G, V	Lt Brown	18	29.6±0.27	+64.3	No
3	Red Oak	Marcado	G, V	Lt Brown	18	STC	n/a	n/a
4	Red Oak	Swiss Dark	G, V	Dk	18	31.7±0.23	+76.1	No
5	Red Oak	Tennessee Cured	G, V	Lt Brown	18	29.7±0.29	+64.9	No
6	Red Oak	Valencia	G, V	Cream	18	24.2±0.34	+34.2	No
7	Red Oak	Wisconsin Frost	G, V	Brown	18	23.8±0.23	+32.2	No
8	JC	Arctic Menthol	P, V, G	Tan	18	22.2±0.30	+23.4	No
9	JC	Black Cherry	P, V, G	Tan	18	20.9±0.13	+16.1	No
10	JC	Chocolate Truffle	P, V, G	Med	18	19.7±0.56	+9.7	No
11	JC	Espresso	P, V, G	Lt Brown	18	21.4±0.11	+18.83	No
12	JC	French Vanilla	P, V, G	Brown	18	20.7±0.11	+14.83	No
13	JC	JC Original	P, V, G	Lt Brown	18	24.9±0.45	+38.5	No
14	JC	Mint Chocolate	P, V, G	Tan	18	24.3±0.47	+35.1	No
15	JC	Simply Strawberry	P, V, G	Tan	18	17.±0.21	-5.8	No
16	JC	Summer Peach	P, V, G	Tan	18	22.0±0.38	+22.1	No
17	JC	Tennessee Cured	P, V, G	Med	18	21.4±0.31	+18.8	No
18	FS-USA	Bubble Gum	U	Orange	24	28.5±0.80	+18.8	No
19	FS-USA	Butterfinger	U	Dark	24	21.9±0.71	-8.6	No
20	FS-USA	Butterscotch FA	U	Yellow	0	0.0	0	n/a
21	FS-USA	Caramel FA	U	Lt Brown	0	0.0	0	n/a
23	FS-USA	Menthol Arctic FA	U	Clear	0	0.0	0	n/a
24	FS-USA	PureNicLiquid	P	Clear (Lt	UK	105.9±3.2	n/a	n/a
25	FS-USA	Vanilla Tahity FA	U	Med	UK	0.0	n/a	n/a
26	FS-USA	Caramel	V	Clear	0	0.0	0	n/a
27	FS-USA	Caramel	U	Yellow-	6	10.2±0.27	+69.5	No
28	FS-USA	Caramel	V	Yellow-	6	10.5±0.72	+75.0	No
29	FS-USA	Butterscotch	V	Yellow-	6	5.6±0.03	-6.0	No
30	FS-USA	Butterscotch	V	Clear	0	0.0	0	n/a
31	JC	Tennessee Cured	P, V	Med	11	15.6±0.85	+42.1	No
34	JC	J.C Original	P, V	Lt Brown	11	17.5±0.55	+58.6	No
35	FS-USA	Chocolate Biscotti	U	Brown	24	34.4±1.59	+43.3	No

#	Manufacturer	Flavor	Hum	Color	[M]*	[Q] (mg/mL)	% Diff From [M]	AEMSA Standard
36	FS-USA	Coconut	U	Clear (Lt Yw Tint)	0	0.0	0	n/a
37	FS-USA	Peanut Butter Cup	U	Dk	24	20.9±1.29	-12.9	No
38	FS-USA	Tiramisu	U	Dk	0	0.0	0	n/a
39	Global Smoke	RY4	U	Yellow	18	26.6±0.63	+47.9	No
40	Global Smoke	Caramel	U	Yellow-	18	23.3±0.86	+29.6	No
41	FS-USA	Butterscotch FA	U	Yellow	0	0.0	0	n/a
49	Red Oak	Marcado	V	Tan	18	STC	n/a	n/a
50	JC	J.C Original	P, V	Tan	18	19.9±0.26	+10.4	No
51	JC	Tennessee Cured	P, V	Lt Brown	11	12.2±0.19	+10.5	No
55	FS-USA	PureNicotineLiquid	P	Clear	UK	134.7±4.0	+34.7	No
56	FS-USA	Butterscotch FA	U	Lt Brown	UK	0.0	n/a	n/a
57	FS-USA	Wyatt Earp	U	Dk	24	31.3±2.55	+30.4	No
68	e-cigexpress	Unflavored PG ase	P	Clear	60	72.9±3.14	+21.5	No
70	Red Oak	Marcado	V	Tan	1.8	27.8±1.36	+54.3	No
71	Red Oak	Domestic	G, V	Tan	18	22.8±0.34	+26.4	No
72	Red Oak	Island	G, V	Tan	18	23.5±0.40	+30.6	No
73	Red Oak	Marcado	G, V	Lt Yellow	18	STC	n/a	n/a
74	Red Oak	Swiss Dark	G, V	Lt Brown	18	28.0±0.28	+55.9	No
75	Red Oak	Tennessee Cured	G, V	Lt Brown	18	28.6±1.04	+59.0	No
76	Red Oak	Valencia	G, V	Clear	18	19.5±0.53	+8.4	No
77	Red Oak	Wisconsin Frost	G, V	Lt Brown	18	34.2±0.72	+89.7	No
78	JC	Arctic Menthol	P, V, G	Lt Brown	18	19.7±0.23	+9.2	No
79	JC	Black Cherry	P, V, G	Tan	18	19.0±0.42	+5.6	No
80	JC	Chocolate Truffle	P, V, G	Brown	18	18.2±0.98	+1.3	Yes
81	JC	Espresso	P, V, G	Brown	18	18.2±0.70	+1.1	Yes
82	JC	French Vanilla	P, V, G	Brown	18	17.8±0.24	-1.3	Yes
83	JC	JC Original	P, V, G	Lt Brown	18	18.40.33	+2.3	Yes
84	JC	Mint Chocolate	P, V, G	Brown	18	19.4±0.44	+9.3	No
85	JC	Spiced Apple Cider	P, V, G	Light	18	16.8±0.52	-6.6	No
86	JC	Summer Peach	P, V, G	Lt Brown	18	17.4±0.30	-3.5	Yes
87	JC	Tennessee Cured	P, V, G	Med	18	19.2±0.34	+6.7	No
88	JC	J.C Original	P, V	Lt Brown	1.8	17.1±0.24	-5.2	No
89	FS-USA	Menthol Arctic FA	U	Clear	UK	0.0	n/a	n/a
90	FS-USA	Caramel	V	Clear	0	0.0	0	n/a

#	Manufacturer	Flavor	Hum	Color	[M]*	[Q] (mg/mL)	% Diff From [M]	AEMSA Standard
91	FS-USA	Butterscotch	V	Clear	6	7.4±0.09	+22.5	No
92	FS-USA	Butterscotch	V	Clear (Lt	0	0.0	0	n/a
93	V2 Cigs	Peppermint	P, V	Lt Yellow	1.8	20.2±0.09	+12.1	No
94	V2 Cigs	Menthol	P	Lt Yellow	18	19.6±0.08	+8.8	No
95	V2 Cigs	Sahara	P	Yellow	18	19.7±0.07	+9.3	No
96	V2 Cigs	V2 Red	P	Yellow	18	18.8±0.07	+4.2	Yes

*Manufacturer's Concentration is in mg unless otherwise stated
JC= Johnson Creek, FS-USA= Freedom Smoke USA
Hum= Humectant Composition, G= Glycerin, V= Vegetable Glycerin, P= Propylene Glycol, U= Unknown
[M]= Manufacturers Concentration, [Q]= Quantified Concentration
Lt= Light, Med= Medium, Dk= Dark, Yw= Yellow
STC= Sticks to Column

Table 1.2 Comparison of Exact Duplicate Refill Fluids

#s	Manufacturer	Flavor	Color Comp	[Q] Comp (mg/mL)	% Diff btwn Duplicates	P value
1/ 71	Red Oak	Domestic	Brown/ Tan	22.76/ 22.76	0	0.9966
2/ 72	Red Oak	Island	Lt Brown/ Tan	29.58/ 23.5	22.91	< 0.0001
3/ 73	Red Oak	Marcado	Lt Brown/ Lt Yellow	STC/ STC	n/a	n/a
4/ 74	Red Oak	Swiss Dark	Dk Brown/ Lt Yellow	31.69/ 28.07	12.12	< 0.0001
5/ 75	Red Oak	Tennessee Cured	Lt Brown/ Lt Brown	29.68/ 28.62	3.64	0.0924
6/ 76	Red Oak	Valencia	Cream/ Clear	24.15/ 19.51	21.26	< 0.0001
7/ 77	Red Oak	Wisconsin Frost	Brown/ Lt Brown	23.8/ 34.15	35.72	< 0.0001
8/ 78	JC	Arctic Menthol	Tan/ Lt Brown	22.21/ 19.66	12.18	< 0.0001
9/ 79	JC	Black Cherry	Tan/ Tan	20.9/ 19.01	9.47	0.0001
10/ 80	JC	Chocolate Truffle	Med Brown/ Brown	19.74/ 18.24	7.9	0.0248
11/ 81	JC	Espresso	Lt Brown/ Brown	21.39/ 18.19	16.17	0.0006
12/ 82	JC	French Vanilla	Brown/ Brown	20.67/ 17.77	15.09	< 0.0001
13/ 83	JC	JC Original	Lt Brown/ Lt Brown	24.93/ 18.41	30.09	< 0.0001
14/ 84	JC	Mint Chocolate	Tan/ Brown	24.31/ 19.67	21.1	< 0.0001
16/ 86	JC	Summer Peach	Tan/ Lt Brown	21.97/ 17.37	23.39	< 0.0001
17/ 87	JC	Tennessee Cured	Med Brown/ Med Brown	21.38/ 19.21	10.69	< 0.0001
23/ 89	FS-USA	Menthol Arctic FA	Clear/ Clear	0/ 0	n/a	n/a
26/ 90	FS-USA	Caramel	Clear/ Clear	0/ 0	n/a	n/a
29/ 91	FS-USA	Butterscotch	Yellow-Orange/ Clear (Yw Tint)	5.64/ 7.35	26.33	< 0.0001
30/ 92	FS-USA	Butterscotch	Clear/ Clear (Lt Yw Tint)	0/ 0	n/a	n/a
31/ 51	JC	Tennessee Cured	Med Brown/ Lt Brown	15.63/ 12.16	24.97	0.0009
49/ 70	Red Oak	Marcado	Tan/ Tan	STC/ 27.78	n/a	n/a
50/ 88	JC	J.C Original	Tan/ Lt Brown	19.87/ 17.06	15.22	< 0.0001

JC= Johnson Creek, FS-USA= Freedom Smoke USA
[Q]= Quantified Concentration
Lt= Light, Med= Medium, Dk= Dark, Yw= Yellow
STC= Sticks to Column

For some products purchased after February 2012, accuracy in labeling appeared to have improved. For example, of the 12 Johnson Creek products purchased in April 2011, only two of the 12 met the ±10% standard. For the 11 Johnson Creek products purchased in February 2012, all 11 were within ±10% of the labeled nicotine concentration, suggesting that manufacturing processes have improved at this company between 2011 and 2012. For Red Oak, a subsidiary of Johnson Creek, only one of the 7 analyzable recent purchases (February 2012) was within 10% of the labeled nicotine concentration, and 6 of the 7 were higher than the labeled concentrations by 26.6 to 89.7%. Only one sample of Freedom Smoke that was purchased in February 2012 had nicotine, and it deviated from the label by 22.5%, which was an increase from the April 2011 purchase that deviated only 6%. In addition, of the four V2 products purchased in May 2012, three of the four products fell within ±10%, suggesting improved manufacturing practices.

Table 1.2 presents longitudinal data comparing refill fluids that were considered exact duplicates, i.e., produced by the same manufacturer and labeled with the same humectant composition, flavor, and nicotine concentration, but purchased on different dates. The Table shows the inventory number of refill fluid duplicate pairs, manufacturer, flavor, coloration, comparisons between the exact duplicates, manufacturer's labeled nicotine concentration, and whether the quantified nicotine concentration was ≤10% from the manufacturer's concentration, as well as the actual percent difference (indicated in parenthesis). The color of the refill fluids, which was evaluated visually, varied within most of the 23 duplicate pairs. The most extreme example was observed between numbers 4 (dark brown) and 74 (light brown), which were duplicate bottles of Red Oak Swiss Dark (Fig. 1.1). Some duplicate samples (e.g., Freedom Smoke #29 and 91) had

very slight differences in coloration (orange-yellow vs. clear with yellow tint), while other duplicates were similar in color (e.g., Johnson Creek #17 and 87) (Fig. 1.1).

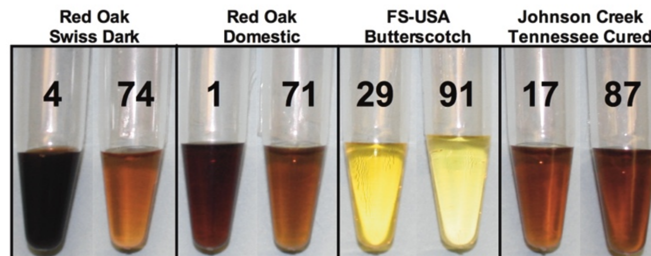


Figure 1.1. Examples of color variation among duplicate refill fluid pairs. Color variation between mates ranged from extreme, Red Oak Swiss Dark, to similar in coloration, Johnson Creek Tennessee Cured.

The 18 pairs of refill fluid that contained nicotine were compared to determine if labeling accuracy improved between purchases. Table 1.2 shows those samples that had measured nicotine concentrations within 10% of the labeled value. Of the18 pairs, only one pair (Red Oak Domestic #s 1, 71) had identical quantified nicotine concentrations and thus showed no change over time from the manufacture's labeled concentration. Only one pair (#s 10, 80) was within 10% of the labeled concentration. For five refill fluids pairs, all from Red Oak, both samples exceeded 10% of the labeled concentration, and of these, one (#s 7, 77) showed an increase in the percent difference over time. Only one refill fluid pair (Freedom Smoke #s 29, 91) showed a diminution in quality with the fluid purchased earlier meeting the criteria and the latter not. 10 pairs (which included 9 from Johnson Creek) showed significant improvements in labeling accuracy over time.

DISCUSSION

The purpose of this study was to evaluate the accuracy of nicotine concentrations that appear on labels of EC refill fluids/DIY products and to test the fidelity of the manufacturing process by evaluating nicotine concentrations in duplicate products purchased on different dates. Of the 71 refill fluids/1 DIY product evaluated, 54 were labeled as containing nicotine and analyzable. Of these, 35 had nicotine concentrations that did not meet the revised AEMSA tolerance level of ±10%. Quantified nicotine concentrations in evaluated fluids varied from as little as 1.1% to as much as 89.7% from the labeled value, with the majority being higher than indicated on the label. Accuracy in labeling improved significantly in more recent samples purchased from one company. We also found significant variation in the color of fluids both between the same flavors from the same manufacturer and between duplicate bottles of the same product. While color variation could be due to the use of different chemicals to create a particular flavor or to changes in color during storage, a dramatic color change would not be expected between products that are considered exact duplicates (i.e. the same product purchased at different times) as was seen with #s 4 and 74. We have observed our products for over 2 years and none have noticeably changed color during storage.

There have been relatively few studies on the accuracy of the labeled nicotine concentrations on EC products [10,13-16] with a subset of studies focusing specifically on refill fluids [8,9,14]. In most of these studies, the quantified nicotine concentrations varied from the labeled concentrations, and the degree of variation was quite diverse. In two studies with relatively small sample sizes, the majority of EC refill fluids contained nicotine concentrations below that of the label [8,14]. In a third study, measured nicotine levels tended to be higher than labeled concentrations; however, the differences

between the labeled and measured concentrations for one manufacturer were minor [10]. A fourth recent study of mainly Western European products found the differences in the labeled and measured nicotine concentrations to be relatively small and suggested that manufacturing practices have improved and may be acceptable [9]. In our study, which is the most comprehensive evaluation of American products to date, most nicotine concentrations were higher than the labeled values, with many being over 20% higher. For longitudinal samples from Johnson Creek, the products that were purchased last showed better accuracy in labeling than those purchase 10 months earlier suggesting an improvement in manufacturing processes for this company. However, a similar improvement was not seen in Red Oak, the Johnson Creek subsidiary. While the trend for at least one company appears to be toward better labeling, it will be important in the future to monitor progress in accuracy of nicotine labeling and to look at multiple products from a spectrum of companies, as there is still variability within and between companies and there is currently no government regulation on these products.

The importance of evaluating longitudinal samples from a manufacturer is demonstrated in our study by the four samples of Johnson Creek J.C. Original (18mg of nicotine /ml) that were purchased at 3 different times and varied only in humectant composition. When calculated nicotine values were compared to labeled values, the deviations from the label were +38.5%, +10.4%, -5.2% and +2.3%. Had only one of these products been evaluated, e.g., the product that differed by +38.5%, the data generated would not be an accurate representation of the product line. Likewise, we have shown for the first time that duplicate samples of the same product can vary in their nicotine concentration. In 7 of 21 samples, nicotine concentration between duplicate

bottles varied by more than 20% (as indicated by stars in the ≤10% Difference column of Table 1.2).

In October 2012, AEMSA was established as a volunteer organization to set responsible and sustainable standards for the safe manufacturing of EC refill fluid products, and their standards are quite stringent. Members, who pay a monthly membership fee, must agree to adhere to these standards and are allowed to display the AEMSA logo on their websites [7]. Although none of the companies evaluated in this study are listed on the AEMSA website as members of this association, refill fluids purchased later in our study were more accurately labeled, with Johnson Creek being the most improved.

Accurate labeling of nicotine concentrations on EC products is important as nicotine is both addictive and toxic [5,17-19], and EC users should have reliable information on nicotine concentrations in these products. Moreover, some people use EC as cessation devices [20-22] and gradually wean themselves off higher doses of nicotine. For this group of EC users, accurately labeled products are important. Also, studies show that decreases in nicotine intake can lead to nicotine withdrawal symptoms and induce compensatory smoking [17]. Another concern with improper labeling is the potential for nicotine overexposure/overdose. Two refill fluids sold as unflavored nicotine in PG had no labeling indicating the nicotine content. Only through HPLC analysis were these products found to contain over 100 mg of nicotine/ml. At these high concentrations, these products may have been intended as DIY products, but this was not stated on the manufacturer's website at the time of purchase nor this was this indicated on the bottles. A consumer would not know these products were DIY without proper labeling. Nicotine doses of 500-1000 mg for adults [23] and 10 mg for children

[5,6,24] can be lethal. Since neither of these bottles had the nicotine concentration or danger warnings printed on their labels, users of these products could be exposed to higher doses of nicotine than they intended. Moreover, bottles with such high concentrations of nicotine present a clear danger to children. When the concentration of a 10 mL bottle of fluid is considered, the total nicotine content would exceed a lethal dose for both children and adults.

We previously showed that EC performance is highly variable both between and within brands of EC [1,25], that cytotoxicity of refill fluids varies among products [3,4], and that puff duration varies among brands [26]. Others have shown significant variability among products in the aerosolization of nicotine and in the concentration of tobacco-specific nitrosamines emitted in EC aerosols [27]. Finally, EC users have sometimes reported symptoms consistent with nicotine overdoses [28]. While this could occur for a number of reasons, accurate labeling would be important to prevent inadvertent overdosing. It is clear from these results that when evaluating the chemical components in EC fluid products and aerosols, multiple products and longitudinal duplicates of products should be tested before any determination can be made on the accuracy of a particular product line of refill fluids. These studies also demonstrate the importance of having regulations governing the accurate labeling of nicotine concentrations on EC products, as well as the need for guidelines to improve the integrity of manufacturing.

REFERENCES:

1 Trtchounian A, Williams M, Talbot P. Conventional and electronic cigarettes (e-cigarettes) have different smoking characteristics. *Nicotine Tob Res* 2010;**12**:905–12. doi:10.1093/ntr/ntq114

2 Trtchounian A, Talbot P. Electronic nicotine delivery systems: is there a need for regulation? *Tobacco Control* 2011;**20**:47–52. doi:10.1136/tc.2010.037259

3 Bahl V, Lin S, Xu N, *et al.* Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models. *Reprod Toxicol* 2012;**34**:529–37. doi:10.1016/j.reprotox.2012.08.001

4 Behar RZ, Davis B, Wang Y, *et al.* Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids. *TOXICOLOGY IN VITRO* 2014;**28**:198–208. doi:10.1016/j.tiv.2013.10.006

5 Solarino B, Rosenbaum F, Rießelmann B, *et al.* Death due to ingestion of nicotine-containing solution: case report and review of the literature. *Forensic Sci Int* 2010;**195**:e19–22. doi:10.1016/j.forsciint.2009.11.003

6 Toxicology Excellence for Risk Assessment (TERA). Derivation Of Human Lethal Doses. U.S. Army Center for Environmental Health Reserach, Fort Detrick MD 2006.

7 AEMSA. E-LIQUID MANUFACTURING STANDARDS. 1st ed. AEMSA 2012.

8 Cameron JM, Howell DN, White JR, *et al.* Variable and potentially fatal amounts of nicotine in e-cigarette nicotine solutions. *Tobacco Control* 2014;**23**:77–8. doi:10.1136/tobaccocontrol-2012-050604

9 Etter J-F, Zäther E, Svensson S. Analysis of refill liquids for electronic cigarettes. *Addiction* 2013;**108**:1671–9. doi:10.1111/add.12235

10 Trehy ML, Ye W, Hadwiger ME, *et al.* Analysis of electronic cigarette cartridges, refill solutions, and smoke for nicotine and nicotine related impurities. *Journal of Liquid Chromatography & Related Technologies* 2011;**34**:1442–58. doi:10.1080/10826076.2011.572213

11 *Time Magazine*. (2013). This Is Not A Cigarette. *Time Magazine*.

12 US Pharmacopeia 2011. 2011;:1–3.

13 Laugesen M, Thornley S, McRobbie H, *et al.* HOW SAFE IS AN E-CIGARETTE? Health New Zealand Ltd 2008. http://www.healthnz.co.nz

14 Goniewicz ML, Kuma T, Gawron M, *et al.* Nicotine levels in electronic cigarettes. *Nicotine Tob Res* 2013;**15**:158–66. doi:10.1093/ntr/nts103

15 Westenberger B. Evaluation of e-cigarettes. Department of Health & Human Services, Center for Drug Evaluation and Research, Food and Drug Administration, Center for Drug Evaluation and Research, Divison of Pharmaceutical Analysis, St. Louis, MO 2009.

16 Goniewicz ML, Knysak J, Gawron M, *et al.* Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. *Tobacco Control* 2014;**23**:133–9. doi:10.1136/tobaccocontrol-2012-050859

17 USDHHS. Risks Associated with Smoking Cigarettes with Low Machine-Measured Yields of Tar and Nicotine. U.S. Department of Health and Human Services, National Institue of Health, National Cancer Institue 2001.

18 Centers for Disease Control and Prevention, Office of Smoking and Health. The Health Consequences Of Smoking Nicotine Addiction. U.S. Department of Health and Human Service, Public Health Service Office of the Surgeon General, Rockville, MD 1988.

19 Centers for Disease Control and Prevention, Office of Smoking and Health. How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease. U.S. Department of Health and Human Service, Public Health Service Office of the Surgeon General, Rockville, MD 2010.

20 Odum LE, O'Dell KA, Schepers JS. Electronic cigarettes: do they have a role in smoking cessation? *J Pharm Pract* 2012;**25**:611–4. doi:10.1177/0897190012451909

21 Polosa R, Caponnetto P, Morjaria JB, *et al.* Effect of an electronic nicotine delivery device (e-Cigarette) on smoking reduction and cessation: a prospective 6-month pilot study. *BMC Public Health* 2011;**11**:786. doi:10.1016/j.tips.2010.12.008

22 Siegel MB, Tanwar KL, Wood KS. Electronic cigarettes as a smoking-cessation: tool results from an online survey. *Am J Prev Med* 2011;**40**:472–5. doi:10.1016/j.amepre.2010.12.006

23 Mayer B. How much nicotine kills a human? Tracing back the generally accepted lethal dose to dubious self-experiments in the nineteenth century. *Arch Toxicol* 2014;**88**:5–7. doi:10.1007/s00204-013-1127-0

24 Nicotine (PIM). inchem.org. http://www.inchem.org/documents/pims/chemical/nicotine.htm (accessed 6 Jul2013).

25 Williams M, Talbot P. Variability among electronic cigarettes in the pressure drop, airflow rate, and aerosol production. *Nicotine Tob Res* 2011;**13**:1276–83. doi:10.1093/ntr/ntr164

26 Hua M, Yip H, Talbot P. Mining data on usage of electronic nicotine delivery systems (ENDS) from YouTube videos. *Tobacco Control* 2013;**22**:103–6. doi:10.1136/tobaccocontrol-2011-050226

27 Kim H-J, Shin H-S. Determination of tobacco-specific nitrosamines in replacement liquids of electronic cigarettes by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* 2013;**1291**:48–55. doi:10.1016/j.chroma.2013.03.035

28 Hua M, Alfi M, Talbot P. Health-Related Effects Reported by Electronic Cigarette Users in Online Forums. *J Med Internet Res* 2013;**15**:e59. doi:10.2196/jmir.2324

Chapter 2

Unexpected Nicotine in Do-it-Yourself Electronic Cigarette Flavorings

Barbara Davis, Aladino Razo, Eugene Nothnagel, Mathew Chen, Prue Talbot

*thnagel, E., Ch*en, M., & Talbot, P. (2015b). Unexpected nicotine in Do

Electronic cigarette (EC) users often create their own refill fluids by blending bottled nicotine/propylene glycol/glycerol mixtures with Do-it-Yourself (DIY) flavourings [1]. Although a complete refill fluid usually contains nicotine, the flavouring solutions themselves are an additive and are presumed to be free of nicotine, which is a known addictive chemical and toxicant [2]. To determine if DIY flavourings are nicotine free, we evaluated 30 products from one vendor, using high performance liquid chromatography (HPLC)(figure 2.1B–D), and confirmed the presence of nicotine via gas chromatography and mass spectrometry (GC-MS) (figure 2.1E, F). HPLC analysis was performed as previously described in detail [3]. Nicotine was extracted from DIY flavorings [4] and GC-MS analysis of the extracts was per-formed using a Hewlett-Packard 5890 Series II gas chromatograph equipped with a Restek Rtx-1MS, 30 m, 0.25 mm column and a Hewlett-Packard 5971A mass selective detector. Samples were analysed from 40°C to 250°C with a total run time of 32.75 min per sample. Of the 30 flavouring products evaluated via HPLC, 4(figure 2.1A) had peaks with the retention time and shape characteristic of nicotine (figure 2.1C, D). GC-MS analysis confirmed the presence of nicotine in all four pro-ducts (figure 2.1B, E, F). The limit of quantification for this method was 10 μg/mL. Nicotine was quantifiable in two bottles, which had concentrations of 14.2 and 95.4 mg/mL (figure 2.1B). Nicotine can be fatal to humans in doses of 500–1000 mg for adults [5] and 10 mg for children [6]. The total nicotine content in these two 5 mL bottles of DIY flavourings was 71 and 477 mg, doses that, if ingested, could be fatal to children and, possibly, to adults. Assuming a dilution factor of 1:10 (http://www.tastypuff.com/product/joosy-froot/), a solution mixed from the Joosy Froot flavour and propylene glycol alone would contain 9.5 mg/mL. These data demonstrate that DIY flavouring products, which are marketed for the purpose of flavour

enhancement, may contain substantial amounts of nicotine. These DIY flavourings are named and described in terms that are attractive to children, have colourful attractive bottles and emit an aroma that may encourage ingestion. Some adult users of ECs are not addicted to nicotine and would avoid the use of these products if nicotine content were indicated on the label.

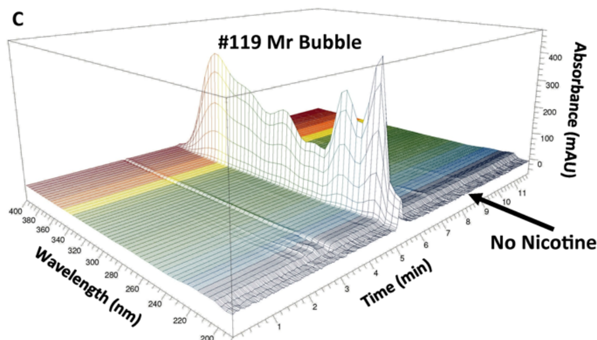
The current finding of nicotine in DIY flavouring products that are expected to be nicotine free and our prior finding that a DIY bottle of nicotine (134.7 mg/mL) was unlabeled [3], are important public health problems. These products, which are presented to the consumer as ‘nicotine free’ (http://www.tastypuff.com/product/joosy-froot/), could lead to unwanted addiction, poisoning, or even death. The simplest solution to this problem would be, at minimum, to require that any products containing nicotine be clearly labelled with the term ‘nicotine’ and an accurate concentration. Consumers who wish to use 0% nicotine products could then avoid unwanted exposure and EC users could protect their children from accidental ingestion of nicotine. The demonstration of nicotine in presumably nicotine-free DIY flavouring solutions should be valuable information for regulatory agencies, physicians, EC users and poison control centres.

A

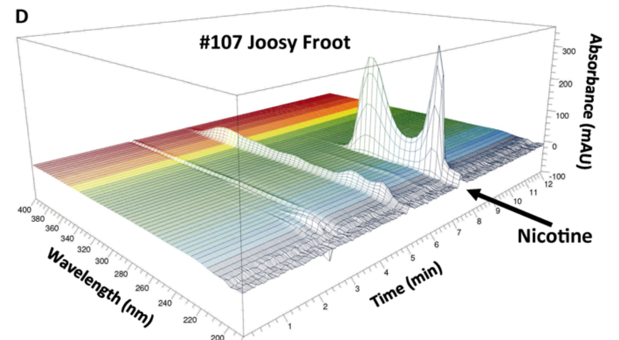
B

#	Flavor	Nicotine Concentration Determined by HPLC (mg/mL)	GC-MS Confirmation of the Presence of Nicotine
53	Sinful Cinnamon	NQ	Yes
69	Sinful Cinnamon	NQ	Yes
106	Nilly Vanilla	14.21±0.47	Yes
107	Joosy Froot	95.43±7.70	Yes

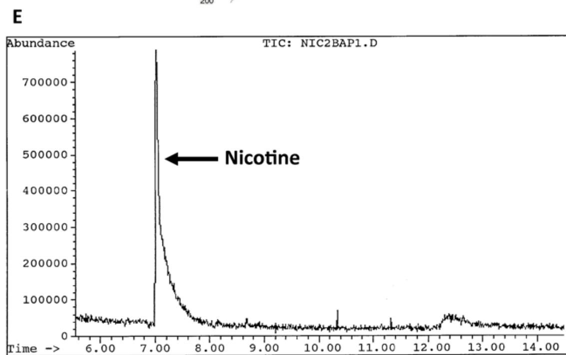
C

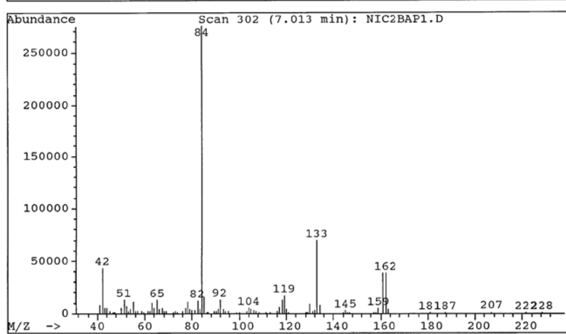


D

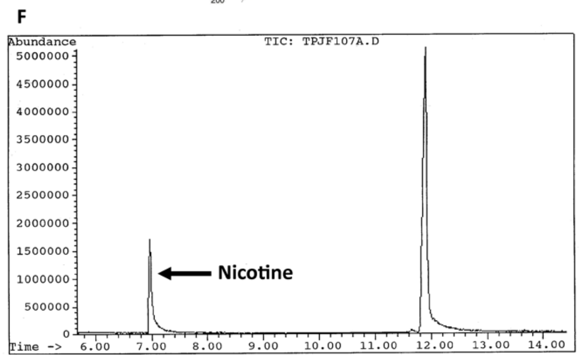


E





F



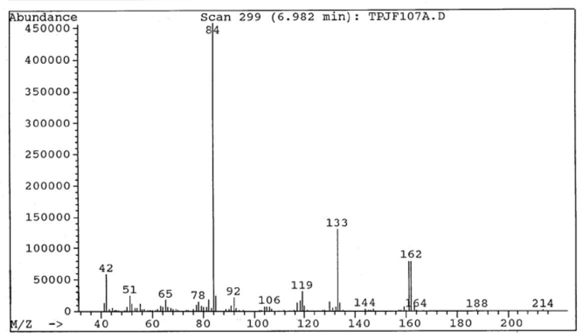


Figure 2.1. Analysis of Tasty Puff Flavorings. (A) Bottles of DIY flavorings in which nicotine was positivity identified. Bottles are indicated by inventory number, #s 53 & 69 are duplicate bottles of Sinful Cinnamon, #106 is Nilly Vanilla, and #107 is Joosy Froot. (B) Summary table of HPLC and GC-MS results. The table indicates inventory number, flavor, quantified nicotine concentration (NQ = nicotine was not quantifiable but was qualitatively determined to be present), and GC-MS confirmation of nicotine. (C and D) 3D Chromatograms of HPLC analyzed flavorings, X axis = time (minutes), Y axis = absorbance (mAu), Z axis = wavelength in nm. (C) is an example of a DIY flavor without nicotine (#119 Mr. Bubble), and (D) is an example of a DIY flavor (#107 Joosy Froot), that contains nicotine, as indicated by the presence of the bimodal peak at approximately 8 min. (E and F) are GC-MS analyses of flavorings. GC data are shown in the two upper graphs, X axis = time (minutes), Y axis =abundance (arbitrary units), and MS data are in the lower graphs, X axis = m/z, Y axis =abundance (arbitrary units). (E) shows the nicotine standard and (F) is an example of a flavoring (#107 Joosy Froot), found to be positive for nicotine.

REFERENCES

1 e-cigarette-forum.com. A Guide to making your own e-juice | E-Cigarette Forum. e-cigarette-forum.com. https://www.e-cigarette-forum.com/forum/threads/a-guide-to-making-your-own-e-juice.218802/ (accessed 5 Mar 2015).

2 National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health. *The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General*. Atlanta (GA): Centers for Disease Control and Prevention (US) 2014.

3 Davis B, Dang M, Kim J, *et al.* Nicotine concentrations in electronic cigarette refill and do-it-yourself fluids. *Nicotine Tob Res* 2015;**17**:134–41. doi:10.1093/ntr/ntu080

4 Wong LS, Green HM, Feugate JE, *et al.* Effects of “second-hand” smoke on structure and function of fibroblasts, cells that are critical for tissue repair and remodeling. *BMC Cell Biol* 2004;**5**:13–3. doi:10.1186/1471-2121-5-13

5 inchem.org. Nicotine (PIM). https://www.inchem.org/documents/pims/chemical/nicotine.htm (accessed 6 July 2015).

6 Mayer B. How much nicotine kills a human? Tracing back the generally accepted lethal dose to dubious self-experiments in the nineteenth century. *Arch Toxicol* 2014;**88**:5–7. doi:10.1007/s00204-013-1127-0

Section II: Heat-Not-Burn

CHAPTER 3

iQOS: Evidence of Pyrolysis and Release of a Toxicant from Plastic

Barbara Davis, Monique Williams, Prue Talbot

Tobacco Control Published Online First: 13 March 2018. doi:10.1136/tobaccocontrol-2017-054104

ABSTRACT

Objective To evaluate performance of the I quit original smoking (iQOS) heat-not-burn system as a function of cleaning and puffing topography, investigate the validity of manufacturer's claims that this device does not burn tobacco and determine if the polymer-film filter is potentially harmful.

Methods iQOS performance was evaluated using five running conditions incorporating two different cleaning protocols. Heatsticks were visually and stereomicroscopically inspected preuse and postuse to determine the extent of tobacco plug charring (from pyrolysis) and polymer-film filter melting, and to elucidate the effects of cleaning on charring. Gas chromatography–mass spectrometry headspace analysis was conducted on unused polymer-film filters to determine if potentially toxic chemicals are emitted from the filter during heating.

Results For all testing protocols, pressure drop decreased as puff number increased. Changes in testing protocols did not affect aerosol density. Charring due to pyrolysis (a form of organic matter thermochemical decomposition) was observed in the tobacco plug after use. When the manufacturer's cleaning instructions were followed, both charring of the tobacco plug and melting of the polymer-film filter increased. Headspace analysis of the polymer-film filter revealed the release of formaldehyde cyanohydrin at 90°C, which is well below the maximum temperature reached during normal usage.

Discussion Device usage limitations may contribute to decreases in interpuff intervals, potentially increasing user's intake of nicotine and other harmful chemicals. This study found that the tobacco plug does char and that charring increases when the device is not cleaned between heatsticks. Release of formaldehyde cyanohydrin is a concern as it is highly toxic at very low concentrations.

INTRODUCTION

With the rise of smoking alternatives, the electronic nicotine delivery systems market has boomed, with electronic cigarettes (EC) being among the most popular worldwide.[1,2] However, there are still a number of conventional (combustible) cigarette smokers who would welcome a cigarette-like tobacco-containing/nicotine-containing product that is devoid of or has a significantly reduced toxicity compared with conventional cigarettes.[1] To appeal to this demographic, Philip Morris International (PMI) has released a new product called the iQOS (I quit original smoking), which is a 'heat-not-burn' system,[3] as an alternative to conventional cigarettes and EC. The iQOS system uses a flange, called the 'heater', which is composed of a silver, gold, platinum, ceramic coating,[4] to heat a rolled, cast-leaf sheet of tobacco impregnated with glycerin, thereby creating an aerosol without combustion.[3] This aerosolization process is proposed to reduce the user's exposure to toxic and carcinogenic chemicals produced by the combustion of tobacco.[5,6] Thus, the consumer gets the 'harm reduction' component of EC along with the mouth/throat feel of a conventional cigarette. The iQOS system has been well received in Japan and Italy. The iQOS is currently sold in 26 markets by PMI with plans to expand to over 30 countries, including the USA.[7] Although this product has been extensively evaluated by the manufacturer,[3,5,6,8–13] these studies appeared in a journal that may have a deficient review process,[14] emphasizing the need for independent evaluation of the iQOS. As our initial study, we have evaluated the performance of the iQOS system under various conditions, tested the effects of cleaning on performance and pyrolysis and determined the composition of and potential health risk from the polymer-film filter.

MATERIALS AND METHODS

iQOS product acquisition and storage

Four iQOS tobacco heating system kits, manufactured by Philip Morris Products S.A. (Switzerland), were purchased online at eBay (https://www. ebay. com/) from sellers with a 98% or higher satisfaction rating. Kits arrived sealed and in excellent condition. Kits were inventoried, and the components of each kit were placed into individual plastic containers and stored in a dry area at 22°C when not in use. Cartons of Marlboro (blue box) heatsticks, manufactured by Philip Morris Brands Sàrl (Italy), were purchased in Japan and shipped to us via a personal shopper. Each carton was individually sealed and in excellent condition. Heatsticks were stored, unopened, in a dry, dark area at 22°C in their cartons until used. Unused heatsticks from opened packs were stored in an airtight bag in their carton.

Cleaning the iQOS

iQOS holders were tested using two cleaning regimens: (1) the ‘per-use’ cleaning protocol in which the device was thoroughly cleaned after each heat-stick using the cleaning sticks to remove residual fluid and tobacco plug debris from the heater and surrounding base and to clean out the cap and (2) the manufacturer’s recommended cleaning instructions in which the cleaning cycle was used after every 20 heatsticks before using the brush cleaners. When heatstick fragments were left behind, the cleaning hook was used to remove these pieces, as necessary, and the holder cap was cleaned by a 5 min warm water immersion. The instructions clearly state that the holder itself is not to be wetted.

Performance evaluation

Pressure drop, which measures the draw resistance of the heat-stick, aerosol absorbance (density), a measure of particulate matter trapped within the aerosol, and puff number were evaluated for iQOS products using equipment and protocols described previously.[15–17] Pressure drop across heatsticks was evaluated using a Cole-Parmer Masterflex L/S peristaltic pump (Vernon Hills, Illinois, USA) connected to a U-tube water manometer to detect the change in differential pressure for each puff. Airflow rates were precalculated/precalibrated to the appropriate pump speed using a conversion factor provided by the pump head manufacturer, and flow rate was verified using a Brooks Instruments Sho-Rate flow meter (Hatfield, Pennsylvania, USA). Aerosol density was evaluated by capturing aerosols in a tubular cuvette, and absorbance was measured immediately at 420 nm using a Bausch & Lomb spectrophotometer (120 V, 0.9 A, Rochester, New York, USA).

iQOS devices were evaluated with five operating conditions; four (conditions 1–4) used the per-use cleaning protocol and one (condition 5) used the manufacturer's recommended cleaning instructions. The pump head, tubing set-up and running conditions were as follows: (1) low airflow rate 2 s protocol—the peristaltic pump was outfitted with a Cole-Parmer Masterflex Model 7015-21 pump head (standard pump head) using Masterflex Tygon E-LFL (tubing size 15) tubing to generate a flow rate of 7 mL/s with a 2 s puff duration for a total puff volume of 14 mL, 14 puffs were taken at 25 s intervals; (2) low airflow rate 4 s protocol—the same pump set-up and running conditions as for condition 1 with a 4 s puff duration generating a 28 mL puff volume; (3) International Organization for Standardization (ISO)—the pump was outfitted with a Cole-Parmer Masterflex L/S Easy-Load II Model 77200-52 high-performance pump head

with Masterflex Tygon E-LFL (tubing size 15) producing a 17.5 mL/s flow rate with a 2 s puff duration, generating a total puff volume of 35 mL, with a total of six puffs taken, one puff every minute; (4) the Health Canada standard (HCI)—a Cole-Parmer Masterflex L/S Easy-Load II Model 77200-52 high-performance pump head was used with Masterflex Tygon E3603 (tubing size 36) tubing for a flow rate of 27.5 mL/s, with a 2 s puff for a total puff volume of 55 mL, 12 puffs were taken at 30 s intervals; (5) manufacturer's recommended cleaning (HCI), the same pump set-up and running conditions as described for condition 4 but in the absence of per-use cleaning; for this protocol the manufacturer's recommended cleaning instructions were followed (table 3.1). For conditions 1–4, three different iQOS devices were evaluated with each device being tested in triplicate, that is, a new heatstick was used for each experiment; condition 5 employed a single device in which 10 heatsticks were tested without cleaning between each stick.

Table 3.1. Performance of iQOS Heat-Not-burn Holders

Holder	Puff Duration)	Puff Interval	Air-flow Rate (mL/s)	Puff Volume (mL)	Total # of Puffs	Average Pressure Drop (mmH_2O)	Average Absorbance
Low Airflow Rate 2 Second Protocol[a]							
A	2	25	7	14	14	13 ± 5	0.42 ± 0.08
B	2	25	7	14	14	13 ± 4	0.45 ± 0.08
C	2	25	7	14	14	18 ± 7	0.46 ± 0.06
Low Airflow Rate 4 Second Protocol[b]							
A	4	25	7	28	14	9 ± 4	0.41 ± 0.05
B	4	25	7	28	14	11 ± 4	0.46 ± 0.09
C	4	25	7	28	14	10 ± 4	0.49 ± 0.04
ISO Standard[c]							
A	2	60	17.5	35	6	62 ± 5	0.49 ± 0.10
B	2	60	17.5	35	6	65 ± 8	0.54 ± 0.09
C	2	60	17.5	35	6	57 ± 5	0.49 ± 0.04
HCI Standard[d]							
A	2	30	27.5	55	12	103 ± 9	0.26 ± 0.03
B	2	30	27.5	55	12	100 ± 9	0.41 ± 0.05
C	2	30	27.5	55	12	105 ±13	0.42 ± 0.05
Manufacturer's Recommended Cleaning (HCI)[e]							
E	2	30	27.5	55	12	103 ± 12	0.46 ± 0.06

Note. [a] Per use cleaning protocol, standard pump head with Tygon 15 tubing.
[b] Per use cleaning protocol, standard pump head with Tygon 15 tubing.
[c] Per use cleaning protocol, high performance pump head with Tygon 15 tubing.
[d] Per use cleaning protocol, high performance pump head with Tygon 36 tubing.
[e] Manufacturer's recommended cleaning, high performance pump head with Tygon 36 tubing.

Effect of use on the tobacco plug and polymer-film filter

The condition of the tobacco plugs was evaluated by visual and microscopic insp ection and imaged using a Nikon C-LEDS stereomicroscope equipped with a Nikon Digital Sight DS-Vi1 camera head (Nikon, Minato, Tokyo, Japan) before and after use. Some heatsticks were dissected before and after use to further evaluate residual char (referred to as 'char' only) of the tobacco plugs and the condition of the polymer-film filter.

Gas chromatography–mass spectrometry analysis of iQOS heatstick polymer-film filters

Gas chromatography–mass spectrometry (GC–MS) using a qualitative wide-scope screening method was performed using an Agilent 7890B GC coupled with a 5977A MSD equipped with a 7698A Headspace Sampler (Santa Clara, California, USA). Evaluation of iQOS aerosols was performed using headspace analysis. Chromatographic separation was accomplished using an Agilent J&W HP-5ms Ultra Inert GC Column (30 mx0.25 mmx0.25 μm) and ultra-pure helium (>99.999% purity) as the carrier gas at a flow rate of 1.5 mL/min. For headspace analysis, three unused heatsticks were dissected, polymer-film filters were removed, and a 3 mm portion (16.7%) closest to the tobacco plug were excised and placed into 20 mL headspace vials. All samples were analyzed with a split ratio of 50:1, a solvent delay of 2 min, with blank analysis between each sample. GC ramp conditions were as follows: 40°C for 5 min, 45°C for 5 min, 90°C for 5 min, 130°C for 5 min, 135°C for 5 min, 165°C for 5 min, 190°C for 2 min, all temperature ramps were at 10°C/min. Ionisation of compounds was performed using electron impact ionisation at 70 eV in positive mode, the ion source

maintained at 250°C and chemicals were identified using the National Institute of Standards and Technology mass spectral library (Gaithersburg, Maryland, USA), only chemicals with an 85% or higher probably match were listed as identifiable.

RESULTS

Components in the iQOS heat-not-burn system

The iQOS kit (figure 3.1A–G) consists of an instruction manual written in German, English, Portuguese and Italian, a pocket charger, the holder (device), a Universal Serial Bus (USB) cable and a European wall adapter plug for charging, moist cleaning sticks to clean the holder and cap, and the cleaner, which contains a long brush for cleaning the inside of the holder, where the heater is housed, a short brush for cleaning the cap and a hook for removing pieces of tobacco plug left in the holder/cap. A universal power adapter was purchased from Amazon (https://www. amazon. com/) and used to charge the pocket charger unit. Each carton of iQOS heatsticks contained 10 individually wrapped packs, and each pack had 20 heatsticks (figure 3.1H).

The iQOS kit components had an overall feel of good craftsmanship. The fabrication of the tobacco plug cast-leaf demonstrates a waste not want not strategy in that the plug is fabricated from pulverised tobacco remnants/waste materials, including tobacco stems, torn leaf material and leaf dust.[18] These items are reconstituted with natural adhesives and glycerin (a solvent that is used in EC fluids to produce aerosol) and processed into sheets forming cast-leaf, which is rolled and used as the tobacco plug.[3]

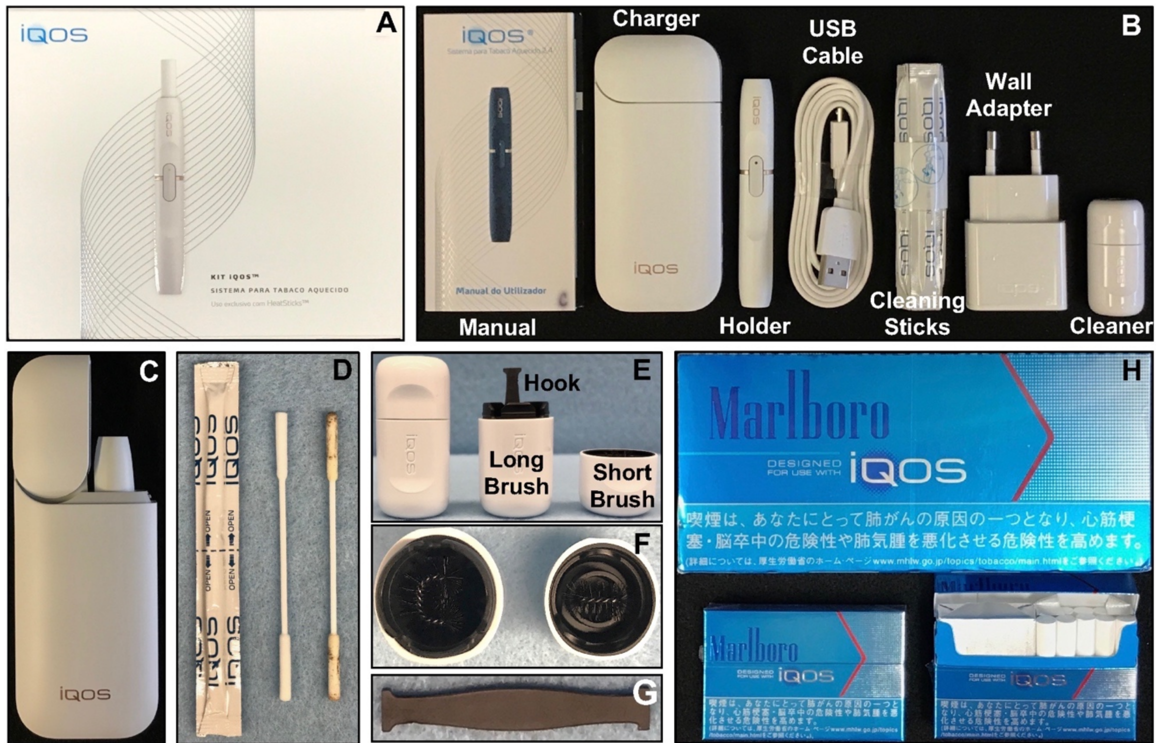


Figure 3.1. The I quit original smoking (iQOS) heat-not-burn system. (A) An iQOS starter kit. (B) The kit consists of an instruction manual, iQOS pocket charger, iQOS holder, USB cable, iQOS cleaning sticks, wall charging adapter and iQOS cleaner. (C) Profile view of iQOS holder inside a pocket charger. (D) Individual pack of iQOS cleaning sticks with an example of an unused stick and a stick after a single use per end. (E) A closed and opened iQOS cleaner; the larger end contains the long brush and protruding cleaning hook, and the shorter end contains the short brush. (F) Internal view of the iQOS cleaner showing the two brushes (long brush on the left, short brush on the right). (G) The cleaning hook removed from the iQOS cleaner. (H) Marlboro iQOS Heat Stick carton (containing 10 individual packs), sealed individual pack and opened pack exposing heatsticks.

Cleaning of iQOS device

The interior chamber of the holder contained a heating element, referred to in the iQOS instruction manual as the silver, gold, platinum, ceramic-coated heater (figure 3.2). Unused holders were clean and debris-free with a white base and white heater with a metallic coil in its centre (figure 3.2A–C). Used holders that were thoroughly cleaned with the cleaning sticks between each heatstick were generally similar to the unused holder, except that the heating element had deposits of hardened dark debris that was not removed by the cleaning stick, cleaning cycle of the pocket charger or long brush (figure 3.2D–F). In the used holder that was not cleaned between heatsticks (manufacturer's recommended cleaning), brown liquid and particulates covered the base, walls and heater (figure 3.2G–I). With continued use in the absence of cleaning, the volume of liquid and debris increased, and the pieces of debris became darker and appeared more charred (figure 3.2D–I were taken after the 10th heatstick was used).

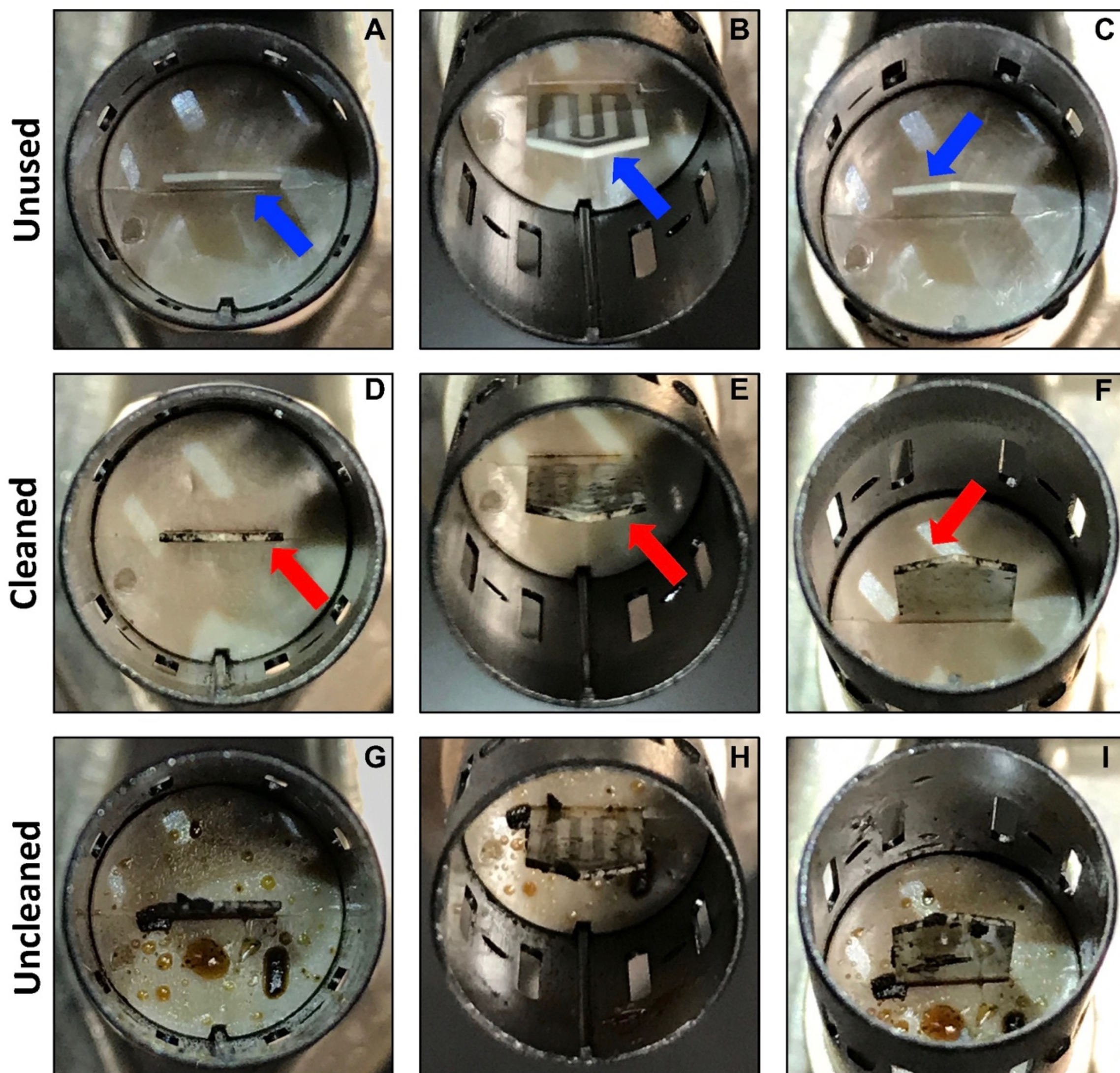


Figure 3.2. Internal view of the I quit original smoking holder. (A–C) Clean, unused holder showing heater (blue arrows). (D–F) Used holder that was cleaned after every use; black residue remains on heater (red arrows). (G–I) Used holder that was not cleaned between uses (10 uses).

iQOS performance

The iQOS gives users a maximum of 14 puffs during a 6 min window per heatstick, after which it must be recharged before it can be used again. Performance of the iQOS was evaluated using five puffing protocols (figure 3.3, table 3.1). For protocols 1–4, three different iQOS devices (holders A, B and C) were tested in triplicate, that is, a new heatstick was used for each experiment, and each device underwent an intensive cleaning between each heatstick. For protocol 5, a single device (holder E) was used, and it was not cleaned between 10 heatsticks (average of the first three heatsticks is shown in figure 3.3I,J). For all testing protocols, pressure drop decreased as puff number increased. Aerosol density readings increased with use, peaking around puffs 7–9 and then begin to decrease. Although pump set-up affected pressure drop, it did not affect aerosol absorbance which remained similar under all running conditions, However, differences in testing conditions may lead to alterations in the chemical constituents present within the aerosol without altering aerosol density. Not cleaning did not affect performance except that pressure drop was more variable during the first four puffs in the uncleaned trials.

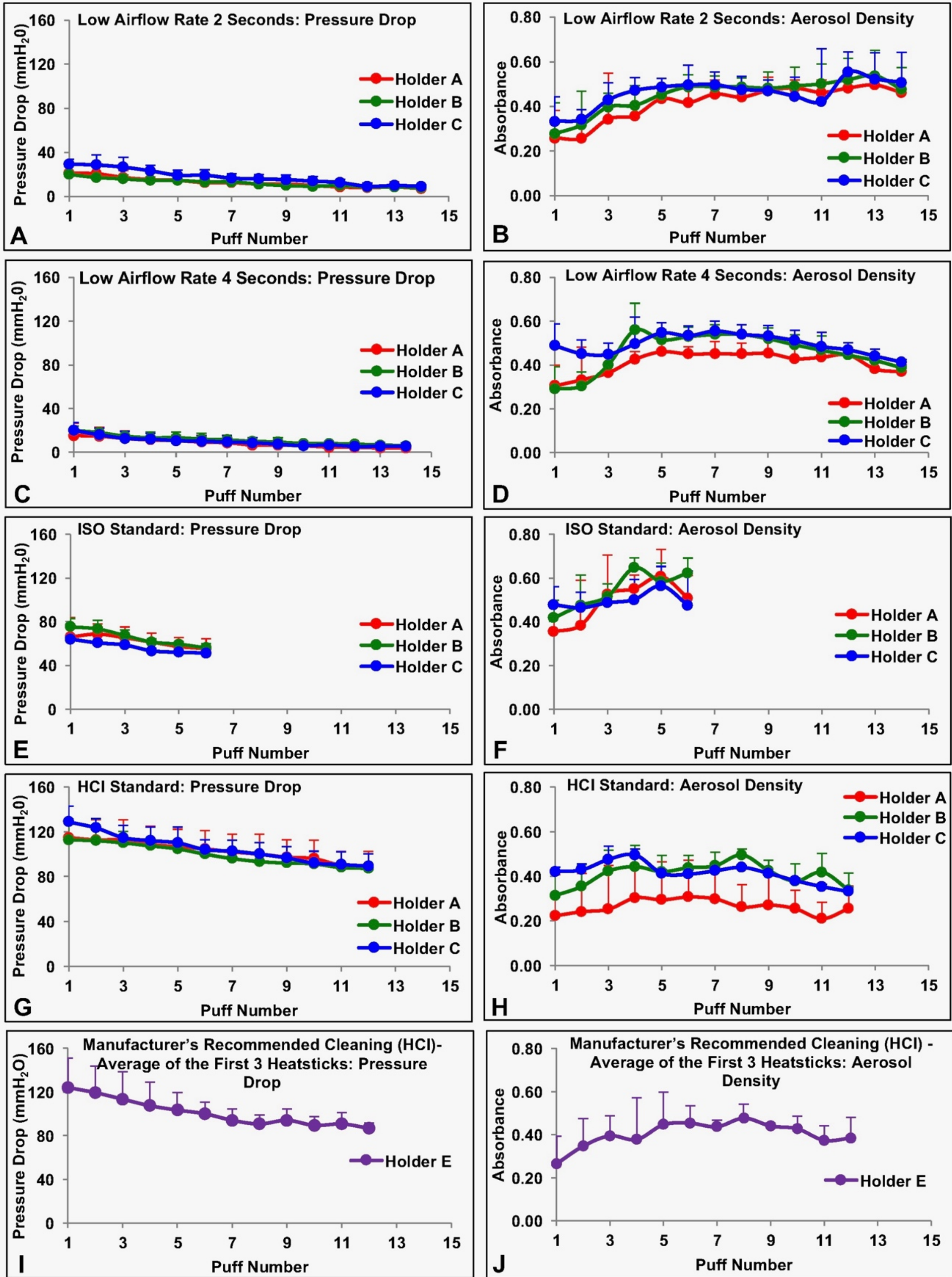

Low Airflow Rate 2 Seconds: Pressure Drop
Pressure Drop (mmH20)
Holder A
Holder B
Holder C
Puff Number
A
Low Airflow Rate 2 Seconds: Aerosol Density
Absorbance
Holder A
Holder B
Holder C
Puff Number
B
Low Airflow Rate 4 Seconds: Pressure Drop
Pressure Drop (mmH20)
Holder A
Holder B
Holder C
Puff Number
C
Low Airflow Rate 4 Seconds: Aerosol Density
Absorbance
Holder A
Holder B
Holder C
Puff Number
D
ISO Standard: Pressure Drop
Pressure Drop (mmH20)
Holder A
Holder B
Holder C
Puff Number
E
ISO Standard: Aerosol Density
Absorbance
Holder A
Holder B
Holder C
Puff Number
F
HCI Standard: Pressure Drop
Pressure Drop (mmH20)
Holder A
Holder B
Holder C
Puff Number
G
HCI Standard: Aerosol Density
Absorbance
Holder A
Holder B
Holder C
Puff Number
H
Manufacturer's Recommended Cleaning (HCI)- Average of the First 3 Heatsticks: Pressure Drop
Pressure Drop (mmH2O)
Holder E
Puff Number
I
Manufacturer's Recommended Cleaning (HCI) - Average of the First 3 Heatsticks: Aerosol Density
Absorbance
Holder E
Puff Number
J

Figure 3.3. Performance characteristics of the I quit original smoking heat-not-burn system. (A, C, E, G and I) Pressure drop is plotted versus the puff number for five puffing protocols. (B, D, F, H and J) Absorbance is plotted versus puff number for the five puffing protocols. Each line of the graph represents the average of three heatsticks for an individual holder (holder A=red, holder B=green, holder C=blue and holder E=purple). HCI, Health Canada Standard; ISO, International Organization for Standardization.

Tobacco plug charring

Dissection of unused and used heatsticks showed tobacco plug charring (figure 3.4A). Stereomicroscopic comparison of unused (figure 3.4B) and used (figure 3.4C) tobacco plugs confirmed charring or blackening of the cast-leaf. Visual and stereomicroscopic inspection of used heatsticks show the effects cleaning had on device heat production. Comparison of the first and 10th used heatstick from holder A (per-use cleaning) shows that with regular cleaning the charred area surrounding the heater, referred to as the zone of char-ring, does not increase with use (figure 3.4D,E). The effects of cleaning on heating were most evident during the course of the manufacturer's recommended cleaning (HCI) testing. Comparison of these heatsticks to unused and per-use cleaned heatsticks showed that in the absence of regular cleaning, the zone of charring increased as the number of heatsticks tested increased (figure 3.4H–L).

Polymer-film melting

Effects of cleaning on heating were not exclusive to the tobacco plug; figure 3.4A shows that the polymer-film filter (labeled 2), which is separated from the tobacco plug (condition 4) by the hollow acetate filter (condition 3), was adversely affected. The aerosol produced by the iQOS was hot enough to melt the polymer-film filter, which could allow release of potentially hazardous chemicals. Melting of the polymer-film filter was evident by slight yellowing of the filter, as well as by narrowing of the end closest to the tobacco plug (figure 3.4A indicated by black arrow). This melting and subsequent cooling of the filter caused it to harden, preventing it from being longitudinally dissected. Comparison of unused and used polymer-film filters from both per-use and manufacturer's recommended cleaning experiments showed the relationship between

cleaning and increased heat generation. First (figure 3.4F) and 10th (figure 3.4G) filters from cleaned devices showed similar discoloration and melting to that of the first filter from the uncleaned device (figure 3.4N). Comparison of these heatsticks to subsequent manufacturer's recommended cleaning used heatsticks showed discoloration and melting of the polymer-film filter increased with increased use (figure 3.4M–Q).

Headspace analysis of unused polymer-film filters

GC–MS headspace analysis of unused polymer-film filters showed the presence of ε-caprolactone and lactide, common components in plastics, as well as 1,2-diacetin, a plasticiser (figure 3.4S). However, of most concern was the presence of form-aldehyde cyanohydrin (glycolonitrile), an acute toxicant often used in the production of synthetic resins and used as a solvent.[19] Formaldehyde cyanohydrin was eluted at 17.97 min, when the column reached 90°C.

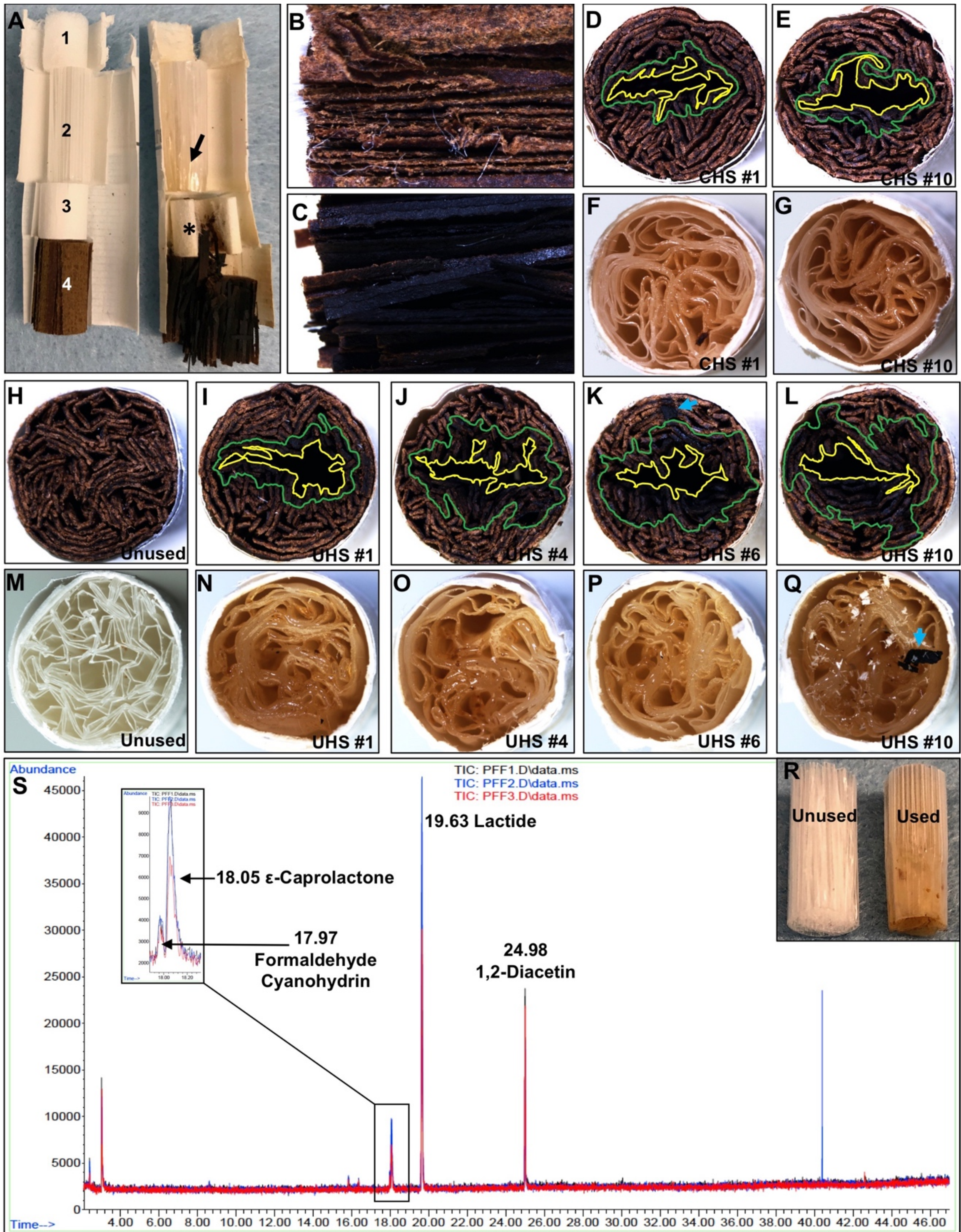

A
1
2
3
4
B
C
D
CHS #1
E
CHS #10
F
CHS #1
G
CHS #10
H
Unused
I
UHS #1
J
UHS #4
K
UHS #6
L
UHS #10
M
Unused
N
UHS #1
O
UHS #4
P
UHS #6
Q
UHS #10
R
Unused
Used
S
Abundance
TIC: PFF1.D\data.ms
TIC: PFF2.D\data.ms
TIC: PFF3.D\data.ms
19.63 Lactide
18.05 ε-Caprolactone
17.97
Formaldehyde
Cyanohydrin
24.98
1,2-Diacetin
Time-->

Figure 3.4. Charring of tobacco plug and melting of polymer-film filter. (A) Dissected heatsticks, each heatstick is composed of: (1) the low-density cellulose mouthpiece filter, (2) polymer-film filter, (3) hollow acetate tube and (4) tobacco plug. Heatsticks from left to right are unused stick with the paper overwrap peeled away, and used stick with the paper overwrap removed with the mouthpiece filter and hollow acetate tube sliced open; black arrow indicates melted region of the polymer-film filter, black asterisk denotes tobacco plug fragments that have been drawn into the hollow acetate tube. (B) An unused tobacco plug. (C) Used tobacco plug showing charring/darkening with use. (D,E) Cross sections of tobacco plugs from the first (D) and 10th (E) heatstick of holder A of the cleaned experiment. Yellow outlined area indicates a void in the cast-leaf left by the heater, the area between the yellow and green outlines are the charred portions of the tobacco plug. (F,G) Cross sections of polymer-film filter from the first (F) and 10th (G) heat stick. Polymer-film filter images shown coincide with tobacco plug images (D) and (E). (For D–G, CHS=cleaned device heatstick.) (H–L) Cross sections of tobacco plugs before use (H) and after use from the first, fourth, sixth and 10th heatstick of the uncleaned experiment (I–L). Yellow outlined area indicates a void in the cast-leaf left by the heater, area between the yellow and green outlines are the charred portions of the tobacco plug. (M–Q) Cross sections of polymer-film filter before (M) and after use (N–Q). Polymer-film filter images shown coincide with tobacco plug images (H–L). Blue arrowheads show charred pieces of cast-leaf that are affixed to the tobacco plug (K) and polymer-film filter (Q). (For (I–L) and (N–Q), UHS=uncleaned device heatstick). (R) Unused and used whole polymer-film filters showing discoloration and film melting, as demonstrated by the narrowing of the used filter. (S) Gas chromatography–mass spectrometry headspace analysis of unused polymer-film filter. Chromatogram shows an overlay of three runs, relative abundance was plotted versus retention time in minutes, unidentifiable peaks were unlabelled. Inset shows a magnified view of peaks with close retention times.

DISCUSSION

Unlike some EC, which often show significant variation in craftsmanship and performance within and between brands,[15,20] the iQOS appearance, design and performance data are consistent with a product that is well manufactured. However, some design features of the iQOS, such the limited time allowed per heatstick and the need to consume the entire heatstick within this time or alternatively waste part of it, will affect user's topography and may lead to unwanted exposure to potentially toxic chemicals emitted from melting plastic and from pyrolysis of tobacco.

In contrast to tobacco and EC, which usually have no constraints on puffing, the iQOS only operates for 6 min, at which time it automatically shuts off and requires charging before it can be used again. Since a maximum of 14 puffs can be taken from each iQOS heatstick, puffing needs to be done at about 25 s intervals to take full advantage of each heatstick; used heat-sticks that have not been fully exhausted cannot be used again as reinsertion would cause the delicate cast-leaf tobacco plug to crumble. This may not appeal to all users, and users who puff less frequently would have a lower number of puffs/heatstick. For users wishing to maximise each heatstick, this limitation will force them to alter their smoking topography by decreasing the interpuff interval and/or accelerating the rate at which they puff, leading to larger volumes of aerosol inhalation.

The manufacturer's cleaning instructions were not fully developed in the instruction manual. The cleaning protocol recommended using the cleaning function of the charger followed by cleaning with the brushes after 20 heatsticks and removing any large fragments of tobacco plug with the hook if necessary. The iQOS kit was equipped with cleaning sticks (figure 3.1B,D), yet their use was not mentioned in the instruction

manual. Our data show that use of one heatstick left a significant amount of debris, fluid and fragments of cast-leaf in the holder (figure 3.2).

While iQOS heatsticks do not produce a flame, they were always charred after use, which we interpret to be a result of pyrolysis. The zone of charring was greater when cleaning was not performed between heatsticks, suggesting that build-up of fluid and debris in the holder increases pyrolytic temperatures. These data are consistent with the idea that despite similarities in performance characteristics, the cleanliness of the device plays a critical role in thermal regulation. Pyrolysis of tobacco is an endothermic reaction which occurs at tempera-tures between 200°C and 600°C, during which the majority of volatile and semivolatile components of cigarette smoke are formed.[21,22] Although the Philip Morris study indicated that the aerosol produced by iQOS devices reduce the amount of chemicals found on the Food and Drug Administration's Harmful and Potentially Harmful Constituents list by limiting tobacco pyrolysis,[5] our study, showing charring, in conjunction with a study by Auer et al, which confirmed the presence of volatile organic compounds, polycyclic aromatic hydrocarbons, carbon dioxide and nitric oxide,[23] contradict the claim that tobacco pyrolysis is minimised in iQOS. Although iQOS operates at temperatures less than 350°C, this does not negate the formation of volatile and semivolatile harmful constituents of tobacco smoke, which tend to have boiling points that range from 70°C to 300°C.[21,22] Heatsticks used in this experiment were dissected and the severity of polymer-film filter melting was examined. The function of the polymer-film filter is to cool the aerosol,[3] thus, it would seem that the polymer composing the film should be heat resistant, although, ε-caprolactone, also known as polycaprolactone, tends to have a low-melting point which is thickness dependent.[24] The intensity of the heat produced by the iQOS, under both cleaned and

uncleaned conditions, was sufficient to melt the polymer-film filter, even though it was not in direct contact with the heater. The amount of damage to the film (increase in melt and alteration of coloration) increased with each heatstick when cleaning was done per the manufacturer's recommended procedure (after 20 heatsticks). Discoloration may be a product of heating and/or staining from the brown fluid that is expelled from the tobacco plug during use.

Our GC–MS data indicate that components of the polymer-film filter are aerosolised at relatively low temperatures. GC–MS headspace analysis of unused filters suggests the polymer-film filter is a combination of ε-caprolactone, lactide, 1,2-diacetin and other unidentified chemicals. The chemicals released from the film filter during heating may not be suitable for inhalation. Thus, it is unknown if the film filter material is safe for use in products where it would undergo intense cycles of heating and cooling. Of greatest concern was the release from the polymer filter of formaldehyde cyanohydrin, a highly toxic chemical that is metabolised in the liver and broken down into formaldehyde and cyanide.[19] Formaldehyde cyanohydrin can be fatal to humans,[19,25,26] with studies showing mouse inhalation LD_{Lo}, the lowest dose of a toxicant that causes the death of an animal,[27] values as 27 ppm/8 hour.[28,29] iQOS holders operate at temperatures between 330°C and 349°C,[3,23] and as a safety feature, the device shuts off when temperatures reach 350°C. The release of formaldehyde cyanohydrin from unused filters during GC–MS analysis occurred at 90°C, a temperature that all users will exceed.

In conclusion, the iQOS appears to be well manufactured, and performance data were consistent between heatsticks. However, the product has limitations that will affect user topography and the application of standard smoking protocols, such as the ISO

3308, which could not be used for more than six puffs with this product. Users may be forced to smoke at a rapid pace in order to fully maximise heatsticks. Decreasing the interpuff interval could lead to an increase in intake of nicotine [30] and carbonyls.[31] This study also showed that the iQOS is not strictly a 'heat-not-burn' tobacco product. The iQOS tobacco appeared to char without ignition, and charring increased when cleaning was not done after each use. This study also showed the potential dangers that the polymer-film filter poses. This thin plastic sheet, readily melts during iQOS use and releases formaldehyde cyanohydrin, a dangerous toxicant. This study has shown that the iQOS system may not be as harm-free as claimed and also emphasises the urgent need for further safety testing as the popularity and user base of this product is growing rapidly.

REFERENCES

1 Marynak KL, Gammon DG, King BA, et al. National and State trends in sales of cigarettes and E-cigarettes, U.S, 2011-2015. Am J Prev Med 2017;53:96–101.

2 CDC Office on Smoking. Smoking and tobacco use; fact sheet; economic facts about U.S. Tobacco production and use.

3 Smith MR, Clark B, Lüdicke F, et al. Evaluation of the tobacco heating system 2.2. Part 1: Description of the system and the scientific assessment program. Regul Toxicol Pharmacol 2016;81(Suppl 2):S17–26.

4 Phillip Morris International. iQOS user manual. 2007;67:37. 2nd ed https://www.pmiscience. com/ sites/ default/ files/ appendix_ 3_-_ ths_ safety_ warnings_ and_ instructions. pdf

5 Schaller JP, Keller D, Poget L, et al. Evaluation of the tobacco heating system 2.2. Part 2: Chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol. Regul Toxicol Pharmacol 2016;81(Suppl 2):S27–47.

6 Schaller JP, Pijnenburg JP, Ajithkumar A, et al. Evaluation of the tobacco heating system 2.2. Part 3: Influence of the tobacco blend on the formation of harmful and potentially harmful constituents of the Tobacco Heating System 2.2 aerosol. Regul Toxicol Pharmacol 2016;81(Suppl 2):S48–58.

7 iQOS. Phillip Morris International. https://www. pmi. com/ smoke- free- products (accessed 8 Oct 2017).

8 Wong ET, Kogel U, Veljkovic E, et al. Evaluation of the tobacco heating system 2.2 Part 4: 90-day OECD 413 rat inhalation study with systems toxicology endpoints demonstrates reduced exposure effects compared with cigarette smoke. Regul Toxicol Pharmacol 2016;81(Suppl 2):S59–81.

9 Sewer A, Kogel U, Talikka M, et al. Evaluation of the tobacco heating system 2.2 (THS2.2). Part 5: microRNA expression from a 90-day rat inhalation study indicates that exposure to THS2.2 aerosol causes reduced effects on lung tissue compared with cigarette smoke. Regul Toxicol Pharmacol 2016;81(Suppl 2):S82–92.

10 Oviedo A, Lebrun S, Kogel U, et al. Evaluation of the tobacco heating system 2.2. Part 6: 90-day OECD 413 rat inhalation study with systems toxicology endpoints demonstrates reduced exposure effects of a mentholated version compared with mentholated and non-mentholated cigarette smoke. Regul Toxicol Pharmacol 2016;81(Suppl 2):S93–122.

11 Kogel U, Titz B, Schlage WK, et al. Evaluation of the tobacco heating system 2.2. Part 7: systems toxicological assessment of a mentholated version revealed reduced cellular and molecular exposure effects compared with mentholated and non-mentholated cigarette smoke. Regul Toxicol Pharmacol 2016;81(Suppl 2):S123–38.

12 Haziza C, de La Bourdonnaye G, Skiada D, et al. Evaluation of the tobacco heating system 2.2. Part 8: 5-day randomized reduced exposure clinical study in Poland. Regul Toxicol Pharmacol 2016;81(Suppl 2):S139–50.

13 Martin F, Talikka M, Ivanov NV, et al. Evaluation of the tobacco heating system 2.2. Part 9: Application of systems pharmacology to identify exposure response markers in peripheral blood of smokers switching to THS2.2. Regul Toxicol Pharmacol 2016;81:S151–7.

14 Velicer C, St Helen G, Glantz SA. Tobacco papers and tobacco industry ties in regulatory toxicology and pharmacology. J Public Health Policy

15 2018;39:e1001145–15.

16 Williams M, Talbot P. Variability among electronic cigarettes in the pressure drop, airflow rate, and aerosol production. Nicotine Tob Res 2011;13:1276–83.

17 Knoll M, Talbot P. Cigarette smoke inhibits oocyte cumulus complex pick-up by the oviduct in vitro independent of ciliary beat frequency. Reprod Toxicol 1998;12:57–68.

18 Knoll M, Shaoulian R, Magers T, et al. Ciliary beat frequency of hamster oviducts is decreased in vitro by exposure to solutions of mainstream and sidestream cigarette smoke. Biol Reprod 1995;53:29–37.

19 Phillip Morris Incorporated, Phillip. PatentNumber: 5,724,998. Phillip Morris Incorporated, ed. 2013;697:1–13.

20 Hall AH, Isom GE, Rockwood GA. Toxicology of cyanides and cyanogens: John Wiley & Sons, 2015.

21 Williams M, Villarreal A, Davis B, et al. Comparison of the Performance of Cartomizer Style Electronic Cigarettes from Major Tobacco and Independent Manufacturers. PLoS One 2016;11:e0149251.

22 Baker RR. A review of pyrolysis studies to unravel reaction steps in burning tobacco. J Anal Appl Pyrolysis 1987;11:555–73.

23 Baker RR, Bishop LJ. The pyrolysis of tobacco ingredients. J Anal Appl Pyrolysis 2004;71:223–311.

24 Auer R, Concha-Lozano N, Jacot-Sadowski I, et al. Heat-not-burn tobacco cigarettes: smoke by any other name. JAMA Intern Med 2017;177:1050–2.

25 Azimi B, Nourpanah P, Rabiee M. null) SA. Poly (ε-caprolactone) fiber: an overview. J Eng Fiber Fabr 2014;9:74–90.

26 Thomas TA, Brooks JW. Accidental cyanide poisoning. Anaesthesia 1970;25:110–4.

27 Zheng S, Yuan X, Wang W, et al. Two fatal intoxications with cyanohydrins. J Anal Toxicol 2016;40:388–95.

28 Tardiff RG, Rodricks JV, eds. Toxic substances and human risk: principles of data interpretation. New York: Springer US, 1987. doi.

29 National Library of Medicine HSDB Database. Glycolonitrile. Toxnet toxicology data network. https:// toxnet. nlm. nih. gov/ cgi- bin/ sis/ search/ a? dbs+ hsdb:@ term+@ DOCNO+ 2123 (accessed 21 Aug 2017).

30 Registry of toxic effects of chemical substances. Acetonitrile, Hydroxy 2017:1–2.

31 Williams JM, Gandhi KK, Lu SE, et al. Shorter interpuff interval is associated with higher nicotine intake in smokers with schizophrenia. Drug Alcohol Depend 2011;118:313–9.

32 Reilly SM, Goel R, Bitzer Z, et al. Effects of topography-related puff parameters on carbonyl delivery in mainstream cigarette smoke. Chem Res Toxicol 2017;30:1463–9.

CHAPTER 4

Comparison of cytotoxicity of IQOS aerosols to smoke from Marlboro Red and 3R4F reference cigarettes

Barbara Davis, Vivian To, Prue Talbot

ABSTRACT

IQOS heat-not-burn cigarette distribution and sales have increased with little understanding of their health effects. This study compared the cytotoxicity of IQOS aerosols to smoke from Marlboro Red (MR) and 3R4F reference cigarettes. Aerosol/smoke solutions were tested as the gas vapor phase (GVP), particulate phase (total particulate matter or TPM), or whole aerosol/smoke (WA). Cytotoxicity was evaluated using the LDH, MTT and neutral red uptake (NRU) assays in conjunction with eight different cell types, mainly from the respiratory system. Most test solutions did not compromise the plasma membranes of cells (LDH). However, mitochondrial activity (MTT), and dye uptake/lysosomal activity (NRU) were depressed by IOQS aerosols and cigarette smoke solutions. 3% TPM, 30% GVP and 30% WA were cytotoxic in most MTT and NRU tests. Embryonic stem cells were among the most sensitive, respiratory tract cells were moderately sensitive, and cancer cells were the least sensitive to treatments. Two cleaning procedures for the IQOS produced similar results in most cases. MR and 3R4F smoke solutions were generally not significantly different from each other. In some cases, IQOS and 3R4F treatments were not significantly different in the MTT and NRU assays, indicating that IQOS was as harmful to some cells as cigarette smoke.

INTRODUCTION

IQOS is a novel heat-not-burn cigarette released by Phillip Morris International (PMI) in 2014. Initially sold only to Japanese and Italian test markets, IQOS is now available in 41 countries and in Duty Free shops worldwide [1]. IQOS functions by heating a cast-leaf tobacco sheet, producing an aerosol without burning of the tobacco [2,3]. Although marketed as a harm reduction product, there is currently little published data on the health effects of IQOS aerosol. The manufacturer has written nine papers evaluating the IQOS system. Of these, only one focused on cytotoxicity [4]. In this study, a filter was used to separate the gas vapor phase (GVP), which passed through the filter and was captured in phosphate-buffered saline solution, from total particulate matter (TPM), which was captured on the filter and solubilized in dimethyl sulfoxide (DMSO). IQOS aerosols were tested for cytotoxicity on mouse embryonic fibroblasts (NIH/3T3) using the neutral red uptake assay (NRU). From these data, it was concluded that IQOS aerosols were less cytotoxic than 3R4F reference cigarettes.

The purpose of our study was to repeat the cytotoxicity tests done by Schaller et al. [4] and to broaden the screen to include eight cell types, three cytotoxicity assays, and Marlboro Red cigarettes in addition to 3R4F research cigarettes. Aerosol/smoke solutions were tested as GVP and TMP, emulating the PMI method, and as whole aerosol/smoke (WA) collected in complete cell culture medium, which better models user exposure. IQOS aerosols were generated using two device cleanliness conditions, C1, in which the Holder was cleaned between each heatstick, and C20, in which the Holder was cleaned after the 20th heatstick, as described in the IQOS instruction manual. In a previous study, a lack of cleanliness in the IQOS Holder increased tobacco plug charring and polymer-film filter melting in the C20 samples [3].

One cell line from the mouse and seven cell types from humans were tested. NIH/3T3 cells are a hardy and fast-growing spontaneously transformed line of mouse embryonic fibroblasts, which were used in the PMI study [4]. In addition, we tested: (1) A-549 cells isolated by others from an epithelial lung carcinoma; (2) BEAS-2B cells, an immortalized human bronchial epithelial cell line, often used for toxicity testing; (3) three primary human bronchial epithelial cell types (NHBE) from a child, an adult nonsmoker, and an adult smoker; (4) normal human lung fibroblasts (NHLF), which play a critical role in lung homeostasis, repair and remodeling and in previous studies have been highly sensitive to toxicant exposure [5,6]; and (5) H9 human embryonic stem cells (H9-hESC) which were used as an *in vitro* human embryo model.

Three assays were used to compare the cytotoxicity of IQOS to that of Marlboro Red (MR) and 3R4F research cigarettes. The assays were: (1) lactate dehydrogenase (LDH), which assesses cell viability/death through leakage of the plasma membrane; (2) (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT), which assesses metabolic activity via mitochondrial reductase function; and (3) NRU, which assesses dye uptake by cells and sequestration in lysosomes. Using multiple cytotoxicity assays is important as treatments may not all affect the same endpoint.

MATERIALS & METHODS

Product Acquisition and Storage

IQOS Heat-not-burn kits (Phillip Morris Products S.A. (Switzerland) and cartons of IQOS Marlboro (blue box) heatsticks (Phillip Morris Brands Sàrl, (Italy) were purchased and stored as previous described [3]. Marlboro Red cigarettes (Philip Morris USA Inc., Richmond, VA) were purchased at Wal-Mart. 3R4F research cigarettes were purchased from the University of Kentucky.

Aerosol and Smoke Solution Production

IQOS aerosols were generated under two conditions, a per-use cleaning protocol (C1), in which the Holder was cleaned between each heatstick, and the manufacturer's recommended cleaning protocol (C20), in which the device was cleaned after the 20th heatstick. Smoke solutions were produced using MR and 3R4F reference cigarettes. Two types of aerosol/smoke solutions were produced, fractionated, which employed a 47 mm Single Stage Filter Assembly (Savillex, Eden Prairie, MN) outfitted with 47 mm Emfab membrane filter (Pall Life Sciences, Ann Arbor, MI), and complete medium, which did not utilize a filter. All aerosol/smoke solutions were made using the following smoking machine configuration: the mouthpiece filter end of the IQOS heatstick (inserted into the IQOS Holder) was inserted into one free end of a 3/8-inch T-Type connector (Thermo Scientific, Rochester, NY). The connector fit tightly and did not allow any air to be pulled into the smoking machine from outside of the heatstick. One end of the T-connector was used to block air flow allowing for the activation of the puff, and the other end was either connected to the filter assembly (fractionated) or connected directly (complete medium) to two in-line glass absorption impingers, custom modified by Kimball Chase (Rockwood,

TN). For fractionated method, the first impinger contained 50 mL of ice-cold Dulbecco's phosphate-buffered saline (DPBS) solution without Ca^{2+} and Mg^{2+} (Lonza, Walkersville, MD), while for WA, the impinger contained 50 mL of cell specific culture medium. For both, the second impinger contained ice-cold deionized water, both impingers were placed into an ice bath during the course of aerosol/smoke collection. The impingers were then connected to a Cole-Palmer Masterflex L/S peristatic pump (Vernon Hills, IL) equipped with a Cole-Palmer Masterflex L/S Easy-Load II Model 77200-52 high performance pump head and utilizing Masterflex Tygon E3603 (Tubing Size 36). This configuration allows for the application of the Health Canada standard (HCI) smoking protocol [7] which requires a 2 second puff that generates a total puff volume of 55 mL (27.5mL/sec), with an interpuff interval of 30 seconds.

Fractionated aerosols/smoke were composed of two parts, a gas vapor phase (GVP), which was immobilized in the DPBS of impinger one, and the total particulate matter (TPM) which was trapped onto the Emfab membrane filters and desorbed by solubilization in 50 mL of dimethyl sulfoxide (DMSO) (Fisher Scientific, Fair Lawn, NJ). Filters were changed after every two heatsticks or cigarettes. For IQOS, a total of 11 Emfab membrane filters were used products (12 puffs per heatstick for a total of 21 heatsticks) and for cigarette products a total of 13 (10 puffs per cigarette for a total of 25 cigarettes). All aerosol/smoke concentrations were expressed as a percent of solution. All solutions were aliquoted into 450 µL volumes and placed in 0.5 mL locking lid microcentrifuge tubes (Fisher Scientific, Fair Lawn, NJ) to reduce headspace, and stored at -80ºC until needed. For each aerosol type, a total of 250 puffs were taken. Heatsticks were designed to have a maximum of 14 puffs but only 12 puffs were taken from each

heatstick because this device automatically shuts off after 6 minutes of use and must be recharged.

MR cigarettes were smoked to a butt length of 35 mm, approximately 10 puffs/cigarette. The overall length of the 3R4F reference cigarettes was longer than MR by 5 mm. The filter portion of the reference cigarette was 8 mm longer than that of the MR and the tobacco filled portion was 3 mm shorter thus it was decided to mimic puff number when smoking the reference cigarette as opposed to butt length.

Cell Types and Cell Culture

NIH/3T3 mouse embryonic fibroblasts (NIH/3T3) (ATCC, Manassas, VA), A-549 human lung carcinoma cells (A-549) (ATCC, Manassas, VA), normal human lung fibroblasts (NHLF) (Lonza, Walkersville, MD), BEAS-2B (ATCC, Manassas, VA), all normal human bronchial epithelial cells (NHBE) (child, adult non-smoker, and adult smoker) (MatTek, Ashland, MA), and H9 human embryonic stem cells (H9-hESC) (WiCell, Madison, WI) were maintained and cultured as described in supplemental text SP4.1.

Cytotoxicity Assays

For all cytotoxicity assays, lactate dehydrogenase (LDH), 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT), 3-amino-7dimethyl-2-methylphenazine hydrochloride (Neutral Red dye Uptake, NRU), cells were plated at cell-type specific densities (Supplemental text SP4.1) and allowed to attach for 24 hrs. Cells were then treated with 3% TPM, 3 and 30% GVP and 30% WA for 24 hrs (Supplemental text SP4.1). After treatment, cells were subjected to either the LDH, MTT or NRU assays.

For LDH, the threshold of cytotoxicity was determined as a 30% increase on the y-axis between the lowest and highest concentration. For MTT and NRU, the threshold of cytotoxicity was set at <70% of the control, as determined by ISO 10993-5 [8]. Each experiment was performed three times. Statistical analyses of concentration-response data were performed using Minitab 18 software (State College, PA). Effects of treatment were determined using a two-way ANOVA followed by Fisher's post-hoc test with a Bonferroni correction. To rank cell type sensitivity, a one-way ANOVA with Fisher's post-hoc test was used.

RESULTS

Overview of Testing

The cytotoxicities of IQOS (C1 and C20) and conventional cigarettes (MR and 3R4F) were evaluated for eight cell types using the MTT, NRU and LDH assays. Three types of aerosol fluid were compared for each IQOS/cigarette group. These were: (1) 3% TPM, (2) 3% GVP, and (3) 30% GVP. Five of the eight cell types (NIH/3T3, A549, NHLF, NHBE-smoker and nonsmoker) were also tested using 30% WA.

Figure 4.1 shows representative concentration-response graphs for WA using NHBE-nonsmoker cells. In the LDH assay (Figure 4.1A), none of the treatments produced cytotoxicity, indicating cell plasma membrane integrity was not compromised. In the MTT assay, the aerosols from conventional cigarettes were more cytotoxic than those from IQOS at the lower concentrations, but cytotoxicities were equivalent at a concentration of 10 and 30% (Figure 4.1B). In the NRU assay, cytotoxicities were similar for all four treatment groups (Figure 4.1C). Concentration-response curves for all aerosol

treatments and cell types are presented in supplemental data SP4.2-SP4.9 for each cytotoxicity assay.

Figures 4.2 through 4.4 report amalgamated data from the LDH, MTT and NRU assays, respectively. Data presented in these figures are the means of the percent of control for the highest tested concentration for each treatment and cell type. Statistical comparisons were done between each group and the 3R4F group, and treatments that were significantly different from the 3RF4 cigarettes are indicated by asterisks. Pale red boxes indicate that a treatment was cytotoxic (i.e., an IC_{70} or >30% reduction from the untreated control was observed), and bright red boxes indicate IQOS values that were not significantly different from the 3R4F group. Green boxes indicate a lack of cytotoxicity. Pale blue boxes indicate cell type(s) that had statistical differences between C1 and C20 for 3% TPM and the bright blue box indicates cell type(s) that had statistical differences between C1 and C20 for 3% TPM and 30% WA. The next sections summarize the data for all treatments and cell types.

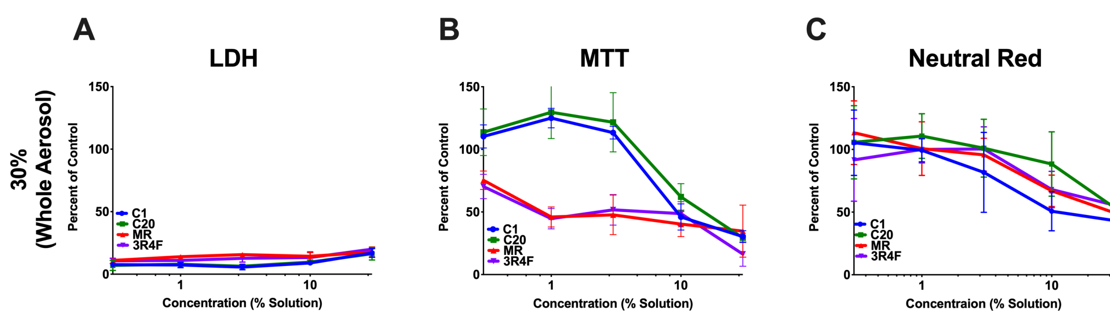


Figure 4.1. Representative concentration-response graphs for NHBE-nonsmoker treated with 30% WA. (A) LDH, (B) MTT, and (C) NRU assays. For all graphs x-axis = concentration in % solution, y-axis = percent of experimental control. Blue = C1, green = C20, red = Marlboro Red, purple = 3R4F. Each line represents the mean ± SD of three independent experiments.

LDH Assay

All but four IQOS treatments and most conventional cigarette treatments were not cytotoxic with the LDH assay (Figure 4.2). The most noteworthy results were: (1) for TPM, only two of 16 conventional cigarette treatments were cytotoxic (30% change from untreated control); (2) for 3% GVP, only one of 16 conventional cigarette treatments were cytotoxic; (3) for 30% GVP, 13 of 32 treatments were cytotoxic and this included IQOS treatments for NHLF and BEAS-2B cells; and (4) for WA, cytotoxicities were only seen in three of 20 treatments (NIH/3T3, A549 and NHLF). Two-way ANOVA results showed no statistical significance when comparing the effects of aerosol/smoke treatment and concentration on untreated control values. These data show that most treatments were not making cell plasma membranes leaky and killing cells. Additional assays were next used to determine if metabolism (MTT) and dye uptake and lysosomal integrity (NRU) were affected by treatments.

LDH Assay

Cell line		3% TPM	3% GVP	30% GVP	30% WA
NIH/3T3	C1	13	17	2	12
	C20	9	9	5	11
	MR	2	7	18	46
	3R4F	4	10	19	30
A549	C1	17	18	17	13
	C20	17	17	19	14
	MR	16	16	15	27
	3R4F	17	16	16	28
NHLF	C1	14	21	43	9
	C20	16	20	44	10
	MR	28	36	37	57
	3R4F	19	26	32	60
NHBE-Smoker	C1	16	16	17	16
	C20	10	18	21	13
	MR	31	17	31	24
	3R4F	27	19	31	19
NHBE-Nonsmoker	C1	9	18	16	17
	C20	11	15	20	16
	MR	23	12	23	18
	3R4F	27	19	22	20
NHBE-Child	C1	8	16	20	n/a
	C20	11	12	23	n/a
	MR	25	14	33	n/a
	3R4F	21	15	30	n/a
BEAS-2B	C1	2	2	35	n/a
	C20	2	2	45	n/a
	MR	8	15	32	n/a
	3R4F	6	3	37	n/a
H9-hESC	C1	26	23	20	n/a
	C20	27	25	23	n/a
	MR	29	26	28	n/a
	3R4F	33	24	46	n/a

Figure 4.2. LDH cytotoxicity data for IQOS and conventional cigarettes. Columns show the four types of aerosol/smoke solution that were tested. Rows show the cell types and product source of the aerosol/smoke solutions. Data are the means of three experiments at the highest concentration tested for each type of aerosol/smoke solution. TPM = total particulate matter of fractionated aerosol/smoke; GVP = gas vapor phase of fractionated aerosol/ smoke; WA = whole aerosol/smoke captured in culture medium. C1 = IQOS cleaned after each use; C20 = IQOS cleaned after MR = Marlboro Red cigarettes; 3R4F = reference cigarette. Pale red boxes = >30% percent of control; green boxes = ≤30% percent of control.

MTT Assay

Most 3% TPM test samples (33 of 40) were cytotoxic in the MTT assay (Figure 4.3). Both conventional cigarettes were cytotoxic to all eight cell types, and IQOS C1 and C20 were cytotoxic to NIH/3T3, NHLF, BEAS-2B, and H9-hESC. The 3% TPM results for A549 (C1), NIH/3T3(C1 and C20) and H9-hESC (C1 and C20) were not significantly different from 3R4F cigarettes (Figure 4.3 red boxes), indicating that for these comparisons, IQOS and 3R4F cigarettes were equivalent in cytotoxicity.

The 3% GVP treatments were in general not cytotoxic. For conventional cigarette treatments, cytotoxicity was only observed for four cell types (NIH/3T3, NHLF, NHBE-child 3R4F and BEAS-2B MR), and IQOS treatments were not cytotoxic to any cell type. In contrast, 30% GVP was cytotoxic to almost all cells, the only exceptions being IQOS C1 and C20 for NIH/3T3 and A549 cells. Results with NHLF, NHBE-child (C20), and BEAS-2B were not significantly different from those with the 3RF4 group (Figure 4.3 red boxes).

30% WA, which was tested with five cell types, was generally cytotoxic. WA from both conventional cigarettes was cytotoxic to all cell types, and IQOS WA was cytotoxic to three of five cell types (NIH/3T3, NHLF, and NHBE-nonsmoker). The effect of WA C1 and C20 on NHBE-nonsmoker was not statistically different from 3R4F (Figure 4.3 red boxes).

The MR and 3R4F groups were not statistically different for any cell type or treatment group except NHLF exposed to 3% GVP for which MR was significantly more cytotoxic than 3R4F ($p < 0.003125$). There were no significant differences between C1 and C20 IQOS for any cell type or treatment group

MTT Assay

		3% TPM	3% GVP	30% GVP	30% WA
NIH/3T3	C1	61	75	87*	41*
	C20	51	91*	104*	43*
	MR	30	66	44	18
	3R4F	49	68	35	8
A549	C1	59	97	88*	75*
	C20	70*	95	93*	73*
	MR	46	87	49	11
	3R4F	41	100	46	9
NHLF	C1	38*	84*	7	62*
	C20	47*	93*	7	67*
	MR	18	23*	5	5
	3R4F	19	51	11	5
NHBE-Smoker	C1	77*	108	53*	83*
	C20	81*	102	41*	96*
	MR	35	94	11	29
	3R4F	33	100	8	25
NHBE-Nonsmoker	C1	77*	107	54*	30
	C20	81*	117	47*	29
	MR	35	96	15	35
	3R4F	33	104	11	16
NHBE-Child	C1	90*	80	50*	n/a
	C20	86*	94	44	n/a
	MR	50	87	16	n/a
	3R4F	44	59	12	n/a
BEAS-2B	C1	40*	89*	12	n/a
	C20	52*	88*	9	n/a
	MR	7	24	2	n/a
	3R4F	6	70	2	n/a
H9-hESC	C1	17	86	11*	n/a
	C20	11	102	13*	n/a
	MR	17	78	3	n/a
	3R4F	12	91	3	n/a

Figure 4.3. MTT cytotoxicity data for IQOS and conventional cigarettes. Columns show the four types of aerosol/smoke solution that were tested. Rows show the cell types and product source of the aerosol/smoke solutions. Data are the means of three experiments at the highest concentration tested for each type of aerosol/smoke solution. C1, C20, and MR red values were statistically compared to 3R4F using two-way ANOVA, * = Bonferroni adjusted $p < 0.003125$. TPM = total particulate matter of fractionated aerosol/smoke; GVP = gas vapor phase of fractionated aerosol/ smoke; WA = whole aerosol/smoke captured in culture medium. C1 = IQOS cleaned after each use; C20 = IQOS cleaned after 20 uses. MR = Marlboro Red cigarettes; 3R4F = reference cigarette. Pale red boxes = toxicity values < 70% of control, green boxes = toxicity values ≥ 70%, bright red boxes = IQOS and R3R4F means were not significantly different.

NRU Assay

For 3 % TPM, conventional cigarettes produced a cytotoxic effect for all eight cell types, while IQOS was cytotoxic to all cell types except NHBE-nonsmoker and NHBE-child. In some cases, either IQOS C1 (NIH 3T3, A549, NHBE-Smoker) or IQOS C20 (BEAS-2B) were cytotoxic. Among IQOS sensitive cells, A549 (C1) and H9-hESC (C1 and C2) were not statistically different from 3R4F treated cells.

3% GVP from IQOS was not cytotoxic to any cell type, while 3% GVP from cigarettes was cytotoxic only to NHLF. 30% GVP from conventional cigarettes was cytotoxic to all eight cell types, and IQOS was toxic to all but A549 cells. Of the seven cell types affected by IQOS, six were not significantly different from 3R4F. Only NIH 3T3 cells, the cell type tested previously by Schaller et al. [4], were significantly less affected by IQOS C1 and C20 than by the 3RF4 research cigarettes.

All 30% WA IQOS and conventional cigarette exposures were cytotoxic. For IQOS, the NBHE-smoker and NBHE-nonsmoker groups were not statistically different from the 3R4F group.

MR was significantly more cytotoxic than 3RF4 for the NHLF. Significant differences between the IQOS C1 and IQOS C20 groups were observed for TPM treatments of A549, NHLF, and NHBE-smoker (pale blue boxes in figure 4.4) and NIH/3T3 had significant differences for TPM and WA treatments (bright blue box in figure 4.4).

Neutral Red Assay

Cell line	Sample	3% TPM	3% GVP	30% GVP	30% WA
NIH/3T3	C1	63*	80	52*	58*
	C20	85*	88*	59*	39*
	MR	24	83	11	15
	3R4F	30	75	13	10
A549	C1	50	106	89*	63*
	C20	79	105	86*	65*
	MR	46	93	43	23
	3R4F	60	95	39	22
NHLF	C1	62*	88*	20	42*
	C20	47*	91*	22	46*
	MR	17	34*	15	7
	3R4F	28	59	17	7
NHBE-Smoker	C1	68*	81	49	38
	C20	96*	80	48	32
	MR	40	95	36	37
	3R4F	35	78	45	39
NHBE-Nonsmoker	C1	109*	95	66	43
	C20	77*	91	43	54
	MR	40	88	47	50
	3R4F	33	77	46	56
NHBE-Child	C1	83	98	62	n/a
	C20	70	100	69	n/a
	MR	47	79	54	n/a
	3R4F	47	82	69	n/a
BEAS-2B	C1	72*	78	17	n/a
	C20	59*	83	16	n/a
	MR	45	73	19	n/a
	3R4F	35	74	13	n/a
H9-hESC	C1	24	96	22	n/a
	C20	27	109	31	n/a
	MR	23	90	12	n/a
	3R4F	19	105	22	n/a

Figure 4.4. NRU cytotoxicity data for IQOS and conventional cigarettes. Columns show the four types of aerosol/smoke solution that were tested. Rows show the cell types and product source of the aerosol/smoke solutions. Data shown are the means of three experiments at the highest concentration tested for each type of aerosol/smoke solution. C1, C20, and MR red values were statistically compared to 3R4F using a two-way ANOVA, * = Bonferroni adjusted $p < 0.003125$. TPM = total particulate matter of fractionated aerosol/smoke; GVP = gas vapor phase of fractionated aerosol/ smoke; WA = whole aerosol/smoke captured in culture medium. C1 = IQOS cleaned after each use; C20 = IQOS cleaned after MR = Marlboro Red cigarettes; 3R4F = reference cigarette. Pale red boxes = toxicity values < 70% of control, green boxes = toxicity values ≥ 70% of the control, bright red boxes = IQOS and R3R4F means are not significantly different. Dark blue box = cell type(s) that had statistical differences between C1 and C20 for 3% TPM and 30% WA. Light blue boxes = cell type(s) that had statistical differences between C1 and C20 for 3% TPM.

Cell Type Sensitivity Hierarchy

Using one-way ANOVA analysis of 30% GVP data, cell types were ranked for overall sensitivity to aerosol/smoke exposure. Figure 4.5 presents the means of three experiments for each cell type in the MTT and LDH assays and their sensitivity to treatment (grouping) based on the ANOVA analysis. In grouping, cells with the different letters were significantly different from each other. Cells were ranked in increasing sensitivity from A through C/D. For MTT data, A549 and NIH/3T3 were the least sensitive, the three NHBE were in the midrange, and NHLF, BEAS-2B and H9-hESC were the most sensitive to treatment (figure 4.5A).

For NRU data, A549 and NHBE-child cells were the least affected by exposure, NHBE-nonsmoker, NHBE-smoker, and NIH/3T3 were mid-range, and NHLF, BEAS-2B and H9-hESC were the most sensitive (figure 4.5B).

To determine an overall hierarchy of sensitivity, the mean values from MTT and NRU were averaged showing that A549 and NIH/3T3 were the least sensitive, the three NHBE were in the midrange, and NHLF, BEAS-2B and H9-hESC were the most sensitive.

Cell Type Sensitivity Hierarchy (30% GVP)

A MTT

Cell Type	Mean	Grouping
A549	68.83	A
NIH/3T3	67.67	A
NHBE-Nonsmoker	30.42	B
NHBE-Child	31.50	B
NHBE-Smoker	28.42	B
NHLF	7.33	C
H9-hESC	7.67	C
BEAS-2B	6.25	C

B Neutral Red

Cell Type	Mean	Grouping
A549	64.17	A
NHBE-Child	63.25	A
NHBE-Nonsmoker	56.08	A B
NHBE-Smoker	44.50	B C
NIH/3T3	34.00	C
H9-hESC	21.17	D
NHLF	18.50	D
BEAS-2B	16.33	D

C Averaged

Cell Type	Average	Grouping
A549	64.17	A
NIH/3T3	63.25	A B
NHBE-Child	56.08	B C
NHBE-Nonsmoker	44.50	C
NHBE-Smoker	34.00	C
H9-hESC	21.17	D
NHLF	18.50	D
BEAS-2B	16.33	D

Figure 4.5. Cell type treatment sensitivity hierarchy. Using one-way ANOVA with Fisher post-hoc test, cells were ranked based on mean percent of control values of three experiments vs. cell type. The grouping column shows the results of the ANOVA. Means that do not share a letter are significantly different. Tables were arranged from least sensitive to most sensitive. (A) MTT data; (B) NRU data; (C) the average of MTT and NRU means was used to determine an overall hierarchy of sensitivity.

DISCUSSION

The cytotoxicities of conventional cigarette smoke and IQOS aerosols were compared in a comprehensive screen using eight cell types, three endpoint assays, and various components of smoke/aerosols. NIH/3T3 cells and the NRU assay were chosen to allow direct comparison to a prior study on IQOS and 3R4F cigarettes [4]. Additional cells from respiratory tissue were included as relevant models for inhalation toxicology, and H9-hESC were studied to determine how embryos and hence prenatal development may be affected by IQOS. Fractions of smoke/aerosol (TMP and GVP) were compared, as was done previously [4], and whole aerosol and smoke, which is what IQOS users actually inhale, was also studied. Our five most significant observations were: (1) IQOS exposure did not lead to cell death (LDH) in most trials but did adversely affect critical cellular functions (MTT & NRU); (2) for some cell types (bright red boxes in Figures 4.3 and 4.4), the toxicity of the IQOS aerosol and 3R4F smoke were not significantly different from each other; (3) cell types varied in their sensitivity to IQOS aerosol and cigarette smoke with cells from human embryos and the respiratory system usually being more sensitive than NIH/3T3 and cancer cells (A549); (4) results between IQOS C1 and C20 were similar for MTT, but NRU analysis showed differences between the treatments in A549 (C1 > C20), NHBE-smoker (C1 > C20), and NHLF (C1 < C20) for TMP and in NIH/3T3 for both TPM (C1 > C20) and WA (C1 < C20); and (5) MR had equivalent cytotoxicities to 3R4F, with the exception of NHLF at 3% GVP.

LDH analysis showed that cells were generally not killed by IQOS, MR or 3R4F treatments. Notable exceptions were the NHLF and BEAS-2B cells, which were affected by all 30% GVP treatments. MTT and NRU data showed that IQOS C1 and C20 did have adverse effects on cell metabolism and dye uptake. These data agree with another

study that investigated the effects of IQOS aerosols using an air-liquid interface (ALI) system and found that exposure did not lead to cell death but rather adversely affected metabolic activity [9]. Comparison of MTT and NRU data showed that both the 3% TPM and 30% GVP were cytotoxic and that the response to GVP was concentration dependent. For WA, both IQOS and conventional cigarettes were cytotoxic to all five cell types with which it was tested. IQOS Holder cleanliness did not significantly affect the outcome for the MTT assay but did sometimes produce a significant effect in the NRU assay, with C20 usually being more cytotoxic than C1. In general MR and R3F4 smoke was equivalent in both the MT and NRU assays.

In a prior study on IQOS cytotoxicity using NIH/3T3 cells [4], the equivalent of IQOS C20 produced very little effect using the NRU, in agreement with our observations (e.g. 85% for 3% TPM and 88% for 3% GVP), and these effects in both studies were significantly different from 3RF4 cigarette smoke, suggesting reduced toxicity with IQOS. However, by expanding evaluations to a broader spectrum of cells that included six types of respiratory cells and by including higher concentrations of GVP as well as WA, it was evident that cytotoxicity was frequently observed with IQOS aerosols. One of the most important observations in our study is the finding that in a number of instances, there was no significant difference in toxicity between IQOS and 3R4F treatments.

A hierarchy of cell sensitivity was created for the MTT and NRU assays and averages of these assays based on the 30% GVP data (figure 4.5). The most sensitive respiratory cells were NHLF, which play a vital role in maintaining proper lung health by producing the extracellular matrix, which is essential for support and normal lung function [10]. H9-hESC were also in the most sensitive group suggesting that IQOS may not be an appropriate product for pregnant women. All three primary, untransformed

NHBE cell types (smoker, nonsmoker, and child) were in the midrange of sensitivity. Within the bronchial epithelial group, the NHBE cells from the smoker were always more sensitive than those from the adult or child. While this hierarchy is based on cells from only one individual per group, these data suggest that the respiratory epithelium from a smoker is less able to tolerate IQOS aerosol exposure than similar cells from non-smokers and children. NIH/3T3 (mouse embryonic fibroblast) and A549 cells (lung carcinoma) were the least sensitive to IQOS treatment, indicating they may not be the best cell choice for testing tobacco products that are inhaled. These data reaffirm that different cell types should be evaluated in cytotoxicity testing and show that cells from the human respiratory system are more sensitive to IQOS treatment than mouse 3T3 cells, which were used in a prior study [4].

Although IQOS aerosol did not kill cells, it did have adverse effects in the MTT and NRU assays. These assays examine different endpoints and provide information on how IQOS aerosol affects cellular functions. A decline in mitochondrial reductase activity, as measured by the MTT assay, can lead to metabolic dysfunction, causing increased ROS production and oxidative damage [11-13]. This dysfunction can adversely affect redox signaling, which regulates cell death and survival pathways [12,14]. Decreases in reductase activity also lowers ATP production by oxidative phosphorylation [14-17], leading to compromised cell health. Dysregulation of succinate dehydrogenase, a key mitochondrial reductase and tumor suppressor, can lead to promotion of malignant cancers [18-20].

NRU data demonstrated that IQOS adversely effected dye uptake through the plasma membrane and/or maintenance of an acidic lysosomal pH. Proper pH gradients across lysosomal membranes require ATP production [21], thus our results with the

NRU assay would be consistent with the observed decline in mitochondrial reductase (MTT assay). While the MTT and NRU assays gave similar results in our study, cytotoxicity was observed somewhat more frequently with the NRU assay (18 out of 32 trials vs 14 out of 32 trials for MTT), suggesting that NRU may be somewhat more sensitive than the MTT assay and may be a better choice if only one assay is used. Lysosomes play a critical role in cellular homeostasis by recycling of macromolecules, but when damaged, hydrolases leak out leading to lysosomal cell death (LCD) [22]. While we did not observe cell death in most trials (LDH), the NRU data suggest that death would have occurred had incubations been longer.

The equivalent cytotoxicity observed with IQOS aerosol and 3R4F research cigarette smoke and the sensitivity of human bronchial epithelial cells and lung fibroblasts to IQOS aerosols is a concern. While our data cannot be directly extrapolated to human health, they clearly show a need for additional studies on IQOS products.

REFERENCES:

1 Hair EC, Bennett M, Sheen E, *et al.* Examining perceptions about IQOS heated tobacco product: consumer studies in Japan and Switzerland. *Tobacco Control* Published Online First: 15 May 2018. doi:10.1136/tobaccocontrol-2018-054322

2 Smith MR, Clark B, Lüdicke F, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 1: Description of the system and the scientific assessment program. *Regul Toxicol Pharmacol* 2016;**81**:S17–S26. doi:10.1016/j.yrtph.2016.07.006

3 Davis B, Williams M, Talbot P. iQOS: evidence of pyrolysis and release of a toxicant from plastic. *Tobacco Control* Published Online First: 13 March 2018. doi:10.1136/tobaccocontrol-2017-054104

4 Schaller J-P, Keller D, Poget L, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 2: Chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol. *Regul Toxicol Pharmacol* 2016;**81**:S27–S47. doi:10.1016/j.yrtph.2016.10.001

5 Bahl V, Lin S, Xu N, *et al.* Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models. *Reprod Toxicol* 2012;**34**:529–37. doi:10.1016/j.reprotox.2012.08.001

6 Behar RZ, Wang Y, Talbot P. Comparing the cytotoxicity of electronic cigarette fluids, aerosols and solvents. *Tobacco Control* 2017;:tobaccocontrol–2016–053472–10. doi:10.1136/tobaccocontrol-2016-053472

7 Health Canada. Tobacco Reporting Regulations. Health Canada 2017.

8 I.S. EN ISO 10993-5: Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity. 2013;:1–52.

9 Leigh NJ, Tran PL, O'Connor RJ, *et al.* Cytotoxic effects of heated tobacco products (HTP) on human bronchial epithelial cells. *Tobacco Control* 2018;:tobaccocontrol–2018–054317–4. doi:10.1136/tobaccocontrol-2018-054317

10 White ES. Lung extracellular matrix and fibroblast function. *Ann Am Thorac Soc* 2015;**12 Suppl 1**:S30–3. doi:10.1513/AnnalsATS.201406-240MG

11 Dröse S. Differential effects of complex II on mitochondrial ROS production and their relation to cardioprotective pre- and postconditioning. *Biochim Biophys Acta* 2013;**1827**:578–87. doi:10.1016/j.bbabio.2013.01.004

12 Korge P, Calmettes G, Weiss JN. Increased reactive oxygen species production during reductive stress: The roles of mitochondrial glutathione and thioredoxin reductases. *Biochim Biophys Acta* 2015;**1847**:514–25. doi:10.1016/j.bbabio.2015.02.012

13 Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J* 2009;**417**:1–13. doi:10.1042/BJ20081386

14 Herst PM, Rowe MR, Carson GM, *et al.* Functional Mitochondria in Health and Disease. *Front Endocrinol* 2017;**8**:265–16. doi:10.3389/fendo.2017.00296

15 Griffiths EJ, Rutter GA. Mitochondrial calcium as a key regulator of mitochondrial ATP production in mammalian cells. *BBA - Bioenergetics* 2009;**1787**:1324–33. doi:10.1016/j.bbabio.2009.01.019

16 Denton RM. Regulation of mitochondrial dehydrogenases by calcium ions. *BBA - Bioenergetics* 2009;**1787**:1309–16. doi:10.1016/j.bbabio.2009.01.005

17 Tarasov AI, Griffiths EJ, Rutter GA. Regulation of ATP production by mitochondrial Ca2. *Cell Calcium* 2012;**52**:28–35. doi:10.1016/j.ceca.2012.03.003

18 King A, Selak MA, Gottlieb E. Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. *Oncogene* 2006;**25**:4675–82. doi:10.1038/sj.onc.1209594

19 Gottlieb E, Tomlinson IPM. Mitochondrial tumour suppressors: a genetic and biochemical update. *Nat Rev Cancer* 2005;**5**:857–66. doi:10.1038/nrc1737

20 Bezawork-Geleta A, Rohlena J, Dong L, *et al.* Mitochondrial Complex II: At the Crossroads. *Trends Biochem Sci* 2017;**42**:312–25. doi:10.1016/j.tibs.2017.01.003

21 Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat Protoc* 2008;**3**:1125–31. doi:10.1038/nprot.2008.75

22 Aits S, Jäättelä M. Lysosomal cell death at a glance. *J Cell Sci* 2013;**126**:1905–12. doi:10.1242/jcs.091181

Chapter 5

IQOS Aerosols Promote an Epithelial-to-Mesenchymal Transition in Lung Cancer Cells

Barbara Davis, Atena Zahedi, Alexander Meuting, Rattapol Phandthong, Preston Sitorus, Prue Talbot

ABSTRACT

Objective: To determine if exposure to IQOS aerosols induces an epithelial-to-mesenchymal transition (EMT) in primary lung carcinoma cells.

Methods: IQOS aerosols were generated under two cleanliness conditions, C1 and C20. A-549 human lung epithelial carcinoma vimentin-RFP reporter cells were aerosol treated for 13 days then evaluated for vimentin and E-cadherin expression. Image processing and video bioinformatics were used to evaluate cell count, morphology, attachment, and motility.

Results: IQOS treatment groups, C1 and C20, showed a reduction in cell counts with a higher enlarged and elongated to cobblestone phenotypic cell ratio than controls. Treatment groups also had increased expression of vimentin and a decrease in E-cadherin. Treated cells lacked defined E-cadherin borders demonstrating a lack of cell-cell attachments. Aerosol-treated cells were more motile than control counterparts with the C20 group being more motile than the C1 group. Analysis of time-lapse videos showed that controls were more proliferative and formed small, stationary colonies, while treated cells did not readily divide. These cells did not form attachments and were much more motile.

Discussion: IQOS aerosol treatment induced an EMT in primary lung cancer cells. EMT is the first step leading to metastasis. These data raise concerns about the safety of IQOS products.

INTRODUCTION

Heat-not-burn products are electronic nicotine delivery devices that claim harm reduction because they do not combust tobacco like conventional cigarettes. In 2014, Phillip Morris International released the IQOS heat-not-burn system to Italian and Japanese's test markets, where it was met with a positive response [1]. Although currently not available in the US, IQOS is being sold in 42 markets worldwide [2,3]. While these devices utilize tobacco as their method of nicotine delivery, studies conducted by Phillip Morris report that the IQOS is less harmful than conventional cigarettes because the tobacco is not ignited thus fewer toxic chemicals are produced [4-12]. Although Phillip Morris has conducted many experiments testing their new product for toxicity, they did not test for its effects on cancer progression.

Lung cancer is the leading cause of cancer-related deaths throughout the world [13]. Death can usually be attributed to the metastasis of cancer cells from the lungs to other organs, making treatment difficult. In order to metastasize, cancer cells must first undergo an epithelial-to-mesenchymal transition (EMT), a process in which primary tumor cells lose cell-cell adhesion and polarity allowing them to dissociate, become motile and invade other tissues [14-17]. This process also increases the resistance of these cells to apoptosis [18]. EMT is accompanied by a reduction in E-cadherin, a cell adhesion molecule and a marker of epithelial cells, and an increase in vimentin, an intermediate filament protein and mesenchymal cell marker [15,17,19,20], making them excellent biomarkers of this transition.

As many IQOS users may be former conventional cigarette smokers, there is a possibility that some of these users have undiagnosed primary lung cancer. Lung cancer is often not detected until later stages, after it has become metastatic [21]. Many

conventional cigarette smokers may switch to IQOS thinking they are reducing their chances of developing cancer. For these reasons there is an urgent need to understand the effects that IQOS have on triggering an EMT, potentially leading to metastatic cancer. The purpose of this study was to determine if exposure to IQOS aerosol can induce an EMT. Experiments were done using an *in-vitro* lung cancer model exposed to IQOS aerosols generated using two operating conditions.

MATERIALS & METHODS:

Product Acquisition and Storage

As described in Davis et al., 2018 [22], IQOS heat-not-burn kits (Phillip Morris Products S.A., Switzerland) were purchased online through Ebay.com, and cartons of IQOS Marlboro (blue box) heatsticks (Phillip Morris Brands Sàrl, Italy) were purchased in Japan and shipped directly to us via a Japanese personal shopper service. All products arrived in excellent condition, were immediately inventoried, and stored in a dry, dark area at 22ºC.

Aerosol Solution Production

IQOS aerosol solutions were generated using two operating conditions, the first employing a per-use cleaning protocol (C1) where the Holder was cleaned between each heatstick and the second, following the manufacturer's recommended cleaning protocol (C20), where the device was cleaned after the 20th heatstick [22]. Aerosols were produced using the following smoking machine configuration: the mouthpiece filter end of the IQOS heatsticks (inserted into the IQOS Holder) was inserted into one free

end of a 3/8-inch T-Type connector (Thermo Scientific, Rochester, NY). The connector fit tightly and did not allow any air to be pulled into the smoking machine from outside of the heatstick. One of the remaining ends of the T-Type connector was used to block air flow allowing for the activation of the puff, and the other was connected to two in-line glass absorption impingers, custom modified by Kimball Chase (Rockwood, TN). The first impinger contained 50 mL of ice-cold F-12K medium (ATCC, Manassas, VA), and the second contained ice-cold deionized water, both impingers were placed into an ice bath during the course of aerosol production. The impingers were then connected to a Cole-Palmer Masterflex L/S peristatic pump (Vernon Hills, IL) equipped with a Cole-Palmer Masterflex L/S Easy-Load II Model 77200-52 high performance pump head and utilized Masterflex Tygon E3603 tubing (Tubing Size 36). This configuration allows for the application of the Health Canada Standard (HCI) [23] smoking protocol which requires a 2 second puff that generates a total puff volume of 55 mL (27.5mL/sec), with an interpuff interval of 30 seconds.

For each aerosol type, a total of 250 puffs was taken. Heatsticks were designed to have a maximum of 14 puffs but due to manufacturer usage time restraints (the device automatically shuts off after 6 minutes of use and must be recharged) only 12 puffs were taken from each heatstick. All solutions were aliquoted into 1.8 mL microcentrifuge tubes (Fisher Scientific, Fair Lawn, NJ) to reduce headspace and stored at -80ºC till needed.

Cell Culture

A-549 VIM RFP (ATCC® CCL-185EMT™) human lung epithelial carcinoma, vimentin-RFP reporter cells (ATCC, Manassas, VA) were cultured on Nunc™ Cell Culture Treated EasYFlasks™ (T-25 flasks) (Fisher Scientific, Fair Lawn, NJ), at 85% relative humidity and 5% CO_2 at 37ºC. Cells were maintained in F-12K medium supplemented with 10% fetal bovine serum (ATCC, Manassas, VA), with medium changes occurring every other day until cells reached a confluence ≤ 80%. For subculture and/or experimental preparation, cells were passaged using the protocol prescribed by ATCC [24]. After detachment, cells were resuspended in fresh culture medium and redistributed into appropriate culture vessels at desired concentrations. For subculture, cells were seeded at 2.5×10^3 cells/cm^2 (6.25×10^4 cells/flask). For all experiments (figure 5.1), cells were seeded at an initial density of 2.5×10^4 cells/well of a 6-well plate (Falcon, Fisher Scientific, Fair Lawn, NJ) and left to attach for 24 hrs. Control cells were given fresh medium and experimental cells were treated with 10% aerosol solution daily (days 1-13). Cells were passaged on day 6 and re-plated at 2.5×10^4 cells/well into a 6-well plate, and passaged again on day 12. These cells were then distributed at specific densities into experiment appropriate vessels. Experiments were performed on day 13. On days 0-12, control and treated cells were imaged daily to monitor changes in cell morphology, vimentin expression, and overall cell density as determined by well confluence.

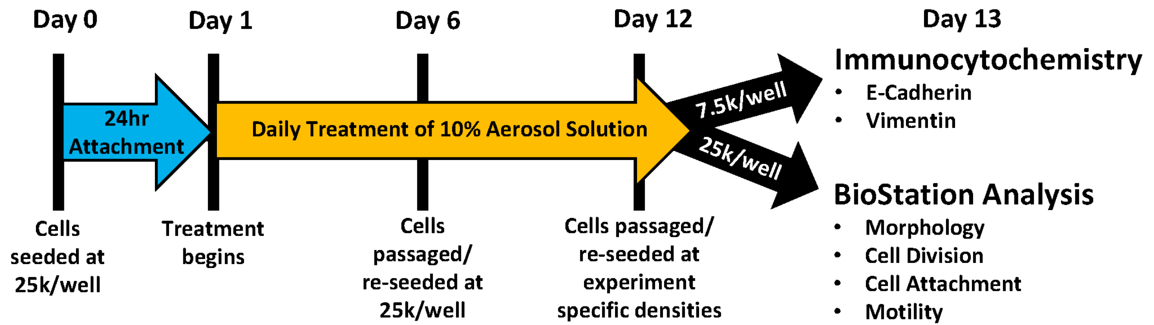


Figure 5.1. Schematic of IQOS experimental timeline. Shows daily breakdown of experimental events and plating densities.

Immunocytochemistry

Cells were passaged into IBIDI Chamber slides (Madison, WI) on day 12, treated cells were plated in the presence of 10% aerosol solution at a seeding density of 7,500 cells/well. Cells were treated for 24 hrs then fixed with 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) for 12 min at room temperature. Cells were blocked in 5% normal goat serum (Gibco, Fisher Scientific, Fair Lawn, NJ) supplemented with 0.3% Triton X-100 (Bio-Rad, Hercules, CA) for 1 hr at room temperature then incubated in anti-E-cadherin antibody (Cell Signaling, Danvers, MA) overnight at 4ºC. Secondary antibody, anti-rabbit IgG Alexa Fluor®488 Conjugate (Cell Signaling), was applied for 1 hr at room temperature, cells were then mounted/nuclear stained with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) and imaged using a Nikon Eclipse Ti-E inverted microscope (Minato, Tokyo, Japan). All solutions were prepared in Dulbecco's phosphate-buffered saline without calcium and magnesium (Lonza, Walkersville, MD), rinse steps were performed between each step, and

antibodies were diluted to a working concentration of 1:200 in a 1% BSA/ 0.3% Triton X-100 solution as described in the Cell Signaling protocol [25].

BioStation Time-lapse Video Generation

Day 12 passaged cells were plated at a seeding density of 5 x 10^4 cells/well in a 6-well plate and left to attach for 24 hrs: treated cells were in the presence of 10% aerosol solution. Cells were then incubated in Hoechst Nucleic Acid Stain (ImmunoChemistry Technologies, Bloomington, MN), at a concentration of 0.5% v/v, for 17 min at 37º C, stain was removed, and fresh medium (control cells) or aerosol solution (treatment cells) was added. Cells were placed in a Nikon BioStation CT (Minato, Tokyo, Japan) and imaged every 10 mins for 24 hrs.

EMT Morphological Analysis and Cell Counting

Morphology analysis was used to classify control and treated cells as cobblestone, enlarged, and elongated. First, individual cells were manually outlined using Adobe PhotoShop software (San Jose, CA). The binary images of the cell segmentations were then imported into CellProfiler image processing software [26]. The number of cells in each morphological group was compared using a one-way ANOVA. Sixty-one morphological features were extracted from each segmented cell. Features that were most useful at classifying cells were determined, such as area, compactness, eccentricity, major axis length, minor axis length and solidity. A training library was developed using 222 cells, which were manually classified into the three morphological groups. This ground truth was used for 10-fold cross-validation, resulting in a 91% classification accuracy using a Naïve Bayes classifier. Datasets were automatically

analyzed using the classifier. Box-Cox transformation was applied to data, and results were statistically compared between the control and treated groups for each morphology class using a one-way ANOVA.

Vimentin Expression

TRITC images, taken using a Nikon Eclipse Ti-E inverted microscope, were masked using a custom procedure created with CL-Quant software (Nikon, Minato, Tokyo, Japan) then manually edited to ensure full cell coverage. Average vimentin fluorescence intensity and mask area were multiplied to determine the integrated density of the vimentin signal. Integrated density of an image was divided by cell count to get average cellular integrated density. Data were analyzed under two conditions, live cell imaging at 10x magnification on days 0, 3, 6, 9, and 12, and fixed and permeabilized cells at a magnification of 60x on day 13. For live cell image analysis, five images were taken per treatment condition at each timepoint, except for day 0 when 3 images were taken. Treatment conditions at each timepoint were compared against controls using a two-way ANOVA with Dunnett's post-hoc test. For day 13 cells, 50 images from across three experiments were used for comparison. Average cellular integrated density of treated cells was compared against the control with a one-way ANOVA using Dunnett's post-hoc test.

E-cadherin Expression

A line scanning method was used to study the expression pattern of E-cadherin in control and treated cells. Lines were drawn on the images using the intensity line

profiler tool of the Nikon Elements Software (Minato, Tokyo, Japan). Pixel intensity data were collected and plotted over the entire line (2000 pixels).

Motility and Migration Analysis

Time-lapse video images generated from day 13 BioStation analysis were analyzed by applying a custom algorithm developed in CL-Quant, as previously described in Zahedi et al, 2018 [27]. Pre-processing procedures were first applied to the blue fluorescent channel to remove noise and boost signal. Cellular debris was removed using small object removal and background subtraction procedures. An individual cell tracking procedure was applied to the final improved signal, resulting in trajectory tracks for each cell. Excel sheets containing the cell tracking data were then imported into MATLAB software (MathWorks Natick, MA, USA). A custom algorithm was applied to display the migratory plots. A threshold of $>$40µm of displacement from a cell's initial starting position was used to classify cells as motile. The percent of motile cells in each treatment was compared using a one-way ANOVA. Populations of motile cells were then sub-divided based on distance traveled, i.e. cells that moved more than 40, 80, and 120 µm; these data were further compared using Fisher's exact test with a Bonferroni correction.

RESULTS

Reduction in Cell Proliferation and Changes to Cell Morphology

Day 12 micrographs (figures 5.2A-C) show a significant reduction in cell number between control and treated cells, however there was no difference between the two

treatments. This finding was confirmed using CellProfiler image processing analysis of day 9 images (figure 5.2G). Also apparent is the change in morphology between control and treated cells. Population morphology classification analysis of day 9 cells (figures 5.2D-F) showed a lack of the small cobblestone phenotype in treatment groups. Within the treatment groups, the primary cell type present was the enlarged cells. Elongated cell populations were sparse in all groups but higher in number in treatments as compared to control. Quantitative analysis of the morphology data (figure 5.2H) confirmed that the treated groups had a significantly lower percentage of cobblestone cells and a corresponding increase in enlarged cells. Data also showed a significant increase in elongated cell populations as compared to controls. Between treatment groups, the C20 group had a slightly higher percentage of enlarged as compared to C1, which was the inverse of the elongated cells, which were higher in the C1 group, however these differences were not significant.

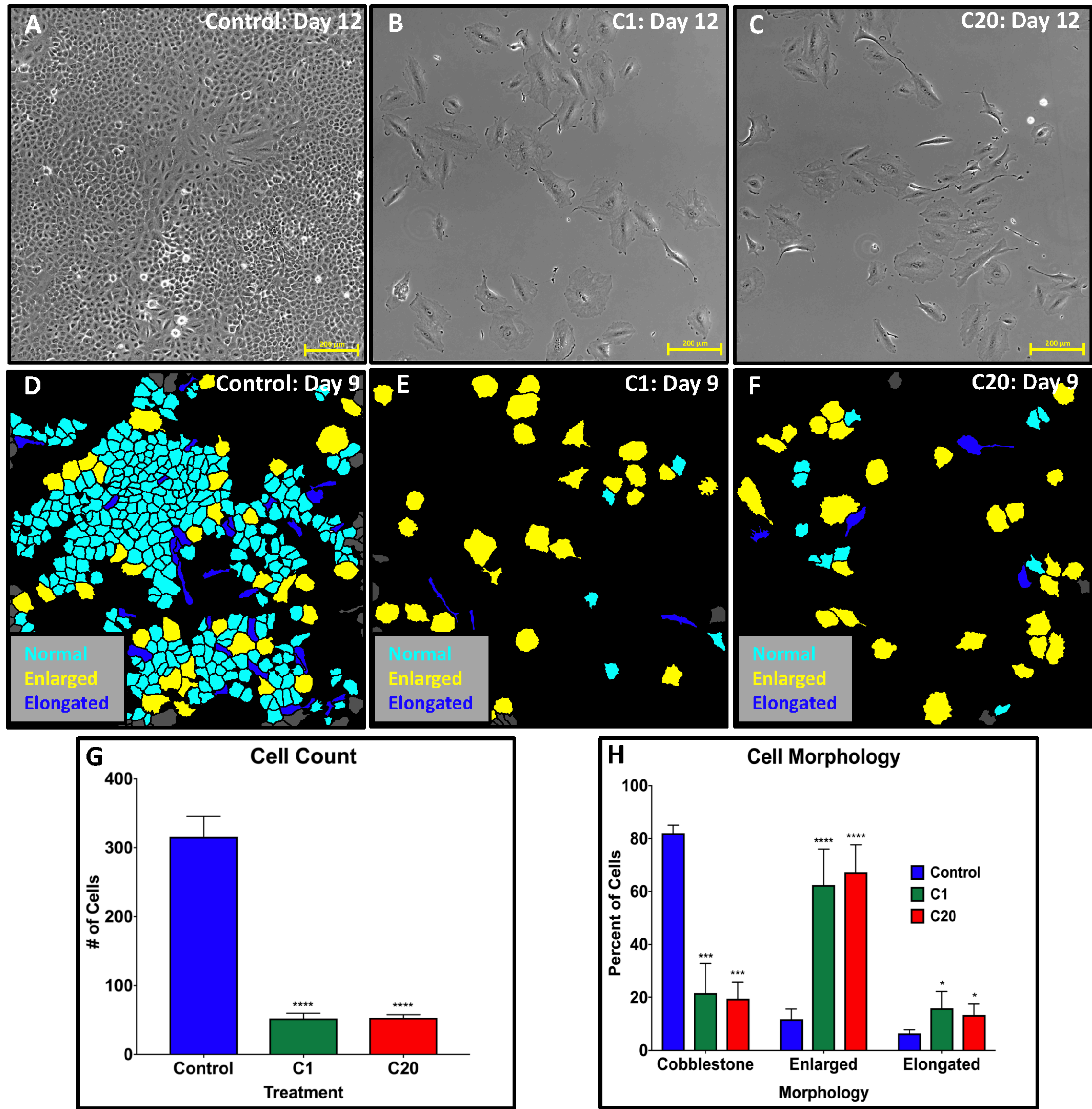


Figure 5.2. Cell count and morphology analysis. (A-C) Day 12 micrographs showing control and IQOS aerosol treated cells. (D-F) Morphology classification segmentation images showing the software ascribed classification of cells, cyan = cobblestone phenotype cells, yellow = enlarged phenotype cells, blue = elongated phenotype cells. (G) Cell count analysis, cell number is plotted versus treatment type. (H) Cell morphology breakdown, percent of cells plotted versus morphology; blue = control, green = C1, red = C20. In G and H, each bar is the mean of 3 experiments ± SEM. * p = < 0.05; *** p = < 0.001; **** p = < 0.0001

Vimentin Expression and Cell-Cell Attachment

Treated cells had larger and more irregularly shaped nuclei than the controls, as evidenced by DAPI staining (figure 5A-C). Visual observation of vimentin expression showed an increase among treated cells and was confirmed by integrated intensity analysis of vimentin (figure 5D). Analysis showed that vimentin was significantly higher in treated cells as compared to controls. There is no significant difference in vimentin expression between treatment types, however C20 cells had a higher initial expression which then dropped at day 12. Day 13 analysis better illuminates this difference (figure 5D inset). Micrographs also showed a reduction of E-cadherin expression in treated cells. Although, punctate E-cadherin is visible in treated cells, there was no cell-cell adhesion, unlike in controls where E-cadherin is clearly forming attachments. Intensity line profiler analysis of E-cadherin validates the finding that E-cadherin expression was higher among control cells (figure 5E). Comparisons between treatment groups showed no difference in E-cadherin expression.

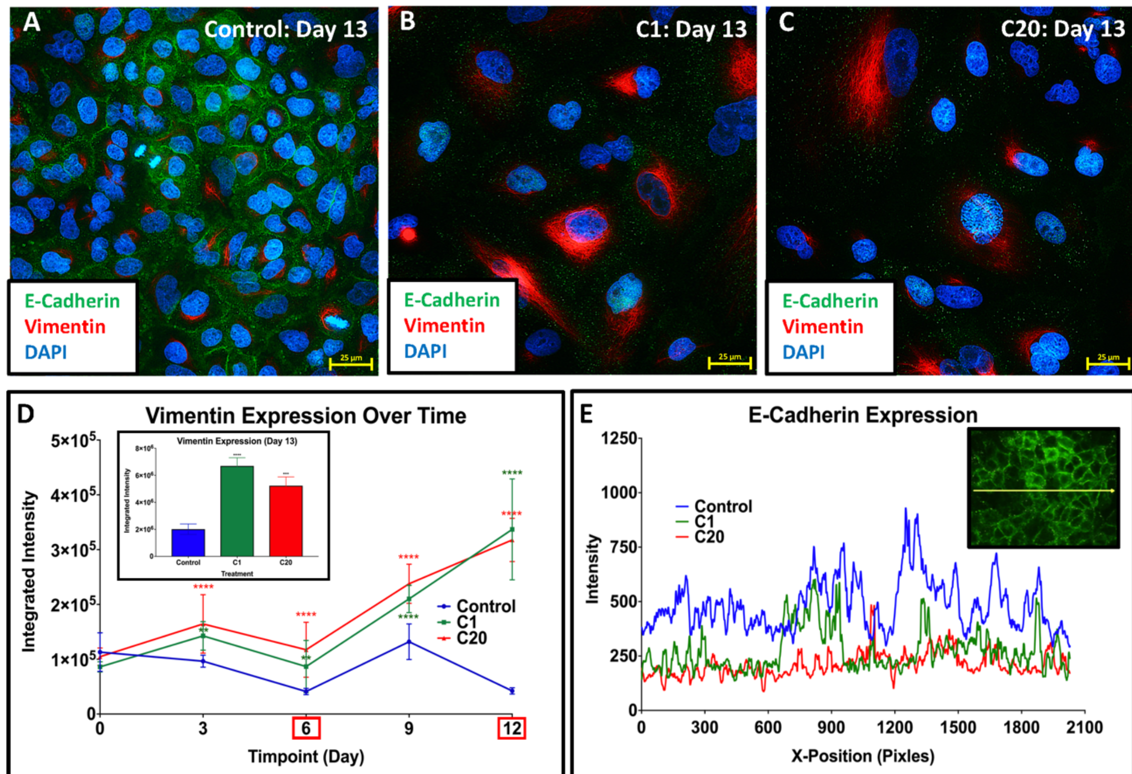
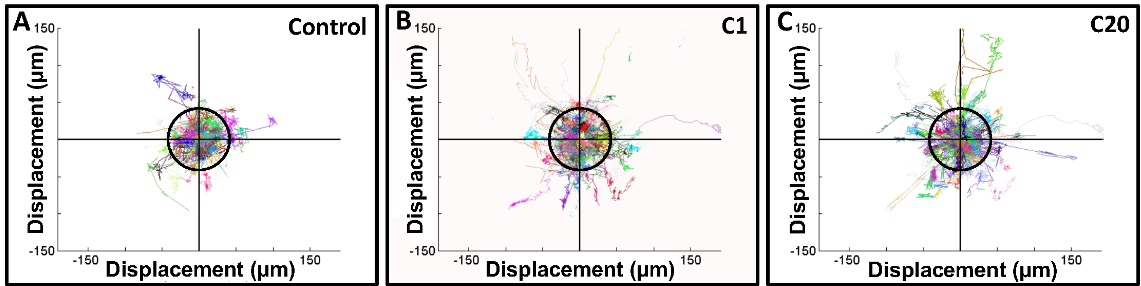


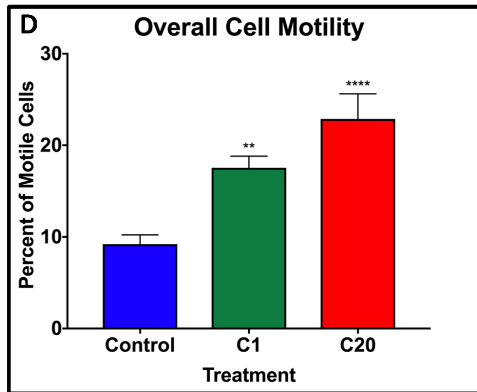
Figure 5.3. Vimentin and E-cadherin expression analysis. (A-C) Fluorescent images of day 13 control and IQOS aerosol treated cells, blue = DAPI, red = vimentin, green = E-cadherin. (D) Vimentin expression over time (live cells, days 0-12), integrated density is plotted versus time in days, red boxes indicate when cells were passaged/re-plated; inset shows day 13 analysis (fixed cells), integrated density plotted versus treatment type. (E) E-cadherin expression of day 13 FITC images, intensity versus x-position in pixels; inset shows an example of the intensity line profiler tool of the Nikon Elements Software (yellow arrow). For all graphs blue = control, green = C1, red = C20. For D, each line, and in the insert, each bar, is the mean of 3 experiments ± SEM. ** $p = < 0.01$; *** $p = < 0.001$; **** $p = < 0.0001$

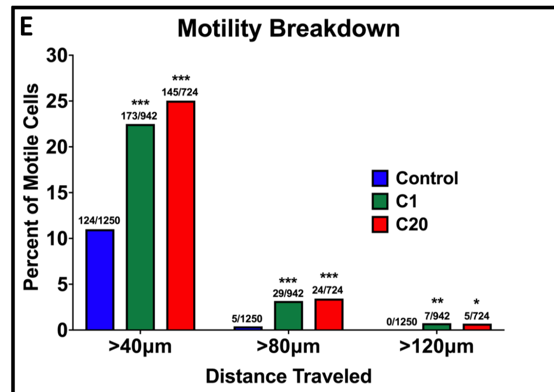
Motility

Migration plot analysis of time-lapse videos (figure 5A-C) revealed that treated cells were more motile and traveled further distances than control cells. A graph of overall cell motility (figure 5D) showed that both treated groups had a significantly larger population of motile cells than the control, with C20 being the more active. A distance travelled breakdown of cell motility (figure 5E) showed that in all distance categories (>40, > 80, and >120 μm), the treated groups were significantly higher than the untreated control. The breakdown also showed that the increased activity among the C20 cells was primarily in the >40 μm range group, there was little difference between treatments in the > 80 and >120 μm ranges.

Evaluation of time-lapse video images generated by the BioStation CT over a 24-hour period (figure 5F-T) showed that control cells divided and formed small cells, which aggregated into colonies that were more stationary. In contrast, treated cells were much more motile than their control counterparts. These did not divide or form cell-cell attachments. When treated cells came in contact with each other, they quickly moved apart, at some points completely separating (Fig 5R & T, see Supplemental figures SP5.1-5.3).


A
Control
B
C1
C
C20
Displacement (μm)
150
-150


D
Overall Cell Motility
Percent of Motile Cells
Control
C1
C20
Treatment
**
****


E
Motility Breakdown
Percent of Motile Cells
124/1250
173/942
145/724
5/1250
29/942
24/724
0/1250
7/942
5/724
Control
C1
C20
>40μm
>80μm
>120μm
Distance Traveled

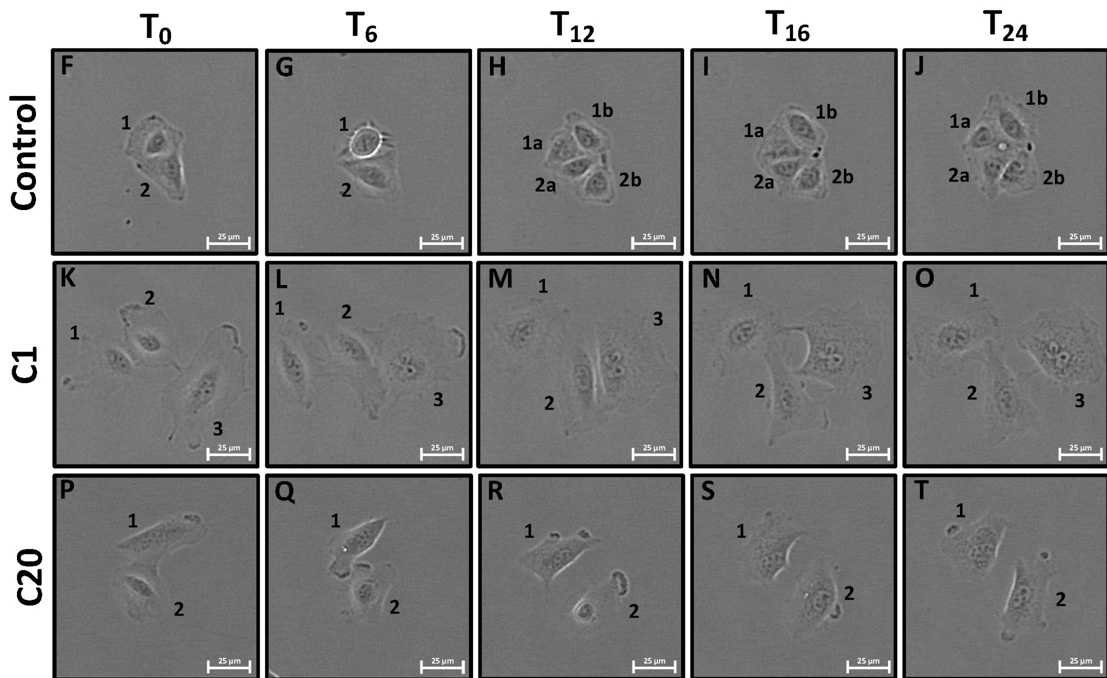

T_0
T_6
T_{12}
T_{16}
T_{24}
Control
C1
C20
25 μm

Figure 5.4. Motility analysis. (A-C) Migration plots of control and IQOS aerosol treated cells, displacement in µm versus displacement in µm. (D) Overall cell motility and percent of motile cells versus treatment type. (E) Motility breakdown, percent of motile cells plotted versus distance traveled in µm. For both E and F blue = control, green = C1, red = C20. (F-T) Stills from time-lapse videos generated over a 24-hour period, (F-J) = control, (K-O) = C1, (P-T) = C20; cells are numbered, divided cells are noted alphanumerically to match the corresponding parent cell number. In D and E, each bar is the mean of 3 experiments ± SEM. * $p = < 0.05$; ** $p = < 0.01$; *** $p = < 0.001$; **** $p = < 0.0001$

DISCUSSION

Harm reduction is enticing to many smokers who are looking for a healthier alternative to conventional cigarettes. Currently there is very little data on the potential adverse health effects that heat-not-burn products may cause in users. Although, according to the manufacturer, IQOS promises a reduction in toxicity, this is little solace if it can promote metastatic lung cancer. Lung cancer has the worst 5-year survival rate of all cancers due to its metastatic potential [13,18,19,28,29]. Understanding the role IQOS products play in effecting a metastatic change in primary lung cancer is imperative. This study is the first to examine IQOS for its potential to induce an EMT in primary lung cancer cells.

One hallmark of an EMT is an increased resistance of cells to apoptotic signaling [15,30,31]. Within treatment groups, only the apoptotic resistant EMT cells remained. This reduction in cobblestone phenotypic cells indicated that these cells died off during treatment (figure 5.2). The remaining enlarged cells adopted a large, flat morphology, unlike the cobblestone cells in the control, which were smaller and raised. These cells were also less prolific. As shown by others, many mesenchymal-like tumor cells, in breast and non-small cell lung cancer, become less proliferative during their migratory phase [32-34], which is reversed when cells revert back to an epithelial state (mesenchymal-to-epithelial transition, MET) upon reaching their target destinations [35-38].

In all groups, there were small populations of elongated cells, which tended to have a classical mesenchymal cell morphology. This change in morphology can be attributed to an increase in vimentin, another hallmark of EMT (figure 5.3). Vimentin plays many rolls in EMT, one of which is to bring about changes in cell morphology

[39,40]. Along with increases in vimentin, another indicator of an EMT is a reduction in E-cadherin. These two proteins work together to bring about an EMT. E-cadherin is an epithelial cell marker and functions as a cell adhesion molecule creating the cell-cell attachments helping in tissue formation and cancer suppression [41-43]. When E-cadherin is present, and vimentin is low, cells form tight cell-cell adhesions leading to the formation of stationary colonies after division (figure 5A, 4F-J). Cells lacking E-cadherin, but with high vimentin, did not divide or form attachments but rather came together transiently and moved apart (figure 5K-T). When similar changes occur *in vivo*, cells can migrate away from the tumor and populate other organs.

Another critical role vimentin plays in EMT is motility [28,39,40,44]. Cells with higher vimentin expression are more motile. Vimentin stiffens the cells and allows for better focal adhesion dynamics [44,45]. In the control group, the cobblestone phenotypic cells make up the predominance of the population, forming small colonies which show little movement (figure 5A, D, E). In comparison, treated cells were significantly more motile with the C20 treatment group being more motile than C1 (figure 5B-E), although this difference was not statistically significant.

Although in this study significant differences in EMT induction were not seen between the C1 and C20 aerosols, in a previous study [22], we showed that the cleanliness of the IQOS played a critical role in heat generation, with the C20 method of cleaning leading to increased tobacco plug charring and polymer-film filter melting. Unlike the C1 aerosol, which was produced by cleaning the device after every heatstick, the C20 aerosol was made using the manufacturers' suggested cleaning instructions, which is a more real-world representation of how a user would clean their device.

In summary, IQOS aerosols, C1 and C20, were able to induce an EMT in primary lung cancer cells. This increase in enlarged, low E-cadherin, high vimentin, motile cells is a cause for concern, as one of the major factors in the lethality of lung cancer is the ability of the cells to travel to and establish new tumors in distant organ systems. With the rapid increase in IQOS popularity and Phillip Morris' push to bring their product to new markets, these findings reinforce the critical need for more extensive testing of IQOS to fully understand its effects on users.

REFERENCES

1. Hair EC, Bennett M, Sheen E, *et al.* Examining perceptions about IQOS heated tobacco product: consumer studies in Japan and Switzerland. *Tobacco Control* Published Online First: 15 May 2018. doi:10.1136/tobaccocontrol-2018-054322

2. *Tobacco Meets Technology*. Phillip Morris International https://www.pmi.com/smoke-free-products/iqos-our-tobacco-heating-system (accessed 25 Sep2018).

3. *What is IQOS?* Phillip Morris International https://www.pmi.com/faq-section/faq/what-is-iqos (accessed 25 Sep2018).

4. Smith MR, Clark B, Lüdicke F, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 1: Description of the system and the scientific assessment program. *Regul Toxicol Pharmacol* 2016;**81**:S17–S26. doi:10.1016/j.yrtph.2016.07.006

5. Schaller J-P, Keller D, Poget L, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 2: Chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol. *Regul Toxicol Pharmacol* 2016;**81**:S27–S47. doi:10.1016/j.yrtph.2016.10.001

6. Schaller J-P, Pijnenburg JPM, Ajithkumar A, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 3: Influence of the tobacco blend on the formation of harmful and potentially harmful constituents of the Tobacco Heating System 2.2 aerosol. *Regul Toxicol Pharmacol* 2016;**81**:S48–S58. doi:10.1016/j.yrtph.2016.10.016

7. Wong ET, Kogel U, Veljkovic E, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 4: 90-day OECD 413 rat inhalation study with systems toxicology endpoints demonstrates reduced exposure effects compared with cigarette smoke. *Regul Toxicol Pharmacol* 2016;**81**:S59–S81. doi:10.1016/j.yrtph.2016.10.015

8. Sewer A, Kogel U, Talikka M, *et al.* Evaluation of the Tobacco Heating System 2.2 (THS2.2). Part 5: microRNA expression from a 90-day rat inhalation study indicates that exposure to THS2.2 aerosol causes reduced effects on lung tissue compared with cigarette smoke. *Regul Toxicol Pharmacol* 2016;**81**:S82–S92. doi:10.1016/j.yrtph.2016.11.018

9. Oviedo A, Lebrun S, Kogel U, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 6: 90-day OECD 413 rat inhalation study with systems toxicology endpoints demonstrates reduced exposure effects of a mentholated version compared with mentholated and non-mentholated cigarette smoke. *Regul Toxicol Pharmacol* 2016;**81**:S93–S122. doi:10.1016/j.yrtph.2016.11.004

10. Kogel U, Titz B, Schlage WK, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 7: Systems toxicological assessment of a mentholated version revealed reduced cellular and molecular exposure effects compared with mentholated and

non-mentholated cigarette smoke. *Regul Toxicol Pharmacol* 2016;**81**:S123–38. doi:10.1016/j.yrtph.2016.11.001

11 Haziza C, La Bourdonnaye de G, Skiada D, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 8: 5-Day randomized reduced exposure clinical study in Poland. *Regul Toxicol Pharmacol* 2016;**81**:S139–50. doi:10.1016/j.yrtph.2016.11.003

12 Martin F, Talikka M, Ivanov NV, *et al.* Evaluation of the tobacco heating system 2.2. Part 9: Application of systems pharmacology to identify exposure response markers in peripheral blood of smokers switching to THS2.2. *Regul Toxicol Pharmacol* 2016;**81**:S151–7. doi:10.1016/j.yrtph.2016.11.011

13 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018;**68**:7–30. doi:10.3322/caac.21442

14 Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009;**119**:1420–8. doi:10.1172/JCI39104

15 Heerboth S, Housman G, Leary M, *et al.* EMT and tumor metastasis. *Clin Transl Med* 2015;**4**:6. doi:10.1186/s40169-015-0048-3

16 Tsai JH, Yang J. Epithelial-mesenchymal plasticity in carcinoma metastasis. *Genes & Development* 2013;**27**:2192–206. doi:10.1101/gad.225334.113

17 Tsai JH, Donaher JL, Murphy DA, *et al.* Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. *Cancer Cell* 2012;**22**:725–36. doi:10.1016/j.ccr.2012.09.022

18 Ahmad A, Gadgeel SM. *Lung Cancer and Personalized Medicine: Novel Therapies and Clinical Management*. Cham: : Springer 2015. doi:10.1007/978-3-319-24932-2

19 Xiao D, He J. Epithelial mesenchymal transition and lung cancer. *Journal of Thoracic Disease* 2011;**2**:154–9. doi:10.3978/j.issn.2072-1439.2010.02.03.7

20 Tsoukalas N, Aravantinou-Fatorou E, Tolia M, *et al.* Epithelial-Mesenchymal Transition in Non Small-cell Lung Cancer. *Anticancer Res* 2017;**37**:1773–8. doi:10.21873/anticanres.11510

21 Popper HH. Progression and metastasis of lung cancer. *Cancer Metastasis Rev* 2016;**35**:75–91. doi:10.1007/s10555-016-9618-0

22 Davis B, Williams M, Talbot P. iQOS: evidence of pyrolysis and release of a toxicant from plastic. *Tobacco Control* Published Online First: 13 March 2018. doi:10.1136/tobaccocontrol-2017-054104

23 Health Canada. Tobacco Reporting Regulations. Health Canada 2017.

24 *ATCC A549-VIM*. ATCC https://www.atcc.org/en/Global/Products/CCL-185EMT.aspx#documentation (accessed 25 Sep2018).

25 *Cell Signaling E-Cad*. Cell Signaling Technology https://www.cellsignal.com/products/primary-antibodies/E-cadherin-24e10-rabbit-mab/3195?site-search-type=Products&N=4294956287&Ntt=E-cadherin+antibody+&fromPage=plp (accessed 25 Sep2018).

26 Carpenter AE, Jones TR, Lamprecht MR, *et al.* CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol* 2006;**7**:R100. doi:10.1186/gb-2006-7-10-r100

27 Zahedi A, Phandthong R, Chaili A, *et al.* Epithelial-to-mesenchymal transition of A549 lung cancer cells exposed to electronic cigarettes. *Lung Cancer* 2018;**122**:224–33. doi:10.1016/j.lungcan.2018.06.010

28 Kidd T. A Novel Role for Cell Adhesion Molecules in Nervous System Development. 2008.

29 Kuchuk M, Addison CL, Clemons M, *et al.* Incidence and consequences of bone metastases in lung cancer patients. *J Bone Oncol* 2013;**2**:22–9. doi:10.1016/j.jbo.2012.12.004

30 Wang W, Goswami S, Lapidus K, *et al.* Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. *Cancer Res* 2004;**64**:8585–94. doi:10.1158/0008-5472.CAN-04-1136

31 Tsai JH, Yang J. Epithelial-mesenchymal plasticity in carcinoma metastasis. *Genes & Development* 2013;**27**:2192–206. doi:10.1101/gad.225334.113

32 Evdokimova V, Tognon C, Ng T, *et al.* Reduced proliferation and enhanced migration: two sides of the same coin? Molecular mechanisms of metastatic progression by YB-1. *Cell Cycle* 2009;**8**:2901–6. doi:10.4161/cc.8.18.9537

33 Salt MB, Bandyopadhyay S, McCormick F. Epithelial-to-mesenchymal transition rewires the molecular path to PI3K-dependent proliferation. *Cancer Discov* 2014;**4**:186–99. doi:10.1158/2159-8290.CD-13-0520

34 Thomson S, Petti F, Sujka-Kwok I, *et al.* A systems view of epithelial-mesenchymal transition signaling states. *Clin Exp Metastasis* 2011;**28**:137–55. doi:10.1007/s10585-010-9367-3

35 Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009;**119**:1420–8. doi:10.1172/JCI39104

36 Brabletz T. EMT and MET in metastasis: where are the cancer stem cells? *Cancer Cell* 2012;**22**:699–701. doi:10.1016/j.ccr.2012.11.009

37 Diepenbruck M, Christofori G. Epithelial-mesenchymal transition (EMT) and metastasis: yes, no, maybe? *Curr Opin Cell Biol* 2016;**43**:7–13. doi:10.1016/j.ceb.2016.06.002

38 Thiery JP, Acloque H, Huang RYJ, *et al.* Epithelial-mesenchymal transitions in development and disease. *Cell* 2009;**139**:871–90. doi:10.1016/j.cell.2009.11.007

39 Lowery J, Kuczmarski ER, Herrmann H, *et al.* Intermediate Filaments Play a Pivotal Role in Regulating Cell Architecture and Function. *J Biol Chem* 2015;**290**:17145–53. doi:10.1074/jbc.R115.640359

40 Mendez MG, Kojima S-I, Goldman RD. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. *FASEB J* 2010;**24**:1838–51. doi:10.1096/fj.09-151639

41 van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. *Cell Mol Life Sci* 2008;**65**:3756–88. doi:10.1007/s00018-008-8281-1

42 Cano A, Pérez-Moreno MA, Rodrigo I, *et al.* The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2000;**2**:76–83. doi:10.1038/35000025

43 Onder TT, Gupta PB, Mani SA, *et al.* Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* 2008;**68**:3645–54. doi:10.1158/0008-5472.CAN-07-2938

44 Messica Y, Laser-Azogui A, Volberg T, *et al.* The role of Vimentin in Regulating Cell Invasive Migration in Dense Cultures of Breast Carcinoma Cells. *Nano Lett* 2017;**17**:6941–8. doi:10.1021/acs.nanolett.7b03358

45 Liu C-Y, Lin H-H, Tang M-J, *et al.* Vimentin contributes to epithelial-mesenchymal transition cancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation. *Oncotarget* 2015;**6**:15966–83. doi:10.18632/oncotarget.386

Chapter 6

Proteomic analysis of normal human bronchial epithelial cells after IQOS aerosol exposure

Barbara Davis, Malcolm Tran, David Volz, Prue Talbot

ABSTRACT

Objective: To determine the effects of acute IQOS aerosol exposure on the proteome of normal human bronchial epithelial cells (NHBE) and to compare the proteome when using different cleaning protocols.

Methods: IQOS aerosol solutions were generated using two cleanliness conditions, C1 (holder cleaned after every heatstick) and C20 (holder cleaned after 20th heatstick). NHBE (nonsmoker) were treated with IQOS aerosol solutions for 24 hours, proteins were isolated and identified using multidimensional protein identification technology analysis. Non-statistically significant data were removed, and the remaining data were evaluated using Database for Annotation, Visualization and Integrated Discovery 6.8 (DAVID) and Ingenuity Pathway Analysis (IPA) software.

Results: A total of 5237 proteins were detected at a 1% false discovery rate (FDR) for control, C1 and C20 treated cells. Of these, 439 and 384 were differentially expressed in C1 versus control (C1vC) and C20 versus control (C20vC), respectively. IPA diseases and functions annotations found 11 common categories in both groups and one additional category in C20vC. DAVID analysis showed five annotation clusters exclusive to C1vC, four to C20vC only, and four clusters common to both groups. IPA analysis identified five pathways for C1vC and three for C20vC that had a $2 < z\text{-score} < -2$ with $p < 0.05$. The three pathways identified in C20vC were among the five identified in C1vC.

Discussion: IQOS aerosol exposures affected expression of proteins involved in metabolic functions. Although there was overlap of annotation categories between C1vC and C20vC, different proteins were modulated, which could lead to different adverse outcomes. IQOS treatment resulted in the activation pathways involved in inflammation, demonstrating that IQOS aerosols may pose health risks to users.

INTRODUCTION

IQOS, a heat-not-burn product, is one of the newest electronic nicotine delivery devices that claims harm reduction [1]. This product is manufactured by Phillip Morris International (PMI) and was released in 2014 to Japanese and Italian test markets where it was positively received [2]. Currently, IQOS is available in 43 countries [3,4]. IQOS uses heated tobacco as its method of nicotine delivery, unlike electronic cigarettes that use a flavored nicotine fluid. IQOS functions by heating a cast-leaf tobacco sheet impregnated with binders and glycerin, producing a nicotine containing aerosol without igniting the tobacco [1,5]. Since the tobacco is not burned, as in a conventional cigarette, fewer toxic chemicals are produced making this product less harmful than conventional cigarettes [1].

IQOS aerosols are derived from tobacco but currently the similarities/differences between tobacco cigarette smoke and IQOS aerosols have not been fully elucidated. One study, by the IQOS manufacturer, compared IQOS aerosol to 3R4F reference cigarette smoke for the presence/reduction of 54 of the 93 chemicals found on the FDA's harmful and potentially harmful constituents (HPHC) list [6]. Moreover, this list is only a fraction of the 7000+ chemicals identified in cigarette smoke [7].

Tobacco cigarette smoke exposure has been linked to many cellular aberrations such as mitochondrial hyperfusion [8], increased reactive oxygen species (ROS) production, oxidative damage, inflammation, altered lipid metabolism, and cellular signaling pathway perturbations [9]. Over time, these dysfunctions may lead to a myriad of health problems/diseases, such as chronic obstructive pulmonary disease (COPD), stroke, coronary heart disease, and/or cancer [9].

The only other proteomics studies on IQOS were done by the manufacturer, and these studies were conducted on rats that had been exposed to mentholated heatsticks for 90 days [10,11].There is a need for independent investigations on IQOS. We previously showed that when the IQOS holder was cleaned after every 20 heatsticks, as recommended by the manufacturer, charring of the tobacco plug was greater than when cleaning was done after each use [5]. Increased heating could produce additional chemical by-products that may contribute to toxicity of the aerosol. The purpose of this study was to determine how exposure to IQOS aerosol affected the proteome of normal human bronchial epithelial cells (NHBE) from a nonsmoker and to compare the proteome of cells treated with aerosol produced using two different cleaning methods.

Materials & Methods:

Product Acquisition and Storage

iQOS Heat-not-burn kits (Phillip Morris Products S.A., Switzerland) were purchased through Ebay.com, and cartons of IQOS Marlboro (blue box) heatsticks (Phillip Morris Brands Sàrl, Italy) were purchased in Japan via a Japanese personal shopper service. All products arrived in excellent condition, were immediately inventoried, and stored in a dry, dark area at 22ºC, as detailed in [5].

Aerosol Solution Production

iQOS aerosols were produced using two operating conditions: (1) C1 in which the Holder was cleaned between each heatstick, and (2) C20 in which the device was not cleaned until after the 20th heatstick, as recommended in IQOS instruction manual.

Aerosol solutions were produced using the following smoking machine configuration: the mouthpiece filter end of the IQOS heatstick (inside of the IQOS Holder) was inserted into one free end of a 3/8-inch T-Type connector (Thermo Scientific, Rochester, NY). The connector fit tightly and did not allow any air to be pulled into the smoking machine from outside of the heatstick. One end of the T-Type was used to block air flow allowing for the activation of the puff and the other end was connected to two in-line glass impingers, custom modified by Kimball Chase (Rockwood, TN). The first impinger contained 50 mL of ice-cold complete cell culture medium and the second impinger contained ice-cold deionized water; both impingers were placed into an ice bath during the course of aerosol production. The impingers were connected to a Cole-Palmer Masterflex L/S peristatic pump (Vernon Hills, IL) outfitted with a Cole-Palmer Masterflex L/S Easy-Load II Model 77200-52 high performance pump head utilizing Masterflex Tygon E3603 (Tubing Size 36) to allow for the application of the Health Canada standard (HCI) [12] smoking protocol. The HCI requires a 2 second puff that generates a total puff volume of 55 mL (27.5mL/sec) with an interpuff interval of 30 seconds. Aerosol solutions were aliquoted into 450 μL volumes and placed into 0.5 mL locking lid microcentrifuge tubes (Fisher Scientific, Fair Lawn, NJ) to reduce headspace, and stored at -80ºC. For each aerosol type (C1 and C20), a total of 250 puffs were taken. Heatsticks were designed to have a maximum of 14 puffs. However, because the device automatically shuts off after 6 minutes of use and must be recharged, only 12 puffs could be taken from each heatstick.

Cell Culture and Exposure

NHBE (MatTek, Ashland, MA) from a 50-year-old, Caucasian, male nonsmoker were maintained and subcultured as prescribed in the MatTEK NHBE protocol. Cells were cultured in Nunc™ Cell Culture Treated EasYFlasks™ (T-25 flasks) and were maintained with NHBE-GM, growth medium (NHBE-BM, basal medium, supplemented with NHBE-GS, growth serum, and NHBE-HCS, hydrocortisone) with medium changes occurring every other day until cells reached a confluence ≤80%. For subculture, cells were seeded at $3.3x10^3$ cells/cm^2 ($8.25x10^4$ cells/flask). For sub-culturing and experiments, cell counts were performed using a hemocytometer (Hausser Scientific, Horsham, PA), and Nikon Eclipse TS100 Inverted Microscope (Tokyo, Japan). For exposure, cells were seeded at $6.6x10^3$ cells/cm^2 ($1.65x10^5$ cells/flask) and left to attach for 24 hrs. Cells were then treated with 3% aerosol solution (C1 or C20) for 24 hours. All cultures were maintained at 37ºC with 5% CO2, and 95% relative humidity.

Protein Isolation

After 24 hr of IQOS aerosol solution exposure, NHBE cells were rinsed and lysed using radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnologies, Dallas, TX) and vortexed at 4ºC for 1 min at 15 min intervals for a total of 45 mins. Lysates were collected, and protein concentration was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Rochester, NY). After quantification, SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 20 μg of protein. The gel was subsequently stained using Coomassie Blue staining (Bio Rad, Hercules, California) to visualize proteins and to ensure equal loading.

Protein Precipitation and MudPIT Analysis

Lysates containing 100 µg of protein were precipitated with cold acetone to a final concentration of 80% overnight at -20 ºC. Samples were centrifuged for 30 mins at 14,000 rpm, forming a pellet. Protein pellets were treated with 1 µg of trypsin overnight at 37 ºC in 100 µL trypsin buffer (50 mM ammonium bicarbonate, pH 8.0) supplemented with 10% acetonitrile. The samples were placed on a vortex mixer for continuous agitation, keeping pellets in suspension. After trypsin digestion, samples were centrifuged, and supernatants were collected and dried down as pellets with a SpeedVac vacuum concentrator and re-dissolved in 20 µL 0.1% formic acid. Products were then subjected to LC/MS analysis.

A multidimensional protein identification technology (MudPIT) approach was employed to analyze the trypsin-treated samples. A two-dimensional nanoAcquity UPLC (Waters, Milford, MA) and an Orbitrap Fusion MS (Thermo Fisher Scientific, San Jose, CA) were configured to perform online 2D-nanoLC/MS/MS analysis. 2D-nanoLC was operated with a 2D-dilution method that is configured with nanoAcquity UPLC. The two mobile phases for the first dimension LC fractionation were 20 mM ammonium formate (pH 10) and acetonitrile, respectively. Online fractionation was achieved by 5-minute elution off a NanoEase trap column (PN# 186003682, Waters) using a stepwise-increased concentration of acetonitrile. A total of five fractions were generated with 11%, 16%, 20%, 25%, and 50% of acetonitrile, respectively. A final flushing step used 80% acetonitrile to clean up the trap column. Each fraction was then analyzed online using a second dimension LC gradient. The second dimension nano-UPLC method was described previously [13].

The Orbitrap Fusion MS method was based on a data-dependent acquisition (DDA) survey. The acquisition time was set from 1-70 min. A Nano ESI source was used with the spray voltage at 2600V, sweep gas at 0, and ion transfer tube temperature at 275°C. An Orbitrap mass analyzer was used for the MS1 scan with resolution set at 60,000. MS mass range was 350-1800 m/z. The AGC target for each scan was set at 500,000 with maximal ion injection time set at 100 ms.

For the MS2 scan, the Orbitrap mass analyzer was used in an auto/normal mode with resolution set at 30,000. Only precursor ions with intensities of 50,000 or higher were selected for the MS2 scan. The sequence of individual MS2 scanning was from most-intense to least-intense precursor ions using a top-speed mode under time control of 4 sec. Higher energy CID (HCD) was used for fragmentation activation with 30% normalized activation energy. Quadrupole was used for precursor isolation with a 2 m/z isolation window. The MS2 mass range was set to auto/normal with the first mass set at 100 m/z. Maximal injection time was 100 ms with the AGC target set at 20,000. Ions were injected for all available parallelizable time. A 20-sec exclusion window was applied to all abundant ions to avoid repetitive MS2 scanning on the same precursor ions using 10 ppm error tolerance. Only charge states from 2 to 6 were allowed for MS2 scan, and undetermined charge states were not included. All MS2 spectra were recorded in the centroid mode.

Raw MS files were processed and analyzed using the Proteome Discoverer version 2.2 (ThermoFisher Scientific, San Jose, CA). The Sequest HT search engine was used to match all MS data to the human UniProt proteome database supplemented with common contaminant proteins such as keratins. The search parameters were the following: trypsin with two missed cleavage, minimal peptide length

for six amino acids, MS1 mass tolerance 10 ppm, MS2 mass tolerance 0.02 Da, Gln→pyro-Glu (N-term Q), oxidation (M), N-terminal acetylation, N-terminal formylation, and XK-acetylation as variable modifications. Only proteins with a 1% false discovery rate (FDR) cut-off were considered in the final result.

Bioinformatics Analysis

Significant abundance ratio adjusted p-value Proteome Discoverer version 2.2 outputs ($p<0.05$) for C1 versus control and C20 versus control (from this point forward will be referred to as C1vC and C20vC, respectively) were considered. The resulting protein lists were uploaded into the Database for Annotation, Visualization and Integrated Discovery 6.8 (DAVID, https://david.ncifcrf.gov/) for enrichment, yielding annotation clusters of effected cellular processes [14].

Log_2(Fold Change) values for significant proteins in C1vC and C20vC were then uploaded into Ingenuity Pathway Analysis (IPA) software (Qiagen, Venlo, Netherlands) to evaluate differentially expressed proteins. Diseases and functions annotations were generated from the IPA database of existing literature. Activation of significant canonical pathways most associated with our proteins were identified using the Ingenuity library.

RESULTS

Protein Identification and Differential Protein Expression

Values in Figure 6.1A represent the number of proteins detected with a FDR controlled at 1%. A total of 5237 proteins were detected among all samples. For individual groups, 106 proteins were detected in control, 73 were exclusive to C1, and 52

were found in only in C20. For the control and C1, 4860 proteins were expressed in common, while a similar number (4814) were expressed in the control and C20. The two IQOS treatments (C1 and C20) had 4806 mutual proteins, and 4737 proteins were shared among all three groups. Fold change evaluations showed that for C1vC, 237 proteins were significantly down-regulated and 202 were upregulated. For C20vC, 210 proteins were down-regulated, while 174 were upregulated (Figure 6.1B). These data show that protein expression was affected by both C1 and C20 aerosol treatment.

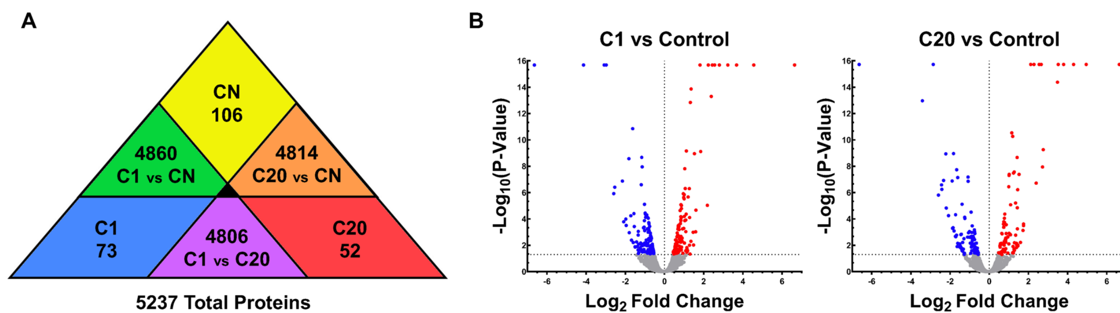


Figure 6.1. Protein identification and differential protein expression in NHBE cells treated with IQOS aerosol. (A) Venn diagram showing proteins identified by Proteome Discoverer version 2.2 as having high FDR. Yellow diamond = controls (CN), blue diamond = C1, and red diamond = C20. Proteins shared between CN and C1 = green triangle, proteins between control and C20 = orange triangle, and between C1 and C20 = purple triangle. Numbers in each color block represent total number of proteins found in each group. (B) Volcano plots of C1 versus control and C20 versus control from Proteome Discoverer version 2.2 data. Plots show differential expression of proteins. x-axis = Log_2 fold change values, y-axis = $-log_{10}$(p-value). Grey dots = proteins found to have a fold value of < $-log_{10}$ (1.3), blue = proteins that were down regulated with a >$-log_{10}$ (1.3) and $-Log_2$ fold change value, red = proteins that were upregulated with a >$-log_{10}$ (1.3) and $+Log_2$ fold change value.

Annotation and Cluster Analysis

Proteomic data with a p-value < 0.05 were analyzed using proteomic analysis software to predict effected biological processes (Figure 6.2). For IPA analysis, software culls data to remove any proteins with a fold-change value from -1 to 1, the remaining data were then grouped by disease and functional categories for both C1vC (Figure 6.2A) and C20vC (Figure 6.2B). Percentages shown are the number of times a particular category was repeated/represented, not the number of enriched proteins within each group. Eleven categories were shared between treatment groups. These were: gene expression, protein synthesis and trafficking; morphology; signaling; inflammation; function and maintenance; movement; death and survival; growth and proliferation; respiratory disease; metabolism; and cancer. In C20vC, one additional category, molecular transport, was affected. The top three most repeated functions were those that involved growth and proliferation (14% for C1vC and 11% for C20vC), metabolism (21% for C1vC and 17% for C20vC), and proteins associated with cancers (29% for C1 and 42% for C20). For both treatment types, inflammation was represented equally at 4%, but of most interest was respiratory diseases, which was more heavily represented in C20vC (6%) than in C1vC (<1%). Identification of enriched proteins effected in the respiratory disease category (Supplemental Table 6.1) found seven individual proteins in C1vC, four of which (TUBB4A, FTL, HTATIP2 and RHOC) together are known effectors in Stage I-II non-small cell lung cancer (NSCLC); and IL1B, IL1RN and GGPS1, which are linked to cystic fibrosis. For C20vC, only three proteins were enriched, IL1B, effecting inflammation of lung cells and TUBBA4A and POLE4, which together appear 32 times, with 24 of the 32 annotations relating to effects on NSCLC. The remaining eight appearances are linked to nasopharyngeal carcinomas. IL1B (grey highlights in

Supplemental table 6.1) and TUBB4A (blue highlights in Supplemental table 6.1) were the only proteins to appear in both C1vC and C20vC.

DAVID functional annotation clustering of C1vC and C20vC data (figure 6.2C) was evaluated to show the distribution of GO terms, based on co-association of genes, categorized by their biological processes and/or molecular functions. The interaction diagram shows groupings that were distinct and shared between the two treatment types. C1vC showed five distinct groups (RNA/mRNA binding, gene expression/mRNA splicing, chromatin remodeling/DNA binding, actin, microtubules), while C20 had four groups (regulation of apoptotic processes, DNA biosynthesis, ubiquitination, and cellular protein metabolic processes). Four groups, calcium binding, mitochondrial transport, oxidation-reduction, and lipid metabolism were affected in both IQOS treatments.

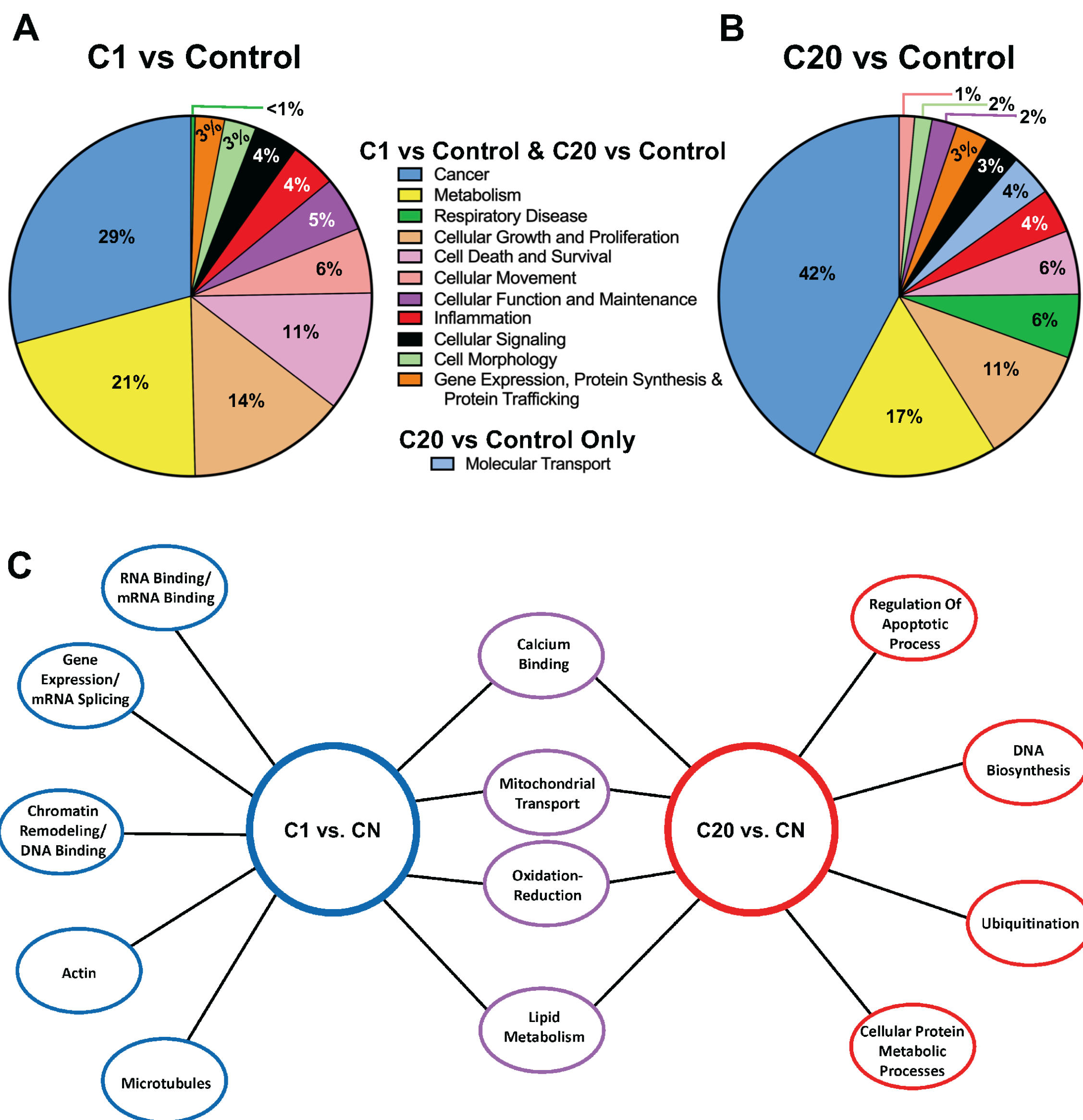


Figure 6.2. Diseases and function annotations. (A) Pie chart of IPA function and disease annotations. Proteomic data with a p-value <0.05 and values that fell outside of 1 < fold-change < -1 were grouped into cell function or disease categories. The percentage of occurrence of each category type for C1vCN and C20vCN are indicated on the respective pie charts. (B) Interaction diagram of DAVID annotation clustering of proteomic data with a p-value <0.05. Red ovals = clusters found in C1vCN, blue ovals = clusters found in C20vCN, purple ovals = clusters found in both groups.

Canonical Pathway Activation

Top enriched canonical pathways were identified for data limited to those that fell outside of 2 < z-score < -2 with a -log(p-value) higher than 1.3 ($p < 0.05$) (figure 6.3A). Pathways are listed in highest to lowest order of z-score (the number of standard deviations from the mean). All identified pathways had positive z-scores, indicating they were upregulated by treatment. Nuclear factor erythroid 2-related factor 2 (NRF2) mediates an oxidative stress response and had the highest z-score for both C1vC (3.317) and C20vC (2.646). In C1vC, significantly enriched networks were associated with 14 proteins, while C20vC had only 9. The adrenomedullin signaling pathway had the second highest z-score for both treatments, 2.646 for C1vC (with seven identified proteins) and 2.236 for C20vC (5 proteins). Gαq Signaling and Rho Family GTPase Signaling, both with a z-score of 2.449 and six identified proteins, were found exclusively in C1vC. Nuclear factor kappa B (NF-κB) signaling had identical z-scores, 2.236, and number of identified proteins, five, for both groups.

For the three pathways shared between IQOS treatment groups, a list of differentially expressed proteins was formulated. Figure 6.3A lists the number of differentially expressed proteins, while figure 6.3B specifically identifies the proteins expressed in both groups (green highlights) and among each individual group (blue highlights are proteins in C1vC only and yellow are for those in C20vC only). The UniProt/Swiss-Prot accession fold change values, cellular location, and protein type were also listed. A total of 14 proteins were identified in the NRF2-mediated oxidative stress response pathway for C1vC, nine of these were also found in C20vC. Among the 14 proteins listed, four had a fold-change >2, three were shared between both groups, while one was only in C1vC. For the adrenomedullin signaling pathway, a total of seven

were found in C1vC with five also being activated in C20vC. All five of the shared proteins had fold-change values >2. NF-κB signaling listed seven proteins, three were shared between both groups and two proteins unique to each group. All three shared proteins had fold-change values >2. Among the unique proteins, C1vC only showed a >2 fold-change for one of the two proteins, while both were >2 for C20vC.

A

Canonical Pathway Name	C1 vs Control			C20 vs Control		
	Number of Proteins Found	p-value	z-score	Number of Proteins Found	p-value	z-score
NRF2-mediated Oxidative Stress Response	14	9.36E-09	3.317	9	4.42E-05	2.646
Adrenomedullin Signaling Pathway	7	3.27E-03	2.646	5	2.50E-02	2.236
Gαq Signaling	6	5.01E-03	2.449	--	--	--
Signaling by Rho Family GTPases	6	3.80E-02	2.449	--	--	--
NF-κB Signaling	5	3.58E-02	2.236	5	2.00E-02	2.236

B

Canonical Pathway	Symbol	Entrez Gene Name	UniProt/Swiss-Prot Accession	Fold Change	Location	Type
NRF2-mediated Oxidative Stress Response	ACTB	actin beta	C9JUM1	100	Cytoplasm	Other
	FTL	ferritin light chain	P02792	1.561	Cytoplasm	Enzyme
	GCLC	glutamate-cysteine ligase catalytic subunit	P48506	1.714	Cytoplasm	Enzyme
	GCLM	glutamate-cysteine ligase modifier subunit	P48507	1.826	Cytoplasm	Enzyme
	GSR	glutathione-disulfide reductase	P00390	1.576	Cytoplasm	Enzyme
	JUNB	JunB proto-oncogene, AP-1 transcription factor subunit	P17275	1.645	Nucleus	Transcription Regulator
	PIK3R1	phosphoinositide-3-kinase regulatory subunit 1	P27986	100	Cytoplasm	Kinase
	SQSTM1	sequestosome 1	Q13501	1.675	Cytoplasm	Transcription Regulator
	TXNRD1	thioredoxin reductase 1	Q16881	2.214	Cytoplasm	Enzyme
	CBR1	carbonyl reductase 1	P16152	1.373	Cytoplasm	Enzyme
	DNAJB2	DnaJ heat shock protein family (Hsp40) member B2	P25686	2.754	Nucleus	Other
	FTH1	ferritin heavy chain 1	P02794	1.62	Cytoplasm	Enzyme
	HMOX1	heme oxygenase 1	P09601	1.538	Cytoplasm	Enzyme
	TXN	thioredoxin	P10599	1.572	Cytoplasm	Enzyme
Adrenomedullin Signaling Pathway	CALM1 (includes others)	calmodulin 1	E7ETZ0	100	Cytoplasm	Other
	IL1B	interleukin 1 beta	P01584	4.704	Extracellular Space	Cytokine
	KCNN4	potassium calcium-activated channel subfamily N member 4	O15554	100	Plasma Membrane	Ion Channel
	PIK3R1	phosphoinositide-3-kinase regulatory subunit 1	P27986	100	Cytoplasm	Kinase
	PLCD4	phospholipase C delta 4	C9JEA7	2.887	Cytoplasm	Enzyme
	IL1RN	interleukin 1 receptor antagonist	P18510	1.438	Extracellular Space	Cytokine
	PTK2B	protein tyrosine kinase 2 beta	Q14289	1.419	Cytoplasm	Kinase
NF-kB	IL1B	interleukin 1 beta	P01584	4.335	Extracellular Space	Cytokine
	PIK3R1	phosphoinositide-3-kinase regulatory subunit 1	P27986	100	Cytoplasm	Kinase
	TAB2	TGF-beta activated kinase 1/MAP3K7 binding protein 2	Q9NYJ8	100	Cytoplasm	Other
	CARD10	caspase recruitment domain family member 10	Q9BWT7	100	Cytoplasm	Other
	IL1RN	interleukin 1 receptor antagonist	P18510	1.438	Extracellular Space	Cytokine
	IKBKB	inhibitor of nuclear factor kappa B kinase subunit beta	O14920	100	Cytoplasm	Kinase
	MALT1	MALT1 paracaspase	Q9UDY8	100	Cytoplasm	Peptidase

Figure 6.3. IPA canonical pathway activation. (A) List of canonical pathways that fell outside of 2 < z-score < -2 with a >-log$_{10}$ (1.3) value. Pathway name, number of enriched proteins, p-value, and z-score for both C1vCN and C20vCN are shown. (B) List of canonical pathways shared between C1vC and C20vC identifying enriched proteins. Gene symbol, Entrez gene name, UniProt/Swiss-Prot accession, fold change values, cellular location and protein type are indicated. Green highlights = proteins found in both C1 versus control and C20 versus control, blue highlights = proteins found in C1 versus control only, and yellow highlight = proteins found in C20 versus control only.

DISCUSSION

IQOS aerosols were produced using two cleanliness conditions and tested on NHBE cells from an adult nonsmoker. IQOS exposure caused significant changes in the proteome of treated cells vs untreated controls and produced differences in the proteome of the C1 vs C20 groups. A total of 5237 proteins were identified, and of these 9% were differentially expressed in C1vC and 8% in C20vC (figure 6.1). Between the two treatment exposure groups (C1vC and C20vC), there was very little difference in the number of differentially expressed proteins. However, evaluations of diseases and functions annotations, annotation clustering, and canonical pathway analysis showed that the profiles of proteins tended to vary between treatment groups.

The treatment affected proteins that play roles in critical cellular functions. Functional annotation outputs of differential expression data (figure 6.2) showed considerable overlap between frequently represented functions/diseases categories for C1vC and C20vC, with growth and proliferation, metabolism, and cancer being the most common. One notable difference between the treatments was the difference in frequency of annotations regarding respiratory disease. For C20vC, respiratory disease was the fourth most often repeated annotation, at 6%, whereas for C1vC, it represented <1%. However, these percentages are not an indicator of protein distribution, as C1vC had seven enriched proteins as opposed to C20vC that had only three. Two of these three, TUBB4A (tubulin beta 4A), a member of the beta tubulin family and is one of two core proteins that heterodimerize form microtubules [15] and POLE4 (DNA polymerase epsilon 4, accessory subunit), a histone-fold protein that interacts with other histone-fold proteins to bind DNA forming larger complexes that function in DNA transcription, replication, and packaging [16], always appeared together. Although there were fewer

proteins affected by C20 treatment, TUBB4A and POLE4 were noted in 34 separate annotations, of which 24 were related to functions associated with NSCLC, indicating that these proteins must play a more integral role in disease function as they appear with more frequency. TUBB4A was also found in C1vC in a complex with three additional proteins that were also annotated in NSCLC, indicating that TUBB4A may play a critical role in lung cancer. IL1B (interleukin 1 beta), a protein in the cytokine family, is an important mediator of inflammatory response; it is also involved in a variety of cellular activities, such as cell growth and proliferation, differentiation, and apoptosis [17]. IL1B was common to both IQOS treatment groups; however, in slightly different fashions. In C1vC, IL1B was found in association with two additional proteins and annotated as having effects in cystic fibrosis. In C20vC, it was found alone, and annotated in lung inflammation. The inflammation category was the seventh (C1vC) and sixth (C20vC) highest represented category (4% for both groups), but this does not exclude inflammation as a risk factor from IQOS use. Acute exposure testing showed a high frequency of annotations in the cellular metabolism category (21% for C1vC and 17% for C20vC). Maintaining proper mitochondrial function is critical and aberrations contribute to inflammation [18-21], i.e. metabolic dysfunction is a precursor to inflammation.

When comparing DAVID outputs between C1vC and C20vC, there were more differences between GO term annotation clustering data than was found within IPA annotation categories. However, the four shared clusters identified by DAVID (mitochondrial transport, oxidation-reduction, lipid metabolism and calcium binding) play a role in inflammation, and chronic inflammation can lead to cancer [22,23]. These results correspond with IPA data, which showed that cancer was the most frequency

represented category for both C1vC (29%) and C20vC (42%), followed by metabolism (21% for C1vC and 17 for C20vC).

When evaluating IPA and DAVID annotation data as a whole, dysregulation of metabolic activities induced by IQOS exposure is the common theme between both treatments. Together, mitochondrial transport, oxidation-reduction, lipid metabolism and calcium binding, all play vital roles in cellular metabolism and maintaining the homeostatic balance of cells by working to regulate inflammatory responses [24-26]. Mitochondria are vital in energy production (ATP synthesis) and metabolism (oxidation-reduction, redox) [27-29]. To maintain proper function, mitochondria rely on mitochondrial transport channels/proteins, such as transferases and ATP binding cassette proteins (Supplemental DAVID data), to allow proteins, metabolites and ATP to cross the inner mitochondrial membrane [29,30]. These channels/proteins also function as sensors and regulators of redox signaling and in balancing reactive oxygen species (ROS) production by releasing excess ROS into the cytosol [31]. Adverse effects on redox reactions, via damage to reductases and/or dehydrogenases (Supplemental DAVID data), leads to increases in ROS production, oxidative damage [18-20], and inflammation, which may eventually lead to cancer [21].

Along with mitochondrial metabolism, lipid metabolism plays vital roles in cell structure maintenance, and the regulation of many cellular processes, such as energy production, cell signaling (functioning as second messengers and as hormones), growth and proliferation, differentiation, death and survival, inflammation, and motility [32,33]. Lipid signaling also plays a role in the regulation of inflammation [34]. Lipid metabolism modulation to mediate inflammation is now being sought as a treatment for inflammatory disease [35] and cancer [36]. Alterations to proteins involved in lipid metabolism, such as

lipid and hormone synthases (Supplemental DAVID data), can lead to altered membrane composition/permeability which can lead to the development of diseases including cancer [32,33].

Calcium is a ubiquitous global second messenger that plays roles in mitochondrial [37] and lipid metabolism [38]. EF-hand calcium binding motif proteins, which are upregulated by treatment (Supplemental DAVID data), a regulated various of cellular functions. They are essential to signaling, cell growth and proliferation, cell cycle, differentiation, and apoptosis (death and survival) [39,40]. EF hand calcium binding proteins, such as calmodulin, calcitonin, S100 family of proteins (Supplemental DAVID data), also play a role in inflammation and tumor cell proliferation and invasion of cancer. S100 family proteins have a higher expression in lung cancer cells making them a good biomarker for the occurrence and prognosis of lung cancer [41,42].

The outcome of these proteomic data are in agreement with cytotoxicity data (previously described in chapter 4) for NHBE (adult nonsmoker cells). Cytotoxicity analysis of lactate dehydrogenase (LDH) data showed IQOS C1 and C20 treatments were not killing cells. However, neutral red uptake (NRU) and 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) assays showed treatments were adversely affecting cellular metabolic functions, such as mitochondrial reductases.

IQOS aerosols activated canonical pathways involved in inflammation (figure 6.3). Three pathways were identified in both C1vC and C20vC, adrenomedullin signaling, NF-κB signaling, and NRF2-mediated oxidative stress, and have all been associated with the inflammation related cancer formation, proliferation, and invasion (metastasis).

Adrenomedullin, a small peptide, has structural similarities to calcitonin gene-related peptide and is expressed in every major organ of the body including the lung; it is also present in specialized cells such as fibroblasts. Adrenomedullin and adrenomedullin signaling play roles in cell growth and proliferation, immune response, apoptosis, angiogenesis, cell adhesion, migration and invasion [43-45]. Adrenomedullin has also been shown to activate NF-κB signaling [46].

The NF-κB family of transcription factors are found in most cell types, and NF-κB signaling plays roles in cell growth and proliferation, differentiation, death and survival, but is most commonly associated with immune and inflammatory response and oncogenesis [47-50]. Stressors, such as tobacco cigarette smoke [51-54], oxidative stress/ROS, and lipids/lipid metabolites increase inflammation and activation of NF-κB signaling leading to the promotion and proliferation of many cancers [55,56]. NF-κB also interacts with the NRF2- mediated oxidative stress pathway, where it negatively regulates NRF2 signaling, thereby promoting inflammation [57].

NRF2, a transcription factor, and the NRF2- mediated oxidative stress pathway activates in response to various intrinsic and extrinsic cell stressors, such as tobacco cigarette smoke, oxidative stress, and inflammation [58-60]. Although the NRF2-mediated oxidative stress pathway is classically known for its role in cytoprotection of normal cells, this same quality has been conferred to cancer cells safeguarding them from chemotherapeutic agents. Many studies have shown that prolonged or overactivation of NRF2 can lead to tumorigenesis and metastasis [61-63], and tobacco cigarette smoke has been implicated in the constitutive activation of NRF2 [64].
The interplay between these pathways and the proteins that mediate and modulate them play critical roles in the proper maintenance of cellular functions, helping to prevent

disease. Aberrations to the expression of these proteins can lead to adverse outcomes, like inflammation. Overall, this study demonstrated that acute IQOS aerosol exposure significantly affected the levels of many proteins necessary for proper homeostatic balance of the cell, primarily those involved in metabolic maintenance, as well as those that participate in respiratory disease, such as NSCLC. This is of concern as previous studies evaluating the effects of tobacco cigarette smoke have also shown adverse effects on cellular metabolism in turn leading to increases in ROS, which promoted oxidative damage and inflammation. These same studies found that inflammation is a promoter of cancer [22,23].

Although there was an overlap between identified annotation categories for both C1 and C20 treatment groups, an in-depth look at protein profiles showed that annotations fell into similar categories, but specific outcomes were affected by different protein groups, demonstrating that the cleanliness of IQOS holders can potentially lead to different adverse effects.

These results suggest that IQOS may not be harm-free and emphasize the need for more extensive testing. Future work could be conducted using a 3D *in vitro* model with exposure at the air-liquid interface, as well as *in vivo* testing. However, data from this investigation will be useful to regulatory agencies, especially those that are currently evaluating the sale of IQOS products in the US and other counties where IQOS is currently unavailable, as well as to physicians and IQOS users.

References

1 Smith MR, Clark B, Lüdicke F, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 1: Description of the system and the scientific assessment program. *Regul Toxicol Pharmacol* 2016;**81**:S17–S26. doi:10.1016/j.yrtph.2016.07.006

2 Hair EC, Bennett M, Sheen E, *et al.* Examining perceptions about IQOS heated tobacco product: consumer studies in Japan and Switzerland. *Tobacco Control* Published Online First: 15 May 2018. doi:10.1136/tobaccocontrol-2018-054322

3 Phillip Morris International. IQOS. IQOS. https://www.pmi.com/smoke-free-products/iqos-our-tobacco-heating-system (accessed 8 Oct2017).

4 Phillip Morris International. What is IQOS? IQOS. https://www.pmi.com/faq-section/faq/what-is-iqos (accessed 27 Jul2018).

5 Davis B, Williams M, Talbot P. iQOS: evidence of pyrolysis and release of a toxicant from plastic. *Tobacco Control* Published Online First: 13 March 2018. doi:10.1136/tobaccocontrol-2017-054104

6 Schaller J-P, Keller D, Poget L, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 2: Chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol. *Regul Toxicol Pharmacol* 2016;**81**:S27–S47. doi:10.1016/j.yrtph.2016.10.001

7 *Harms of Cigarette Smoking and Health Benefits of Quitting*. National Cancer Institue https://www.cancer.gov/about-cancer/causes-prevention/risk/tobacco/cessation-fact-sheet (accessed 3 Nov2018).

8 Bahl V, Johnson K, Phandthong R, *et al.* Thirdhand Cigarette Smoke Causes Stress-Induced Mitochondrial Hyperfusion and Alters the Transcriptional Profile of Stem Cells. *Toxicol Sci* 2016;:kfw102–15. doi:10.1093/toxsci/kfw102

9 Services USDOHAH. The Health Consequences of Smoking - 50 Years of Progress: A Report of the Surgeon General. 2014;:1–1081.

10 Oviedo A, Lebrun S, Kogel U, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 6: 90-day OECD 413 rat inhalation study with systems toxicology endpoints demonstrates reduced exposure effects of a mentholated version compared with mentholated and non-mentholated cigarette smoke. *Regul Toxicol Pharmacol* 2016;**81**:S93–S122. doi:10.1016/j.yrtph.2016.11.004

11 Kogel U, Titz B, Schlage WK, *et al.* Evaluation of the Tobacco Heating System 2.2. Part 7: Systems toxicological assessment of a mentholated version revealed reduced cellular and molecular exposure effects compared with mentholated and non-mentholated cigarette smoke. *Regul Toxicol Pharmacol* 2016;**81**:S123–38. doi:10.1016/j.yrtph.2016.11.001

12 Health Canada. Tobacco Reporting Regulations. Health Canada 2017.

13 Drakakaki G, van de Ven W, Pan S, *et al.* Isolation and proteomic analysis of the SYP61 compartment reveal its role in exocytic trafficking in Arabidopsis. *Cell Res* 2012;**22**:413–24. doi:10.1038/cr.2011.129

14 Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009;**4**:44–57. doi:10.1038/nprot.2008.211

15 *TUBB4A tubulin beta 4A class IVa [Homo sapiens (human)]*. NCBI https://www.ncbi.nlm.nih.gov/gene/10382 (accessed 12 Nov2018).

16 *POLE4 DNA polymerase epsilon 4, accessory subunit [Homo sapiens (human)]*. NCBI https://www.ncbi.nlm.nih.gov/gene/56655 (accessed 12 Nov2018).

17 *IL1B*. NCBI https://www.ncbi.nlm.nih.gov/gene/3553 (accessed 12 Nov2018).

18 Dröse S. Differential effects of complex II on mitochondrial ROS production and their relation to cardioprotective pre- and postconditioning. *Biochim Biophys Acta* 2013;**1827**:578–87. doi:10.1016/j.bbabio.2013.01.004

19 Korge P, Calmettes G, Weiss JN. Increased reactive oxygen species production during reductive stress: The roles of mitochondrial glutathione and thioredoxin reductases. *Biochim Biophys Acta* 2015;**1847**:514–25. doi:10.1016/j.bbabio.2015.02.012

20 Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J* 2009;**417**:1–13. doi:10.1042/BJ20081386

21 Liu X, Chen Z. The pathophysiological role of mitochondrial oxidative stress in lung diseases. *J Transl Med* 2017;**15**:207. doi:10.1186/s12967-017-1306-5

22 Cantin AM. Cellular response to cigarette smoke and oxidants: adapting to survive. *Proc Am Thorac Soc* 2010;**7**:368–75. doi:10.1513/pats.201001-014AW

23 D'Anna C, Cigna D, Costanzo G, *et al.* Cigarette smoke alters the proteomic profile of lung fibroblasts. *Mol Biosyst* 2015;**11**:1644–52. doi:10.1039/c5mb00188a

24 van Vliet AR, Verfaillie T, Agostinis P. New functions of mitochondria associated membranes in cellular signaling. *Biochim Biophys Acta* 2014;**1843**:2253–62. doi:10.1016/j.bbamcr.2014.03.009

25 Raturi A, Simmen T. Where the endoplasmic reticulum and the mitochondrion tie the knot: the mitochondria-associated membrane (MAM). *Biochim Biophys Acta* 2013;**1833**:213–24. doi:10.1016/j.bbamcr.2012.04.013

26 Szymański J, Janikiewicz J, Michalska B, *et al.* Interaction of Mitochondria with the Endoplasmic Reticulum and Plasma Membrane in Calcium Homeostasis, Lipid Trafficking and Mitochondrial Structure. *Int J Mol Sci* 2017;**18**. doi:10.3390/ijms18071576

27 Aravamudan B, Thompson MA, Pabelick CM, *et al.* Mitochondria in lung diseases. *Expert Rev Respir Med* 2013;**7**:631–46. doi:10.1586/17476348.2013.834252

28 Handy DE, Loscalzo J. Redox regulation of mitochondrial function. *Antioxid Redox Signal* 2012;**16**:1323–67. doi:10.1089/ars.2011.4123

29 Wohlrab H. Transport proteins (carriers) of mitochondria. *IUBMB Life* 2009;**61**:40–6. doi:10.1002/iub.139

30 Alberts B, Johnson A, Lewis J, *et al.* The Transport of Proteins into Mitochondria and Chloroplasts. In: *Molecular Biology of the Cell. 4th edition*. Garland Science 2002.

31 O-Uchi J, Ryu S-Y, Jhun BS, *et al.* Mitochondrial ion channels/transporters as sensors and regulators of cellular redox signaling. *Antioxid Redox Signal* 2014;**21**:987–1006. doi:10.1089/ars.2013.5681

32 Huang C, Freter C. Lipid metabolism, apoptosis and cancer therapy. *Int J Mol Sci* 2015;**16**:924–49. doi:10.3390/ijms16010924

33 Santos CR, Schulze A. Lipid metabolism in cancer. *FEBS J* 2012;**279**:2610–23. doi:10.1111/j.1742-4658.2012.08644.x

34 Glass CK, Olefsky JM. Inflammation and lipid signaling in the etiology of insulin resistance. *Cell Metab* 2012;**15**:635–45. doi:10.1016/j.cmet.2012.04.001

35 van Diepen JA, Berbée JFP, Havekes LM, *et al.* Interactions between inflammation and lipid metabolism: relevance for efficacy of anti-inflammatory drugs in the treatment of atherosclerosis. *Atherosclerosis* 2013;**228**:306–15. doi:10.1016/j.atherosclerosis.2013.02.028

36 Prevete N, Liotti F, Amoresano A, *et al.* New perspectives in cancer: Modulation of lipid metabolism and inflammation resolution. *Pharmacol Res* 2018;**128**:80–7. doi:10.1016/j.phrs.2017.09.024

37 Denton RM, McCORMACK JG. The role of calcium in the regulation of mitochondrial metabolism. *Biochemical Society Transactions* 1980;**8**:266–8. doi:10.1042/bst0080266

38 Ritz E, Heuck CC, and RBPAMIH, *et al.* Phosphate, calcium and lipid metabolism. *Springer*

39 Yang H, Zhang Q, He J, *et al.* Regulation of calcium signaling in lung cancer. *Journal of Thoracic Disease* 2010;**2**:52–6.

40 Yáñez M, Gil-Longo J, Campos-Toimil M. Calcium binding proteins. *Adv Exp Med Biol* 2012;**740**:461–82. doi:10.1007/978-94-007-2888-2_19

41 Huang H, Huang Q, Tang T, *et al.* Clinical significance of calcium-binding protein S100A8 and S100A9 expression in non-small cell lung cancer. *Thorac Cancer* 2018;**9**:800–4. doi:10.1111/1759-7714.12649

42 Vogl T, Gharibyan AL, Morozova-Roche LA. Pro-inflammatory S100A8 and S100A9 proteins: self-assembly into multifunctional native and amyloid complexes. *Int J Mol Sci* 2012;**13**:2893–917. doi:10.3390/ijms13032893

43 Cuttitta F, Martinez A. Adrenomedullin. In: Kastin AJ, ed. *Handbook of Biologically Active Peptides*. Elsevier. 2013. 489–93. doi:10.1016/B978-0-12-385095-9.00066

44 Nishikimi T, Kuwahara K, cutti, *et al.* Adrenomedullin. In: Schisler JC, Lang CH, Willis MS, eds. *Endocrinology of the Heart in Health and Disease*. Elsevier. 2017. 41–58. doi:10.1016/B978-0-12-803111-7.00002-6

45 Zudaire E, Martínez A, Cuttitta F. Adrenomedullin and cancer. *Regulatory Peptides* 2003;:175–83. doi:10.1016/S0167-0115(03)00037-5

46 Pleguezuelos O, Hagi-Pavli E, Crowther G, *et al.* Adrenomedullin signals through NF-kappaB in epithelial cells. *FEBS Lett* 2004;**577**:249–54. doi:10.1016/j.febslet.2004.10.019

47 Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* 2004;**25**:280–8. doi:10.1016/j.it.2004.03.008

48 Oeckinghaus A, Ghosh S. The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol* 2009;**1**:a000034. doi:10.1101/cshperspect.a000034

49 Tornatore L, Thotakura AK, Bennett J, *et al.* The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. *Trends Cell Biol* 2012;**22**:557–66. doi:10.1016/j.tcb.2012.08.001

50 Liu T, Zhang L, Joo D, *et al.* NF-κB signaling in inflammation. *Signal Transduct Target Ther* 2017;**2**:17023. doi:10.1038/sigtrans.2017.23

51 McMillan DH, Baglole CJ, Thatcher TH, *et al.* Lung-targeted overexpression of the NF-κB member RelB inhibits cigarette smoke-induced inflammation. *The American Journal of Pathology* 2011;**179**:125–33. doi:10.1016/j.ajpath.2011.03.030

52 Hasnis E, Bar-Shai M, Burbea Z, *et al.* Mechanisms underlying cigarette smoke-induced NF-kappaB activation in human lymphocytes: the role of reactive nitrogen species. *J Physiol Pharmacol* 2007;**58 Suppl 5**:275–87.

53 Anto RJ, Mukhopadhyay A, Shishodia S, *et al.* Cigarette smoke condensate activates nuclear transcription factor-kappaB through phosphorylation and degradation of IkappaB(alpha): correlation with induction of cyclooxygenase-2. *Carcinogenesis* 2002;**23**:1511–8.

54 Manzel LJ, Shi L, O'Shaughnessy PT, *et al.* Inhibition by cigarette smoke of nuclear factor-κB-dependent response to bacteria in the airway. *Am J Respir Cell Mol Biol* 2011;**44**:155–65. doi:10.1165/rcmb.2009-0454OC

55 Xia Y, Shen S, Verma IM. NF-κB, an active player in human cancers. *Cancer Immunol Res* 2014;**2**:823–30. doi:10.1158/2326-6066.CIR-14-0112

56 Karin M. NF-kappaB as a critical link between inflammation and cancer. *Cold Spring Harb Perspect Biol* 2009;**1**:a000141. doi:10.1101/cshperspect.a000141

57 Wardyn JD, Ponsford AH, Sanderson CM. Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. *Biochemical Society Transactions* 2015;**43**:621–6. doi:10.1042/BST20150014

58 Wang Y-Y, Chen J, Liu X-M, *et al.* Nrf2-Mediated Metabolic Reprogramming in Cancer. *Oxidative Medicine and Cellular Longevity* 2018;**2018**:9304091. doi:10.1155/2018/9304091

59 Pandey P, Singh AK, Singh M, *et al.* The see-saw of Keap1-Nrf2 pathway in cancer. *Crit Rev Oncol Hematol* 2017;**116**:89–98. doi:10.1016/j.critrevonc.2017.02.006

60 Hayes JD, Dinkova-Kostova AT. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem Sci* 2014;**39**:199–218. doi:10.1016/j.tibs.2014.02.002

61 Rojo de la Vega M, Chapman E, Zhang DD. NRF2 and the Hallmarks of Cancer. *Cancer Cell* 2018;**34**:21–43. doi:10.1016/j.ccell.2018.03.022

62 Jaramillo MC, Zhang DD. The emerging role of the Nrf2-Keap1 signaling pathway in cancer. *Genes & Development* 2013;**27**:2179–91. doi:10.1101/gad.225680.113

63 Lau A, Villeneuve NF, Sun Z, *et al.* Dual roles of Nrf2 in cancer. *Pharmacol Res* 2008;**58**:262–70. doi:10.1016/j.phrs.2008.09.003

64 Müller T, Hengstermann A. Nrf2: friend and foe in preventing cigarette smoking-dependent lung disease. *Chem Res Toxicol* 2012;**25**:1805–24. doi:10.1021/tx300145n

CONCLUSION

Chapter 1

Nicotine concentration labeling on EC refill fluid products was often inaccurate with a majority of fluids containing nicotine in excess of the manufacturer's concentration labeling. Evaluation of products lacking any labeling of nicotine content proved to be either nicotine free or have concentrations of nicotine in excess of 100 mg/mL and may have been intended for DIY use. Generally, products labeled as zero nicotine contained no detectable nicotine. Comparison of duplicate refills fluids showed a lack of fidelity in manufacturing. Duplicate products varied greatly in their nicotine concentrations as well as coloration. However, one of the five bands tested showed significant improvement in labeling accuracy over time. These data demonstrate the need for regulations governing the accurate labeling of nicotine concentrations on refill fluids, as well as the need for manufacturing guidelines to improve product integrity.

Chapter 2

DIY flavoring products from a single company contained substantial amounts of nicotine. These products are additives to EC refill fluids and are presumed to be free of nicotine. Among the 30 flavoring products tested, two were found to contain nicotine at concentrations of 14.2 and 95.4 mg/mL with the total nicotine content of a 5 mL volume bottle being 71 and 477 mg, respectively. These are concentrations that, if ingested, could be fatal to children and possibly adults. These data reinforce the need for stringent manufacturing guidelines for EC products and for nicotine handling and accurate labeling.

Chapter 3

This study has shown that the iQOS system may not be as harm-free as claimed. IQOS is a well manufactured product with performance data being consistent between heatsticks. However, product usage limitations, the device shuts off after 6 mins and must be recharged before it can be used again, affected the application of standard ISO and HCI smoking protocols. These limitations can also affect user topography, forcing them to smoke at a rapid pace in order to fully maximize heatsticks. Decreasing the interpuff interval could lead to an increase in intake of nicotine and carbonyl compounds. This study also showed that the iQOS tobacco plugs appeared charred without ignition and charring increased when cleaning was not performed after each use. This study also showed that the polymer-film filter, a thin plastic sheet, melts during iQOS use and releases formaldehyde cyanohydrin. Results emphasize the urgent need for further safety testing as the popularity and user base of this product is growing rapidly.

Chapter 4

Cytotoxicity evaluations of IQOS aerosols showed that exposures did not lead to cell death but did adversely affect cellular function, specifically mitochondrial reductase function and lysosomal integrity. IQOS aerosol and 3R4F smoke were not significantly different from each other in many cell types and cell types varied in their sensitivity to IQOS aerosol and cigarette smoke with NHLF, BEAS-2B, and H9-hESC being the most sensitive. NIH/3T3 and A549 were the least sensitive with A549, a cancer cell line, being the most impervious to treatment. NRU indicated differences in C1 and C20 aerosols for NIH/3T3, A549, NHLF, and NHBE (smoker), showing that cleanliness can affect toxicity results but are cell type specific. While these data cannot be directly correlated to

specific adverse human health conditions, they reinforce that IQOS is not a harm free and clearly demonstrate the need for additional studies.

Chapter 5

IQOS aerosol treatment successfully induced an EMT in primary lung cancer cells. Exposure increased the number of in enlarged phenotypic, low E-cadherin, high vimentin, motile cells. EMT is the first step leading to cancer metastasis. The biggest factor in the deadliness of lung cancer is the ability of the cells to travel to new location and establish tumors in multiple organ systems. These data raise concerns about the safety of IQOS products, and as IQOS popularity and worldwide distribution increases, these findings reinforce the critical need for more extensive testing of IQOS to fully understand the potential adverse health effects it may have on users.

Chapter 6

IQOS aerosol exposure was able to effect changes in protein expression. Classifying protein expression differences into categories, it was demonstrated that IQOS exposure greatly effects proteins involved in metabolic functions. There was overlap of annotation categories between both aerosol types. However, protein expression changes caused by C1 and C20 exposures had different protein expression profiles. These data show that the cleanliness of IQOS holders affects aerosol quality and modulates different proteins, which can potentially lead to different adverse effects. These results suggest that IQOS poses health risks to users and emphasize the need for more extensive testing. As PMI pushes to increase sales of their product through

introduction into new markets, the data from this investigation will be extremely useful to regulatory agencies, as well as to physicians and IQOS users.

APPENDIX A

Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models

Vasundhra Bahl, Sabrina Lin, Nicole Xu, Barbara Davis, Yu-huan Wang, Prue Talbot

Reprod Toxicol 2012;**34**:529–37. doi:10.1016/j.reprotox.2012.08.001

Reproductive Toxicology 34 (2012) 529–537

Contents lists available at SciVerse ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models

Vasundhra Bahl[a,b,c,1], Sabrina Lin[b,c,1], Nicole Xu[b,c,1,2], Barbara Davis[c], Yu-huan Wang[b,c], Prue Talbot[a,b,c,*,1]

[a] *Environmental Toxicology Graduate Program, University of California, Riverside, CA 92521, United States*
[b] *UCR Stem Cell Center, University of California, Riverside, CA 92521, United States*
[c] *Department of Cell Biology and Neuroscience, University of California, Riverside, CA 92521, United States*

ARTICLE INFO

Article history:
Received 21 March 2012
Received in revised form 5 August 2012
Accepted 11 August 2012
Available online xxx

Keywords:
Embryonic stem cells
Neural stem cells
Pulmonary fibroblasts
Electronic cigarettes
E-juice
Nicotine
Cytotoxicity
Tobacco-related disease

ABSTRACT

Electronic cigarettes (EC) and refill fluids are distributed with little information on their pre- and postnatal health effects. This study compares the cytotoxicity of EC refill fluids using embryonic and adult cells and examines the chemical characteristics of refill fluids using HPLC. Refill solutions were tested on human embryonic stem cells (hESC), mouse neural stem cells (mNSC), and human pulmonary fibroblasts (hPF) using the MTT assay, and NOAELs and IC_{50}s were determined from dose–response curves. Spectral analysis was performed when products of the same flavor had different MTT outcomes. hESC and mNSC were generally more sensitive to refill solutions than hPF. All products from one company were cytotoxic to hESC and mNSC, but non-cytotoxic to hPF. Cytotoxicity was not due to nicotine, but was correlated with the number and concentration of chemicals used to flavor fluids. Additional studies are needed to fully assess the prenatal effect of refill fluids.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Electronic cigarettes (EC) are nicotine delivery devices that are rapidly gaining acceptance as an alternative to conventional cigarettes with little knowledge regarding their effects on prenatal development or adult health [1–3]. EC have a mouthpiece containing a fluid-filled cartridge, an atomizer used to vaporize the cartridge fluid, and a battery that powers the atomizer [3]. The cartridge fluid usually contains nicotine, flavorings, and a humectant that when heated by the atomizer creates an inhalable aerosol. In some EC, the cartridge and atomizer are combined into a single unit called a “cartomizer” [3,4]. Refill fluid, also known as E-juice or E-liquid, contains flavoring, nicotine, and a humectant(s), such as propylene glycol (PG) and/or vegetable glycerin (VG). Used EC cartridges or cartomizers can be refilled with drops of refill fluid, which is readily available often from third party vendors on the Internet or in shopping malls.

While the detrimental effects of conventional cigarette smoke on both adult and prenatal health are well documented [5–9], little direct work has been done on the health effects of EC products, in spite of a recognized need for such information [10]. It has been proposed that EC are less harmful than conventional tobacco products due to their lower total number of chemicals and lower concentration of carcinogens [11,12]. EC refill fluids are often sold by vendors other than the EC manufacturers, and they have received even less evaluation than EC devices themselves. As a step toward better understanding the health effects of EC, we evaluated the cytotoxicity of 40 samples of EC refill fluid using cells that model both embryonic and adult stages of the life cycle. With the introduction of human embryonic stem cells (hESC) [13], it is now possible to examine effects of consumer products and environmental chemicals on cells that model an early stage of prenatal development [14]. Recent studies have shown that hESC when cultured in vitro have the characteristics of the epiblast cells present in young

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EC, electronic cigarette; DPBS, Dulbecco's phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; hESC, human embryonic stem cells; hPF, human pulmonary fibroblasts; HPLC, high pressure liquid chromatography; IC_{50}, concentration that produces a 50% inhibition when compared to a control; mNSC, mouse neural stem cells; MTT, 3-(45-dimethylthiazol-2-yl)-25-diphenyltetrazolium bromide; NOAEL, no observed adverse effect level; PG, propylene glycol; VG, vegetable glycerin.

* Corresponding author at: Department of Cell Biology and Neuroscience, 900 University Avenue, University of California, Riverside, CA 92521, United States. Tel.: +1 951 827 3768; fax: +1 951 827 4286.
E-mail address: talbot@ucr.edu (P. Talbot).
[1] These authors contributed equally to this paper.
[2] Current address: University of Pennsylvania, Philadelphia, PA 19104, United States.

0890-6238/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.reprotox.2012.08.001

Table 1
NOAELs, and IC_{50}s for refill fluid products in the screen.

Inv. no	Refill fluid	Company	Nicotine (mg/ml)	hESC		mNSC		hPF	
				IC_{50}	NOAEL	IC_{50}	NOAEL	IC_{50}	NOAEL
32	Propylene glycol	FS-USA[a]		>1	0.3	>1	0.3	>1	>1
33	Vegetable glycerin	FS-USA		>1	>1	>1	>1	>1	>1
18	Bubblegum	FS-USA	24	>1	0.3	>1	0.3	>1	>1
30	Butterscotch	FS-USA	0	>1	0.3	>1	0.1	>1	0.001
29	Butterscotch	FS-USA	6	>1	0.1	>1	0.1	>1	>1
26	Caramel	FS-USA	0	>1	0.3	>1	0.1	>1	>1
27	Caramel	FS-USA	6	>1	0.3	>1	0.3	>1	0.1
28	Caramel	FS-USA	6	>1	0.3	>1	0.3	>1	0.3
40	Caramel	Global Smoke	18	0.75	0.1	>1	0.3	0.41	0.01
19	Butterfinger	FS-USA	24	0.51	0.1	>1	0.3	>1	>1
23	Menthol Arctic	FS-USA	0	0.45	0.3	>1	>1	0.45	0.3
7	Wisconsin frost	Red Oak	18	0.37	0.1	0.61	0.3	>1	>1
1	Domestic	Red Oak	18	0.37	0.1	0.31	0.1	>1	>1
13	JC original	Johnson Creek	18	0.38	0.03	0.45	0.3	>1	>1
12	French vanilla	Johnson Creek	18	0.34	0.1	0.37	0.1	0.97	0.3
25	Vanilla Tahity	FS-USA	0	0.36	0.1	0.35	0.1	0.19	0.03
17	Tennessee cured	Johnson Creek	18	0.26	0.01	0.32	0.1	>1	0.3
5	Tennessee cured	Red Oak	18	0.32	0.1	0.09	>1	>1	0.03
2	Island	Red Oak	18	0.24	0.01	0.30	0.1	>1	>1
24	Pure nicotine	FS-USA	100	0.23	0.01	0.31	0.1	0.35	0.001
6	Valencia	Red Oak	18	0.22	0.03	0.31	0.1	>1	0.03
14	Mint chocolate	Johnson Creek	18	0.12	0.01	0.28	0.1	>1	0.1
4	Swiss Dark	Red Oak	18	0.11	0.03	0.16	0.03	0.30	0.1
21	Caramel	FS-USA	0	0.1	0.03	0.14	0.03	0.22	0.01
11	Espresso	Johnson Creek	18	0.08	0.01	0.30	0.1	>1	0.3
3	Mercado	Red Oak	18	0.08	0.01	0.09	0.03	0.82	0.3
15	Simply strawberry	Johnson Creek	18	0.06	0.01	0.43	0.3	>1	0.1
8	Arctic Menthol	Johnson Creek	18	0.05	0.01	0.19	0.1	>1	0.3
20	Butterscotch	FS-USA	0	0.06	0.03	0.22	0.03	0.26	0.03
16	Summer peach	Johnson Creek	18	0.04	0.01	0.45	0.1	>1	0.3
9	Black cherry	Johnson Creek	18	0.05	0.01	0.16	0.1	>1	0.3
34	JC original	Johnson Creek	11	0.04	0.01	0.46	0.1	>1	>1
10	Chocolate truffle	Johnson Creek	18	0.03	0.01	0.26	0.03	>1	>1
31	Tennessee cured	Johnson Creek	11	0.03	0.01	0.30	0.1	>1	0.001
22	Cinnamon Ceylon	FS-USA	0	0.01	0.01	0.04	0.01	0.07	0.03
41	Butterscotch[b]	Freedom Smoke FlavourArt	0	–		0.58	0.3	0.26	0.03

[a] FS-USA, Freedom Smoke USA.
[b] This was not a part of the original screen.

implantation embryos [15,16]. Although some toxicological work has been done previously using hESC [17,18], adaptation of these cells to standard toxicological studies has been slow because they grow in colonies that are difficult to count and plate accurately. We recently developed a method that is amenable to studying hESC in 96-well plate assays, such as the MTT assay. In the current study, we have taken advantage of this method to perform dose–response cytotoxicity experiments using: (1) hESC, which model the epiblast stage of development [15,16], (2) mouse neural stem cells (mNSC) isolated from the brain of a newborn, and (3) human pulmonary fibroblasts (hPF), which represent an adult cell from one of the initial points of contact for inhaled EC aerosol. The purpose of our study was to compare the sensitivity of embryonic and adult cells to a range of EC refill products and to test the hypothesis that embryonic cells are more sensitive to EC product exposure than adult lung cells. The study included two humectants, 29 different flavors of refill fluid, products from four vendors, five concentrations of nicotine, and six samples that may have caused adverse health effects in users. HPLC spectral analysis was also done to determine if chemicals varied between products with the same flavor or between bottles of the same product.

2. Materials and methods

2.1. Sources of refill fluids

A convenience sampling procedure was adopted to select products for analysis. Products were manufactured by Freedom Smoke USA (Tucson, AZ), Global Smoke (Los Angeles, CA), Johnson Creek (Johnson Creek, WI), and Red Oak (a subsidiary of Johnson Creek). These manufacturers were chosen as they represent popular domestic companies whose products are readily available to e-cigarette users on the Internet. Thirty-six bottles of refill fluid containing various flavorings and nicotine concentrations were evaluated (Table 1). Thirty-four refill bottles were purchased from the manufacturers via the Internet, the Global Smoke product was purchased at a local mall (Riverside, CA), and one bottle was sent to us by a user who thought the refill sample had made her ill. The bottle from the user had been opened when we received it, and we cannot eliminate the possibility that the contents were modified. The bottles that we purchased were chosen to give a range of manufacturers, humectants, nicotine concentrations, and flavors. All bottles were given an inventory number.

2.2. Culturing hESC, mNSC, and hPF

H9-Oct4-GFP hESC, obtained from the Stem Cell Core at the University of California, Riverside, and H9 hESC obtained from WiCell (Madison, WI) were cultured in a 5% CO_2 incubator at 37 °C and 95% relative humidity using methods previously described in detail [19]. hESC were maintained on Matrigel (Fisher Scientific, Bedford, MA) coated 6-well plates (Falcon, Fisher Scientific, Chino, CA) containing complete mTeSR®1 Medium (Stem Cell Technologies, Vancouver, BC, Canada) and were used for experimentation when wells were 60–80% confluent. Each day, cultures were observed for normal morphology, and medium was changed. To subculture or prepare hESC for experiments, wells were washed with Dulbecco's phosphate buffered saline (DPBS) (GIBCO, Invitrogen, Carlsbad, CA), colonies were enzymatically detached using Accutase (eBioscience, San Diego, CA), and large cell clumps were mechanically dispersed using sterile glass beads. For MTT experiments, cell concentration was adjusted spectrophotometrically to produce 20,000 cells/well using a BioMate 3S Spectrophotometer (Thermo Fisher Scientific, Chino, CA).

mNSC were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD) containing 10% fetal bovine serum, 5% horse serum, 1% sodium pyruvate (Lonza, Walkersville, MD) and 1% penicillin–streptomycin (GIBCO, Invitrogen, Carlsbad, CA). The cells were cultured in Nunc T-25 tissue culture flasks (Fisher

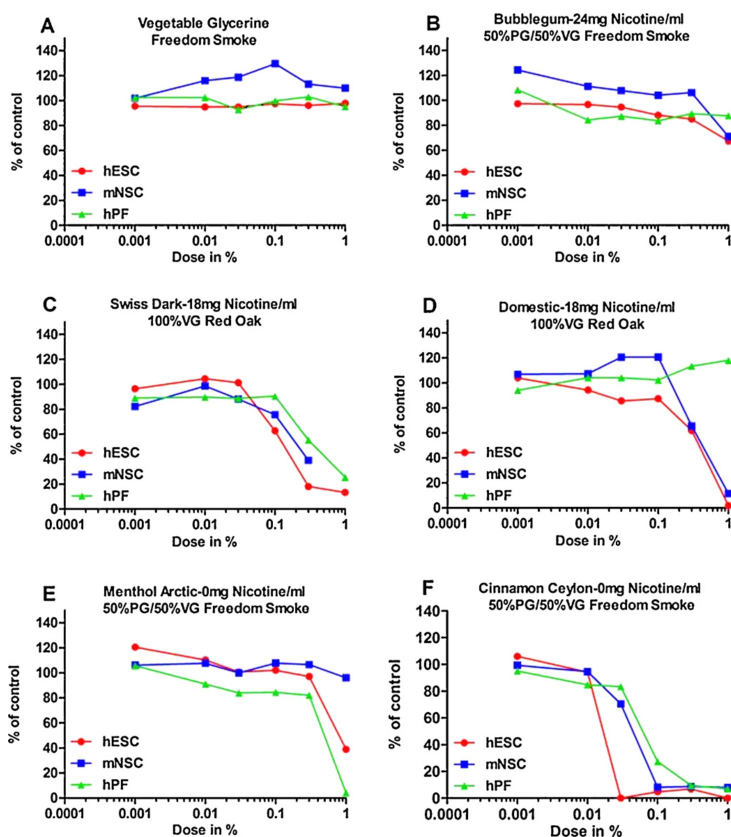


Fig. 1. Dose–response curves showing representative examples of data obtained in the MTT cytotoxicity assay. Absorbance (percentage of the control) from the MTT assay is plotted as a function of the refill fluid dose. (A) Vegetable glycerin (non-cytotoxic), (B) Bubblegum (non-cytotoxic), (c) Swiss Dark (moderately cytotoxic), (D) Domestic (moderately cytotoxic to the stem cells), (E) Menthol Arctic (moderately cytotoxic the hPF), (F) Cinnamon Ceylon (highly cytotoxic).

Scientific, Tustin, CA), medium was replaced on alternate days, and when confluency reached about 80%, cells were used in an experiment. To detach cells for testing, wells were washed with DPBS then treated with 0.05% trypsin EDTA/DPBS (GIBCO, Invitrogen, Carlsbad, CA) for 1 min at 37 °C. For the MTT assay, cells were plated at 2500 cells/well in 96-well plates.

Human pulmonary fibroblasts (hPF) (ScienCell, Carlsbad, CA) were cultured using the suppliers protocol in complete fibroblast medium containing 2% fetal bovine serum, 1% fibroblast growth serum, and 1% penicillin/streptomycin. hPF were grown on poly-L-lysine (15 μl/10 ml) coated T-25 flasks, which were prepared and incubated overnight prior to use. hPF were examined microscopically daily, and medium was changed every other day. hPF were cultured in 5% CO_2 at 37 °C and 95% relative humidity until 85% confluent, at which time they were used for MTT testing. For sub-culturing and experimental set up, cells were washed with DPBS and detached with 0.01% trypsin diluted in DPBS for 1 min at 37 °C.

2.3. Testing refill solutions for cytotoxicity using hESC, mNSC and hPF in the MTT assay

Thirty-five refill products were evaluated for cytotoxicity in 96-well plates using the MTT assay with hESC, mNSC and hPF. The 96-well plates were laid out to have negative controls in columns 1 and 2, followed by various doses of refill solution (0.001%, 0.01%, 0.03%, 0.1%, 0.3%, and 1%) in ascending order from left to right, followed by two additional negative controls in columns 10 and 11. The latter two controls were used to determine if any of the 1% doses produced vapor that impaired cell survival in adjacent wells lacking refill solution.

To set up an experiment with hESC, wells were coated with Matrigel, and then 50 μl of either mTeSR or mTeSR with varying doses of refill solution were added to each well. 50 μl of cell suspension in mTeSR (20,000 cells/well) were added to each well. Experiments with mNSC and hPF were set up in a similar manner except that mNSC were plated directly onto non-coated plates at 2500 cells/well and hPF were plated on poly-L-lysine coated plates (20 μl/10 ml) at 20,000 cells/well. After incubation at 37 °C, 5% CO_2 and 95% relative humidity for 48 h, the MTT assay was performed.

The MTT assay measures conversion of a yellow tetrazole (MTT) to a purple formazan that can be quantified spectrophotometrically at 570 nm [20]. Conversion to the colored formazan occurs in healthy cells with active mitochondria. After plates incubated 48 h, MTT (Sigma–Aldrich, St. Louis, MO) (5 mg/ml in DPBS with calcium and magnesium) (Fisher Scientific, Chino, CA) was added to each well, and the plates were rocked at least 5 min to disperse MTT, then incubated for 2 h at 37 °C, 95% relative humidity, and 5% CO_2. Plates were then drained of solution, and 100 μl of dimethyl sulfoxide (DMSO) (Fisher, Chino, CA) were added and mixed evenly with a pipette to form a uniformly colored solution. Absorbance was read at 570 nm using a Victor2 (PerkinElmer, Waltham, MA, USA) or Epoch (Biotek, Winooski, VT) microplate reader.

2.4. HPLC analysis of Butterscotch and Caramel flavored refill solution

Three Butterscotch (#20, #29, #30) and five Caramel (#21,#26, #28, #40, #27) flavored refill products (Table 1) were analyzed by HPLC. After performing the MTT assays, one additional Butterscotch flavored sample (#41) was received from

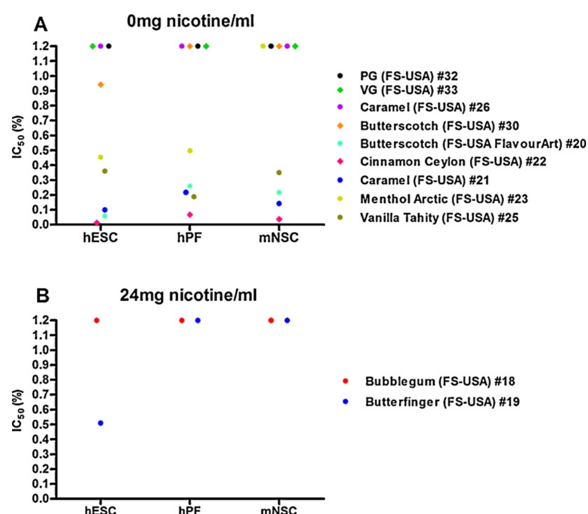


Fig. 2. Relationship between cytotoxicity and nicotine. The IC_{50}s (dose in percent) are plotted for each cell type for each product in a category. Points plotted at 1.2 were non-cytotoxic in the MTT assay. (A) IC_{50}s for cells treated with refill fluid containing 0 mg of nicotine. (B) IC_{50}s for cells treated with refill fluid containing 24 mg of nicotine/ml. There was no correlation between nicotine concentration and cytotoxicity.

Freedom Smoke USA, analyzed using HPLC, and tested for cytotoxicity using mNSC and hPF. Phosphoric acid (85%) and HPLC grade chemicals (triethylamine, water, and acetonitrile) were purchased from Fischer Scientific (Fair Lawn, NJ). Sodium hydroxide was purchased from EM Scientific (Gibbstown, NJ). Samples were analyzed using a Hewlett Packard Series 1100 HPLC, consisting of a quaternary pump, degasser, column thermostat and manual injector. A 200 mm × 4.6 mm Thermo Scientific Hypersil ODS C18 column with a particle size of 5 μm was used at 35 °C with a flow rate of 0.8 mL/min. The diode array detector signal was set to 260 nm with a bandwidth of 40 nm and a reference signal of 380 nm and bandwidth of 10 nm. The injection volume was 5 μL. An isocratic method was used with a buffered mobile phase consisting of 76.9% water, 23% acetonitrile, and 0.1% triethylamine. The pH of the mobile phase was adjusted daily to 7.6 using phosphoric acid and sodium hydroxide. A 5% stock solution of refill fluid in non-buffered mobile phase, consisting of 77% water and 23% acetonitrile was produced for each sample. The working concentration of refill fluids was 0.5%. Three-dimensional spectra were analyzed for each sample to determine the number of peaks and their elution time and relative height.

2.5. Data analysis

MTT absorbance data were normalized by setting the negative control group (column 2) in each row to 100%. All other wells in each row were expressed as a percentage of the negative control. IC_{50}s were computed with Prism software (GraphPad, San Diego, CA) using the log inhibitor vs. normalized response-variable slope with the top and bottom constraints set to 100% and 0%, respectively. When a sigmoidal curve could not be fit to the data using GraphPad, IC_{50}s were determined by eye to obtain a best fit. No observed adverse effect levels (NOAEL) were determined by reading directly off the dose–response curves.

3. Results

3.1. Dose–response of 35 refill products using the MTT assay

Refill solutions had various effects on cell survival in the MTT assay ranging from no evidence of cytotoxicity to high levels of toxicity (representative graphs are shown in Fig. 1; additional data are shown in Table 1 and Supplement Figs. 1–3). Products listed in Table 1 are grouped in a hierarchy of potency based on their IC_{50}s for hESC, which, in general, were more sensitive to refill solutions than the other two cell types. Table 1 also gives information on the NOAELs for each cell type and refill solution tested.

Refill products were grouped in three major categories: low cytotoxicity ($IC_{50} > 1\%$) (Fig. 1A and B and Supplement Fig. 1), moderate cytotoxicity (IC_{50} between 0.1 and 1%) (Fig. 1C–E and Supplement Fig. 2), and high cytotoxicity ($IC_{50} < 0.1\%$) (Fig. 1F and Supplement Fig. 3). The two humectants most often used in refill solutions, vegetable glycerin (VG) (Fig. 1A) and propylene glycol (PG) (Supplement Fig. 1A; Table 1), were non-cytotoxic for all cell types. An example of a non-cytotoxic refill fluid (Bubblegum #18) is shown in Fig. 1B. Five additional samples, which were Butterscotch or Caramel flavored, were also non-cytotoxic at the highest dose tested (Supplement Fig. 1).

Fifteen refill samples were moderately cytotoxic to hESC, and in general, mNSC responded similarly to these samples (Fig. 1C–E; Table 1, and Supplement Fig. 2). For most refill samples in this group, hESC and mNSC were killed by the 1% dose. In contrast, most (10 of 15) refill samples in this group had little or no effect on hPF (Supplement Fig. 2B–E, G–I, K–L). However, Freedom Smoke Menthol Arctic (Fig. 1E) and Global Smoke Caramel (Supplement Fig. 2 A) produced stronger cytotoxic effects on hPF than on the other two cells.

Twelve refill samples were highly cytotoxic to hESC (Fig. 1F, Table 1, Supplement Fig. 3), and all samples in this group affected mNSC. In contrast, the effect was not as strong for hPF, and 7 of 12 samples in this group did not affect hPF at the highest dose (Supplement Fig. 3B–E, G, H, J, K). Cinnamon Ceylon was the most potent sample tested and the only sample that produced strong cytotoxic effects on all three cell types (Fig. 1F).

3.2. Relationship between nicotine concentration and potency

In the samples studied, nicotine concentration ranged from 0 to 24 mg/ml. The IC_{50}s for samples within each nicotine concentration were compared for the three cell types to determine if nicotine concentration correlated with potency (Fig. 2). Points plotted at 1.2 on the *Y*-axis in Fig. 2 had IC_{50}s greater than 1% and were considered non-cytotoxic.

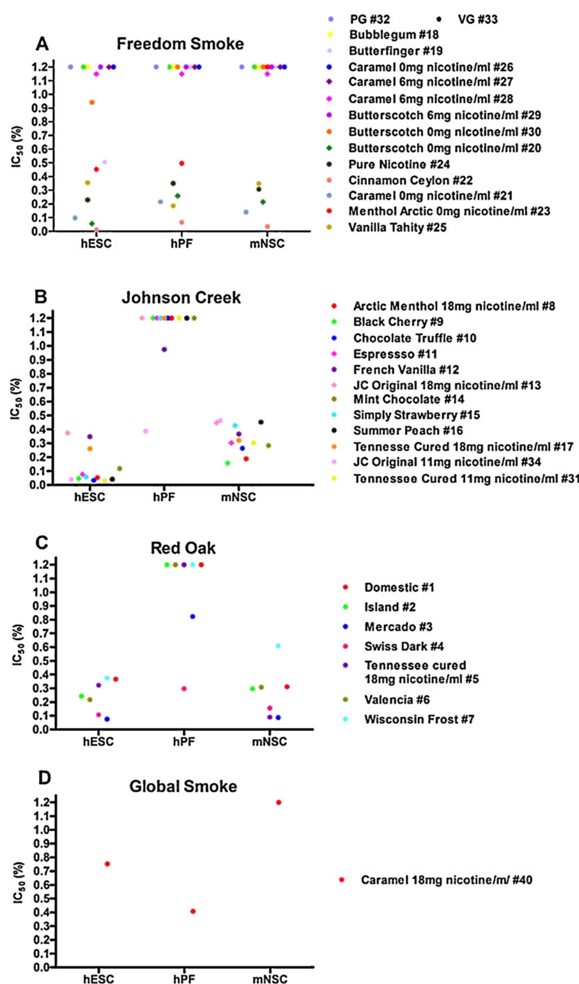


Fig. 3. Relationship between brand and cytotoxicity: The IC_{50}s (dose in percent) are plotted for each cell type for each product in a category. Points plotted at 1.2 were non-cytotoxic in the MTT assay. (A) IC_{50}s for cells treated with Freedom Smoke products. (B) IC_{50}s for cells treated with Johnson Creek products. (C) IC_{50}s for cells treated with Red Oak products. (D) IC_{50}s for the Global Smoke product.

Nine refill samples, including PG and VG, contained no nicotine and fell into all three categories of potency (low, moderate, and high cytotoxicity) (Fig. 2A), indicating cells did not survive better in samples lacking nicotine. Two samples contained 24 mg nicotine/ml, and were either non-cytotoxic or moderately cytotoxic (Fig. 2B), indicating high levels of nicotine were not correlated with high cytotoxicity.

3.3. Relationship between company of origin and potency

Graphs comparing potency among refill products from four companies and comparing sensitivity of each cell type to each product are shown in Fig. 3. Most samples ($N = 15$) came from Freedom Smoke USA, and potency ranged from non-cytotoxic to highly cytotoxic (Fig. 3A). Cinnamon Ceylon was the only sample that was highly cytotoxic to all cell types.

The cytotoxic response was very different for the Johnson Creek samples ($N = 12$), most of which were highly or moderately cytotoxic to hESC and mNSC, with mNSC being slightly less sensitive than the hESC. In contrast, all but one sample was non-cytotoxic to hPF (Fig. 3B). A similar pattern was seen for Red Oak products (Fig. 3C), which were moderately or highly cytotoxic to hESC and mNSC, while most were non-cytotoxic to hPF.

The IC_{50}s for the sample obtained from Global Smoke ranged from non-cytotoxic (mNSC) to moderately cytotoxic (hESC and hPF) (Fig. 3D).

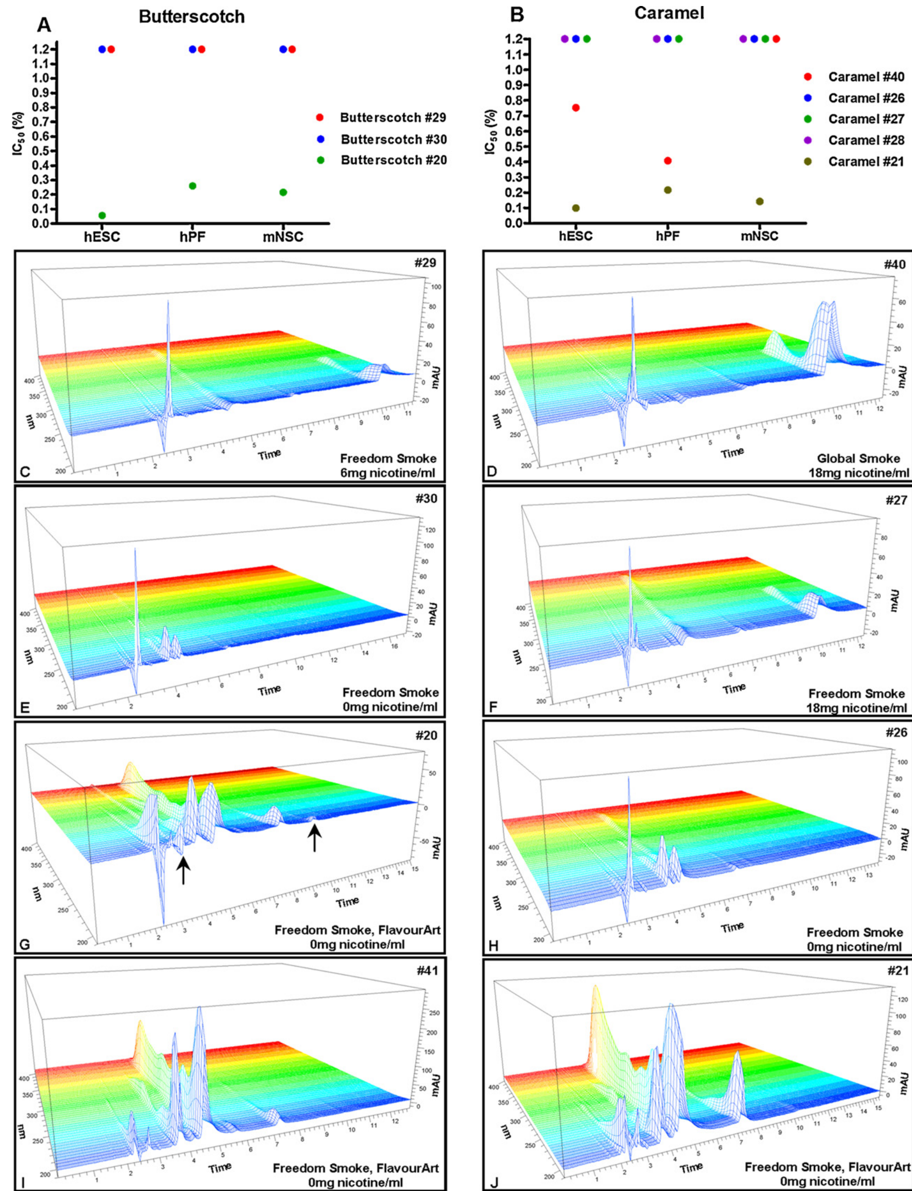


Fig. 4. Relationship between flavors and cytotoxicity: (A) IC_{50}s for cells treated with Butterscotch flavored refill fluid. (B) IC_{50}s for cells treated with Caramel flavored refill fluid. For A and B, the IC_{50}s (dose in percent) are plotted for each cell type for each product in a category. Points plotted at 1.2 were non-cytotoxic in the MTT assay. (C, E, G, I) Three-dimensional HPLC spectra for four samples of Butterscotch flavored refill fluid. (D, F, H, J) Three-dimensional HPLC spectra for four samples of Caramel flavored refill fluid. *X* axis = time (minutes), *Y* axis = absorbance (mAu), *Z* axis = wavelength in nm. First peaks are humectants, peak between 10 and 11 min in some spectra is nicotine, and peaks between the humectants and nicotine are flavoring peaks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Relationship between flavors and potency

The IC_{50}s for the three Butterscotch and five Caramel samples included in the study ranged from non-cytotoxic to highly cytotoxic (Fig. 4A and B). To determine if chemical differences in these samples could account for the cytotoxic differences, three-dimensional HPLC spectra of the Butterscotch and Caramel samples were analyzed.

HPLC spectra for the three Butterscotch samples from Freedom Smoke USA differed from each other (Fig. 4C, E, G). The first peaks to elute were the humectants, the peak eluting between 10 and 11 min is nicotine, and the peaks between the humectant and nicotine are flavorings. The two Butterscotch samples that were non-cytotoxic (Fig. 4C and D) had few flavoring peaks with low heights. In contrast, Butterscotch (FlavourArt #20) (Fig. 4G), which was cytotoxic to all cell types (Fig. 4A), had a complex spectrum with more flavor peaks that were higher than in the non-cytotoxic samples. The highly cytotoxic sample (bottle #20) was received from a user and therefore could have been altered after manufacture. A bottle (#41) which had an identical label to that in Fig. 4G (bottle #20) was received directly from the vendor after the 35-sample study was completed and was tested in the MTT assay and by HPLC (Fig. 4I). The original sample (Fig. 4G, bottle #20) had two minor peaks (arrows) that were not present in the new sample (Fig. 4I, bottle #41). Moreover, the two major peaks in the new sample (Fig. 4I) were 4–5 times higher than the corresponding peaks in the original sample (Fig. 4G), indicating a much higher concentration of these chemicals in bottle #41. The refill solution in both bottles was moderately to highly cytotoxic (Fig. 4A and Supplement Fig. 4).

Spectra for four Caramel samples (Fig. 4D, F, H, J) were different from each other (#27 is not shown). Global Smoke #40 contained mainly humectant (PG) and nicotine with virtually no flavoring peaks (Fig. 4D). Freedom Smoke USA #27 contained humectant (VG), nicotine, and a small flavoring peak (Fig. 4F). Freedom Smoke USA #26 had humectant (VG) and two flavoring peaks of small height (Fig. 4H). Freedom Smoke USA FlavourArt #21 contained three flavoring peaks that were 5–6 times higher than peaks in the other three samples (Fig. 4J). The Caramel product that had the largest number of peaks and the highest peaks (Freedom Smoke, #21) (Fig. 4J) was also the most cytotoxic (Fig. 4B).

4. Discussion

Understanding the health effects of EC refill fluid is important as these products have become widely distributed without much prior testing. Refill fluid is handled by users, manufacturers, and potentially by children living in homes where EC are used. As a step to understanding how EC products affect human health, we compared the cytotoxicity of 35 refill fluid samples using embryonic and adult cells. Refill products varied significantly in their potency over the dose range tested. In general, stem cells from embryos (hESC) and newborns (mNSC) were more sensitive to refill solutions than differentiated adult lung fibroblasts, as shown clearly in the Johnson Creek/Red Oak data. Of 35 products tested, only Caramel #40 and Menthol Arctic #23 had stronger effects on hPF than on the stem cells. These data support our hypothesis that cells from embryos and newborns are more sensitive to EC products than adult cells and are consistent with the concept that embryos are usually more sensitive to environmental chemicals than adults [21]. Our data further demonstrate the importance of using multiple cell types, including embryonic cells, when evaluating EC products. The cytotoxic effects that some refill fluids produced on stem cells could translate into embryonic loss or developmental defects during pregnancy. While it is not yet known what dose of refill fluid reaches an embryo or fetus when a pregnant woman receives dermal, oral, or pulmonary exposure, our data indicate that further work should be done on the effects of these products during pregnancy.

It is possible that our data underestimate the cytotoxicity of refill fluids to lung cells. In a preliminary trial, vapors from 10% doses of some refill fluids killed control cells in adjacent wells. To avoid vapor effects, assays were performed at a maximum concentration of 1%. This would be 100 times less than a user would receive on their skin or inhale into their mouth/lungs. The NOAELs and IC_{50}s should therefore be interpreted with this dose range in mind. Exposure of lung cells to full strength aerosol, which is heated, may have stronger effects than reported in our study, and even samples we found to have low cytotoxicity with lung fibroblasts may be cytotoxic in vivo at full strength.

The potency of refill products varied greatly, demonstrating the importance of evaluating multiple products. Some products had no effect at the doses tested, while others killed all cells at doses lower than a user may receive. Cinnamon Ceylon (#22) was the most potent of the refill fluids tested and strongly inhibited survival of all cell types. Refill fluid users have expressed caution about cinnamon flavored products on Internet blogs and have mentioned mouth, throat, and lung problems when using cinnamon flavored refill fluid (http://www.e-cigarette-forum.com/forum/health-safety-e-smoking/212870-do-you-vape-cinnamon-flavors-read.html).

Cytotoxicity studies on EC products are rare. When various European refill fluid aerosols were tested in the MTT assay using mouse 3T3 fibroblasts, only 1 out of 15 products showed cytotoxicity at the highest doses tested [22,23]. We found more cytotoxic samples in our set of 35 refill products; however, the European study is not directly comparable to ours due to differences in products, sample preparation, experimental design, and method of analysis.

Several major conclusions can be drawn from our study. First, hESC were generally more sensitive to refill fluids than the other two cell types, and mNSC were generally more sensitive than hPF. Secondly, no company emerged as having all non-cytotoxic or all cytotoxic refill products. However, an interesting pattern was observed for samples from Johnson Creek and Red Oak, which were generally cytotoxic to stem cells and non-cytotoxic to lung fibroblasts. Third, there was no correlation between cytotoxicity and nicotine concentration for the dose range used. Fourth, each refill product needs individual evaluation to determine cytotoxicity, preferably using multiple cell types. Fifth, the refill fluid provided to us by a user who thought the sample had made her ill was moderately to highly cytotoxic, as was a duplicate bottle purchased directly from the vendor. Sixth, within a particular flavor, cytotoxicity was highly variable, even when the flavor came from a single manufacturer, as was seen with the Butterscotch and Caramel samples from Freedom Smoke. For example, one Butterscotch sample (#41) received directly from the company was highly toxic, while two other Butterscotch flavors (#29 and #30) from the same company had low toxicity. HPLC analysis showed that increased cytotoxicity within a flavor was correlated with an increase in the number and height of the flavoring peaks (Fig. 4C, E, G, I). In addition, two different bottles from the same manufacturer with identical Butterscotch labels (#20 and 41) had slightly different chemical composition and significantly different amounts of the two major flavoring chemicals (Fig. 4G and I). Since one of these bottles was supplied to us by a user, we cannot eliminate the possibility that the two additional peaks were added after manufacture. However, the bottle we purchased from the company had much higher concentrations of the two major flavoring peaks. Since it is unlikely the user could have removed flavoring from the bottle, the difference in amount of flavoring between bottles #20 and #41 probably represents a true difference in the contents of a single product from this company. Similar differences in the amount of added flavorings were seen in caramel flavored refill fluid from Freedom Smoke (e.g., bottles

#26 and #21) These data show that users cannot assume that the chemicals or the concentration of the chemicals used to create a particular flavor will be identical in all products having the same flavor. We are currently identifying the chemicals in those products that were cytotoxic, so that in the future refill products can be improved by using only non-cytotoxic flavorings at relatively low concentration.

Our data may help refill fluid users identify and avoid products that could pose health risks to themselves and their offspring. For example, Cinnamon Ceylon (#22) was highly potent for the three cell types and would likely present more risk than flavors such as Bubblegum (#18) which had low cytotoxicity for all cells. However, even products we found to be non-cytotoxic may produce different, possibly stronger, effects when used repeatedly at full strength doses. As related examples, PG, which is "generally regarded as safe" and was non-cytotoxic for all cell types in the MTT assay, increased respiratory, throat and nasal symptoms, and cause vocal cord inflammation with prolonged inhalation by theater workers [24], and chronic exposure to PG in indoor air may induce or exacerbate allergic symptoms, asthma, and rhinitis [25].

Lung fibroblasts were relatively robust and often not affected by doses of refill fluid that were cytotoxic to the two stem cell groups. However, lungs contain progenitor cells and stem cells that are critical to lung tissue regeneration and repair [26,27]. Further studies are needed to determine how lung stem cells and other lung cell types respond to refill fluid and if chronic exposure to inhaled refill fluid affects lung health. A recent human study showed that 5 min of EC inhalation significantly altered several measures of lung physiology [28]. The MTT assay used in our study measured cytotoxicity, while the latter study by Vardavas et al. measured physiological responses that do not include cell death, but could be important to the overall lung health.

5. Conclusions

Embryonic and neonatal stem cells were generally more sensitive to refill products than adult lung fibroblasts. Refill fluid users should be aware that: (1) the low doses and one time exposure used in our study may underestimate cytotoxicity, and (2) within a flavor, such as Butterscotch or Caramel, chemical composition and cytotoxicity were variable. The latter point demonstrates that it cannot be assumed that a specific flavor, such as Butterscotch, will always be non-cytotoxic. The results of this study, while preliminary, may be helpful to individuals who are considering using EC, to EC users who are trying to identify refill brands that have low cytotoxicity, to refill fluid suppliers concerned with user safety, to health care workers and physicians who advise EC users, and to policy makers involved in health and environmental issues relating to EC regulation.

Funding

Funding was provided by the California Tobacco-Related Disease Research Program (XT-0118), the California Institute for Regenerative Medicine (NE-A0005A-IE), the UCR Academic Senate, the UCR Graduate Division (Dean's Fellowship to V.B.), a STEM Pathway Project award to Barbara Davis, and a TRDRP Postdoctoral Fellowship (20FT-0084) to S.L.

Conflict of interest

None declared.

Acknowledgements

We are grateful to Dr. Evan Snyder (Stanford-Burnham Medical Institute, San Diego, CA) for providing the mNSC. We also thank Monique Williams, R. Talbot, Kamlesh Asotra, and Rachel Behar for their comments on the manuscript, Laurie Graham for her assistance with equipment, Frank Monreal for help preparing Fig. 4, Crystal Hua for performing literature searching, and Jisso Kim and Michael Dang for assisting with the HPLC.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2012.08.001.

References

[1] Ayers JW, Ribisl KM, Brownstein JS. Tracking the rise in popularity of electronic nicotine delivery systems (electronic cigarettes) using search query surveillance. American Journal of Preventive Medicine 2011;40:448–53.
[2] Noel JK, Rees VW, Connolly GN. Electronic cigarettes: a new 'tobacco' industry? Tobacco Control 2011;20:81.
[3] Trtchounian A, Talbot P. Electronic nicotine delivery systems – is there a need for regulation. Tobacco Control 2010;20:47–52.
[4] Williams M, Talbot P. Variability among electronic cigarettes in the pressure drop, airflow rate, and aerosol production. Nicotine & Tobacco Research 2011;13:1276–83.
[5] U.S. Department of Health and Human Services. How tobacco smoke causes disease: the biology and behavioral basis for smoking-attributable disease: a report of the surgeon general. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health; 2010.
[6] Higgins S. Smoking in pregnancy. Current Opinion in Obstetrics and Gynecology 2002;14:145–51.
[7] Shiverick KT, Salafia C. Cigarette smoking and pregnancy. I: Ovarian, uterine and placental effects. Placenta 1999;20:265–72.
[8] Talbot P. In vitro assessment of reproductive toxicity of tobacco smoke and its constituents. Birth Defects Research Part C: Embryo Today 2008;84:61–72.
[9] Talbot P, Riveles K. Smoking and reproduction: the oviduct as a target of cigarette smoke. Reproductive Biology and Endocrinology 2005;3:52.
[10] Etter J-F, Bullen C, Flouris AD, Laugesen M, Eissenberg T. Electronic nicotine delivery systems: a research agenda. Tobacco Control 2011;20:243–8.
[11] Cahn Z, Siegel M. Electronic cigarettes as a harm reduction strategy for tobacco control: a step forward or a repeat of past mistakes. Journal of Public Health Policy 2011;32:16–31.
[12] Laugesen M. Safety report on the Ruyan e-cigarette cartridge and inhaled aerosol. Christchurch: Health New Zealand; 2008. p. 1–22.
[13] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–7.
[14] Talbot P, Lin S. Mouse and human embryonic stem cells: can they improve human health by preventing disease. Current Topics in Medicinal Chemistry 2011;11:1638–52.
[15] Nichols J, Smith A. Naive and primed pluripotent states. Cell Stem Cell 2009;4:487–92.
[16] Nichols J, Smith A. The origin and identity of embryonic stem cells. Development 2011;138:3–8.
[17] Lin S, Fonteno S, Weng JH, Talbot P. Comparison of the toxicity of smoke from conventional and harm reduction cigarettes using human embryonic stem cells. Toxicological Sciences 2010;118:202–12.
[18] Zdravkovic T, Genbacev O, LaRocque N, McMaster M, Fisher S. Human embryonic stem cells as a model system for studying the effects of smoke exposure on the embryo. Reproductive Toxicology 2008;26:86–93.
[19] Lin S, Talbot P. Methods for culturing mouse and human embryonic stem cells. Methods in Molecular Biology 2011;690:31–56.
[20] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 1983;65:55–63.
[21] Grandjean P, Bellinger D, Bergman A, Cordier S, Davey-Smith G, Eskenazi B, et al. The Faroes statement human health effects of developmental exposure to chemicals in our environment. Basic and Clinical Pharmacology and Toxicology 2007;102:73–5.
[22] Romagna G. Clear Stream Project: citotoxicity assessment of an electronic cigarette vapour on 3T3 fibroblasts. Data review and comment. http://clearstream.flavourart.it/site/?p=128&lang=en.
[23] Romagna G. Clear Stream Project: citotoxicity assessment of an electronic cigarette vapour on 3T3 fibroblasts. Data review and comment.

Updated data from the third laboratory analysis; 2011. http://clearstream.flavourart.it/site/?p=431&lang=en.
[24] Propylene gylcol. The Center for the Evaluation of Risks to Human Reproduction; 2003.
[25] Choi H, Schmidbauer N, Sundell J, Hasselgren M, Spengler J, Bornehag CG. Common household chemicals and the allergy risks in pre-school age children. PLoS One 2010;5:e13423.
[26] Kajstura J, Rota M, Hall SR, Hosoda T, D'Amario D, Sanada F, et al. Evidence for human lung stem cells. New England Journal of Medicine 2011;364:1795–806.
[27] Kotton D, Fine A. Lung stem cells. Cell and Tissue Research 2008;331:145–56.
[28] Vardavas CI, Anagnostopoulos N, Kougias M, Evangelopoulou V, Connolly GN, Behrakis PK. Short-term pulmonary effects of using an electronic cigarette: impact on respiratory flow resistance, impedance, and exhaled nitric oxide. Chest 2012;141(6):1400–6.

APPENDIX B

Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids.

Rachel Behar, Barbara Davis, Vasundhra Bahl, Prue Talbot

Toxicology in Vitro 2014;28:198–208. doi:10.1016/j.tiv.2013.10.006

Toxicology in Vitro 28 (2014) 198–208

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids

R.Z. Behar [a,b,c], B. Davis [a,b,c], Y. Wang [b,c], V. Bahl [b,c,d], S. Lin [b,c], P. Talbot [b,c,*]

[a] Cell Molecular and Developmental Biology Graduate Program, University of California, Riverside, CA 92521, United States
[b] UCR Stem Cell Center, University of California, Riverside, CA 92521, United States
[c] Department of Cell Biology and Neuroscience, University of California, Riverside, CA 92521, United States
[d] Environmental Toxicology Graduate Program, University of California, Riverside, CA 92521, United States

ARTICLE INFO

Article history:
Received 28 August 2013
Accepted 11 October 2013
Available online 25 October 2013

Keywords:
Electronic cigarettes
Cinnamaldehyde
Human embryonic stem cells
In vitro screen
Cytotoxicity
Tobacco products

ABSTRACT

In a prior study on electronic cigarette (EC) refill fluids, Cinnamon Ceylon was the most cytotoxic of 36 products tested. The purpose of the current study was to determine if high cytotoxicity is a general feature of cinnamon-flavored EC refill fluids and to identify the toxicant(s) in Cinnamon Ceylon. Eight cinnamon-flavored refill fluids, which were screened using the MTT assay, varied in their cytotoxicity with most being cytotoxic. Human embryonic stem cells were generally more sensitive than human adult pulmonary fibroblasts. Most products were highly volatile and produced vapors that impaired survival of cells in adjacent wells. Cinnamaldehyde (CAD), 2-methoxycinnamaldehyde (2MOCA), dipropylene glycol, and vanillin were identified in the cinnamon-flavored refill fluids using gas chromatography–mass spectrometry and high-pressure liquid chromatography (HPLC). When authentic standards of each chemical were tested using the MTT assay, only CAD and 2MOCA were highly cytotoxic. The amount of each chemical in the refill fluids was quantified using HPLC, and cytotoxicity correlated with the amount of CAD/product. Duplicate bottles of the same product were similar, but varied in their concentrations of 2MOCA. These data show that the cinnamon flavorings in refill fluids are linked to cytotoxicity, which could adversely affect EC users.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Electronic cigarettes (EC), which deliver nicotine to users without burning tobacco, are rapidly gaining popularity worldwide (Ayers et al., 2011; Etter et al., 2011; McQueen et al., 2011). The original EC consisted of a cartridge with nicotine-containing fluid and an atomizer which aerosolized the cartridge fluid when heated by a battery (Trtchounian et al., 2010). In many newer models, the cartridge and atomizer are combined into a single unit, termed a "cartomizer" (Williams and Talbot, 2011). Cartridge/cartomizer fluid contains nicotine, flavorings, and a humectant, such as propylene glycol (Bahl et al., 2012; Laugesen, 2008). Nicotine concentrations usually range from 0 to 24 mg/ml. Used cartomizers can be replaced or refilled with fresh fluid, referred to as refill fluid (Bahl et al., 2012). Although the basic design of EC is similar across brands, significant variation in performance exists between and within brands (Trtchounian et al., 2010; Williams and Talbot, 2011). EC and their associated products are sold in shops, malls, and online where age verification is not always needed, making these products relatively accessible.

Several recent online surveys and interviews found that EC may help users limit or stop smoking conventional cigarettes (Etter, 2010; Etter and Bullen, 2011; Goniewicz et al., 2013; McQueen et al., 2011). Nevertheless, some users are concerned about the toxicity of EC (Etter, 2010; Etter and Bullen, 2011), while others acknowledge that EC are addictive and may not be completely safe, but consider them less harmful than conventional cigarettes (Goniewicz et al., 2013).

EC aerosol contains relatively few chemicals (Goniewicz et al., 2012; Laugesen, 2008; Westenberger, 2009), suggesting they are safer to use than conventional cigarettes. However, significant amounts of tin were present in the fluid of one brand of EC, and the corresponding aerosol contained metals, including metal nanoparticles (Williams et al., 2013). In a clinical case report, a woman was diagnosed with exogenous lipoid pneumonia seven months after she started using EC (McCauley et al., 2012), and her condition improved when she stopped EC use. Lipoid pneumonia was thought to be caused by inhaling aerosolized EC oil-based humectants, which lead to dyspnea, productive cough, and subjective fevers. A second recent study examined the effect of EC use on

* Corresponding author at: Department of Cell Biology and Neuroscience, University of California, Riverside, CA 92521, United States. Tel.: +1 951 827 3768; fax: +1 951 827 4286.
E-mail addresses: rbeha001@ucr.edu (R.Z. Behar), barbara.davis@email.ucr.edu (B. Davis), yuhuan_wang@yahoo.com (Y. Wang), vbahl001@ucr.edu (V. Bahl), sabrina.c.lin@gmail.com (S. Lin), Talbot@ucr.edu (P. Talbot).

0887-2333/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tiv.2013.10.006

respiratory mechanics and the fraction of exhaled nitric oxide in healthy smokers. Individuals ad-lib puffed for 5 min, during which time EC use caused an increase in impedance, peripheral airway flow resistance, and oxidative stress (Vardavas et al., 2012). In a recent infodemiological study, numerous symptoms attributed to EC were self-reported in Internet forums by EC users (Hua et al., 2013). These studies show that the safety of EC cannot be assumed and that EC may cause their own set of health problems, which are not necessarily found with conventional cigarette use.

Recent *in vitro* studies of cytotoxicity suggest that EC products differ in their potential to adversely affect health. In our prior *in vitro* screen, EC refill fluids varied widely in their cytotoxicity when tested with human embryonic stem cells (hESC), mouse neural stem cells (mNSC), and human pulmonary fibroblasts (hPF) (Bahl et al., 2012). The stem cells were generally more sensitive to refill fluids than differentiated adult lung cells. The same study also showed that the flavoring chemicals and their concentrations varied among refill fluids of the same flavor both within and between manufacturers. In addition, the cytotoxicity of EC refill fluids correlated with the number and concentration of chemicals used for flavoring.

In our prior refill fluid screen, Cinnamon Ceylon was the most cytotoxic of 36 products that were tested (Bahl et al., 2012). The purpose of the current study was to determine if cinnamon-flavored EC refill fluids are generally cytotoxic and to identify the toxicant(s) in Cinnamon Ceylon. Eight additional cinnamon-flavored refill fluids were screened for cytotoxicity. The chemicals in Cinnamon Ceylon were determined using GC–MS, and authentic standards of the identified chemicals were tested to establish the potency of each. The amount of each chemical in the cinnamon-flavored refill products was quantified with HPLC, and correlations were made between the concentrations of the chemicals and the cytotoxicity of each product tested.

Two cell types were used to evaluate cytotoxicity. hESC, which resemble post-implantation epiblast cells (Nichols and Smith, 2009), were chosen as a model for an early stage of prenatal development and could therefore be useful in identifying products that may be embryotoxic. hPF were used to model effects that could occur in lungs following inhalation of EC refill fluid vapors. It is well established that conventional cigarette products can effect lung fibroblasts and lead to disease development (Hallgren et al., 2010; Selman and Pardo, 2002; Kitamura et al., 2011; Togo et al., 2008). These cell types were also used in our prior study (Bahl et al., 2012) and therefore allow comparison to prior our work and to planned future work involving aerosols.

2. Materials and methods

2.1. Sources of refill fluids and chemicals

Ten cinnamon-flavored EC refill products (inventory numbers = #22, #42, #53, #54, #58, #60, #61, #62, #65, #69) were purchased from online vendors. Refill fluid #53 and #69, Sinful Cinnamon, are duplicate purchases from Tasty Puff (Albuquerque, NM). Refill fluid #60, Cinnamon, and #61, Cinnabun, were both purchased from e-cigexpress (Orlando, FL). Refill fluids #22, Cinnamon Ceylon FlavourArt, #42 Cinnamon, and #54, Cinnamon FlavourArt, were purchased from Freedom Smoke USA (Tucson, AZ), #58, Cinna-Bomb x2, was purchased from Vaporbomb.com (Barberton, OH), #62, Cinnamon, was purchased from Vapormaxx (Richmond, VA), and #65, Cinnamon e-liquid, was purchased from DIY Flavor Shack (Las Vegas, NV). Bottles contained various concentrations of nicotine, cinnamon flavoring, and percentages of propylene glycol and/or vegetable glycerin. Trans-cinnamaldehyde (referred to as CAD) was purchased from TCI (Tokyo, Japan), 2-methoxycinnamaldehyde (2MOCA), and dipropylene glycol were purchased from Sigma Aldrich (St. Louis, MO), and vanillin was purchased from Fisher Scientific (Fair Lawn, NJ).

2.2. Culturing hESC and hPF

hESC (H9) were obtained from WiCell (Madison, WI) and cultured in a 5% CO_2 incubator at 37 °C and 95% relative humidity using methods previously described in detail (Lin and Talbot, 2011). hESC were seeded on Matrigel (Fisher Scientific, Bedford, MA) coated 6-well plates (Falcon, Fisher Scientific, Chino, CA) in mTeSR®1 medium (Stem Cell Technologies, Vancouver, BC, Canada). Each day, cultures were observed using a phase contrast microscope and medium was changed. To prepare cells for experimentation, wells at 60–80% confluency were washed with Dulbecco's phosphate buffered saline (DPBS) (GIBCO, Invitrogen, Carlsbad, CA) to remove excess medium, and then cells were enzymatically detached using Accutase (eBioscience, San Diego, CA). Large cell clumps were mechanically dispersed with sterile glass beads to form small colonies of 2–10 cells. For MTT experiments, cell concentration was adjusted using a BioMate 3S Spectrophotometer (Thermo Fisher Scientific, Chino, CA) to produce 40,000 cells/well in a 96-well plate, as previously described in detail (Behar et al., 2012a,b).

Human pulmonary fibroblasts (hPF), purchased from ScienCell (Carlsbad, CA), were cultured using the manufacturer's protocol in complete fibroblast medium containing 2% fetal bovine serum, 1% fibroblast growth serum, and 1% penicillin/streptomycin. hPF were grown on poly-L-lysine (15 μl/10 ml) (ScienCell, Carlsbad, CA) coated T-25 flasks that were prepared then incubated overnight prior to use. hPF cells were examined daily using an inverted phase contrast microscope, and medium was changed every other day. hPF were cultured in 5% CO_2 at 37 °C and 95% relative humidity and prepared for experimentation once reaching 80–90% confluency. Stock 0.25% trypsin (Gibco by Life Technologies, Grand Island, NY) was diluted in calcium/magnesium free DPBS to form a working concentration of 0.01%, which was then used to remove cells from the poly-L-lysine coated surfaces. hPF were dispersed into single cells and plated at 5000 cell/well in 96-well plates.

2.3. Testing for a vapor effect using Cinnamon Ceylon

2.3.1. Spectrophotometric quantification of transfer of Cinnamon Ceylon between adjacent wells in 96-well plates

1% and 0.3% doses of Cinnamon Ceylon refill fluid were prepared using autoclaved water. The absorbance of these dilutions was recorded at 295 nm using a BioMate 3S spectrophotometer with water as the blank. 1% and 0.3% were chosen as the concentrations to study the vapor effect of this product in a 96-well plate. 1% Cinnamon Ceylon solution was prepared in water and 200 μl was added to one of the central wells in a 96-well plate; no other wells contained Cinnamon Ceylon. Wells above, below, to the left and to the right of the central well were filled with 200 μl/well of water forming a cross pattern. The plate was incubated at 37 °C with 5% CO_2 and 95% relative humidity for 48 h. At the end of 48 h, the absorbance of the Cinnamon Ceylon containing well and of the wells containing only water were recorded at 295 nm. These absorbance values were compared to the absorbance values at the beginning of the experiment to determine if Cinnamon Ceylon transferred between adjacent wells.

2.3.2. Demonstrating cytotoxicity of vapors transferred between wells

To determine if the Cinnamon Ceylon that transferred between adjacent wells caused cytotoxicity, 40,000 hESC or 5000 hPF/well were plated in a 96-well plate using a cross pattern in which the central well contained a known dose of Cinnamon Ceylon and

the neighboring wells contained only hESC or hPF culture medium. After 48 h of incubation, an MTT assay was run to determine if the neighboring wells were adversely affected by the central well, which contained Cinnamon Ceylon. The MTT assay was performed as described in detail previously (Behar et al., 2012b). Vapor effects were considered to have occurred if absorbances in the wells immediately adjacent to the central well had lower absorbances than wells further from the central well. If the highest dose initially tested (1%) created a vapor effect, then 0.3% was tested and so on until a high dose was found that did not produce a vapor effect.

2.4. Screening refill fluid and authentic standards for cytotoxicity using hESC and hPF in the MTT assay

Nine refill products and four authentic standards were screened in 96-well plates in dose response experiments using the MTT assay to observe cytotoxic effects on hESC and hPF. The doses of refill fluid were 0.001%, 0.01%, 0.03%, 0.1%, 0.3%, and 1.0%. An initial screening to determine dose range showed that doses higher than 1% often created a vapor effect in 96-well plates causing neighboring wells to become adversely effected. When refill fluids and authentic standards were tested, the 96-well plates were laid out to have negative controls to the right (C1) and left (C2) of the dose range. The C1 control was always adjacent to the lowest dose. Comparison of the C1 and C2 controls were used to determine if any of the high doses produced vapor that impaired cell survival in adjacent wells. To set up an experiment with hESC, all wells were first coated with Matrigel, and then either mTeSR or mTeSR with varying doses of refill fluid or chemical(s) was added to each well to give 50 μl of medium/well. 50 μl of cells in mTeSR (40,000 cells/well) were added to each well, and the plate was incubated at 37 °C, 95% relative humidity, and 5% CO_2 for 48 h. To ensure that equal numbers of hESC were added to each well, a method that gives uniform plating was used (Behar et al., 2012a,b). Micrographs were taken at 48 h with a Nikon Eclipse TE200 inverted microscope equipped with Hoffman modulation optics and a Diagnostic Instruments Inc. (Model #3.2.0). Experiments with hPF were set up in a similar manner with hPF plated at 5000 cells/well on poly-L-lysine coated plates (20 μl/10 ml). After incubation at 37 °C, 95% relative humidity, and 5% CO_2 for 48 h, the MTT assay was performed. Cinnamon Ceylon and the authentic standards were tested in three independent experiments and means and standard deviations were used to produce dose response curves for each cell type. The eight cinnamon flavored refill fluids were evaluated in an iterative dose response screen that was performed at successively decreasing high doses until a vapor effect was not observed. We have previously shown that a similar dose response screen enables reliable comparison of relative cytotoxic among EC products (Bahl et al., 2012).

2.5. Gas chromatography–mass spectrometry

A Waters GCT Gas Chromatography Mass Spectrometer, located at the UCR Analytical Chemistry Instrumentation Facility, was used to identify individual chemicals found in Freedom Smoke USA Cinnamon Ceylon FlavourArt EC refill fluid. The analysis was performed using a 30 m, 0.25 μm DB-5 column, and the sample was diluted in methanol at the ratio of 1:80. For analysis, 1 μl of the diluted Cinnamon Ceylon EC refill fluid was injected into the instrument at an initial temperature of 50 °C. The temperature was then increased to 100 °C over a period of 4 min, 300 °C over 8 min, and finally 350 °C over 15 min. The time required to complete analysis for one sample was approximately 30 min. Masslynx software was used to process GC–MS data, and comparison to a spectral library enabled identification of three peaks.

2.6. HPLC analysis

HPLC Grade methanol and water were purchased from Fischer Scientific (Fair Lawn, NJ). Samples were analyzed using a Hewlett Packard Series 1100 HPLC, consisting of a quaternary pump, degasser, column thermostat and manual injector. A 200 mm × 4.6 mm

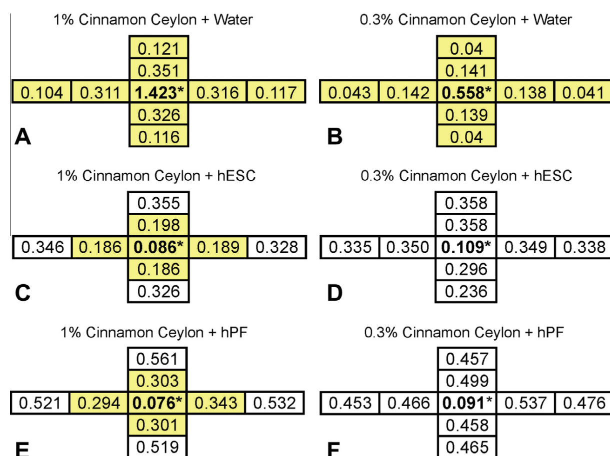


Fig. 1. The detection and elimination of Cinnamon Ceylon vapor effect. Transfer of Cinnamon Ceylon vapor from a central well containing (A) 1% or (B) 0.3% Cinnamon Ceylon in a 96-well plate to adjacent wells in a cross pattern containing only water was demonstrated by reading the absorbance of Cinnamon Ceylon after 48 h. In a 96-well plate, hESC (C–D) and hPF (E–F) were plated in a cross pattern, and a single dose of Cinnamon Ceylon was added to the center well of the cross, and cell viability was accessed with the MTT assay after 48 h. Absorbances are given for each well. 1%, but not 0.3%, solutions of Cinnamon Ceylon refill fluid produced a vapor effect that killed cells in adjacent wells. The central well of the cross pattern that contains either a 1% dose or a 0.3% dose of Cinnamon Ceylon is indicated by the bold text with an *. Yellow wells indicate a vapor effect occurred. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Thermo Scientific Hypersil ODS C18 column with a particle size of 5 μm was used at 35 °C with a flow rate of 0.8 ml/min. The diode array detector sample signals were set to 232 nm with a bandwidth of 10 nm for vanillin, 290 nm with a bandwidth of 4 nm for CAD, and 288 nm with a bandwidth of 4 nm for 2MOCA. The reference signal for all three compounds was set to 380 nm with a bandwidth of 100 nm. The injection volume was 5 μl. An isocratic method was used with a mobile phase consisting of 70% methanol and 30% water. A 5% stock solution of refill fluid in 100% methanol was produced for each sample. The injection concentration of refill fluids was 0.5%. Vanillin, CAD, and 2MOCA were identified in refill samples and quantified from standard curves using their elution time and relative peak height. Three-dimensional chromatograms were also analyzed for each sample.

2.7. Data analysis

MTT absorbance data were normalized by setting the negative control group (C1), furthest from the highest dose, in each row to 100%. All other wells in each row were expressed as a percentage of the negative control. The control closest to the highest dose was defined as the vapor effect control (C2). If the mean of the vapor effect control was less than 85% of the negative control (C1), a vapor effect was interpreted to have occurred and the test sample was rescreened at a lower high dose. For the Cinnamon Ceylon and authentic standard dose response experiments, IC_{50} values were computed with Prism software (GraphPad, San Diego, CA) using the log inhibitor vs normalized response-variable slope with the top and bottom constraints set to 100% and 0%, respectively. For the iterative screen data, IC_{50} values were determined by eye. The no observed adverse effect levels (NOAEL) were determined by reading directly off of the graphs. Statistical analysis on three replicate experiments of the dose response curves for Cinnamon Ceylon and for the chemicals identified in cinnamon-flavored refill fluids were done using Graph Pad Prism. Statistical significance was determined using an analysis of variance (ANOVA). When significance was found, treated groups were compared to C1 controls using Dunnett's post hoc test, and means were considered significantly different for $p < 0.05$.

3. Results

3.1. Cinnamon Ceylon produced a vapor effect

EC refill fluids contain volatile organic chemicals that can transfer to adjacent wells and effect cell viability (Behar et al., 2012a), thus causing an erroneous leftward shift in dose response curves. In the initial MTT screen with Cinnamon Ceylon, a vapor effect was observed (not shown). To quantify how much Cinnamon Ceylon was transferred to adjacent wells, wells containing 1% or 0.3% Cinnamon Ceylon solution and wells containing only water in a cross pattern in a 96-well plate were read at 295 nm in a spectrophotometer at the start of an experiment and again after 48 h of incubation at 37 °C (Fig. 1A and B). At time 0, the absorbance of 1% and 0.3% Cinnamon Ceylon was 3.65 and 2.57, respectively. After 48 h of incubation, the absorbance of 1% Cinnamon Ceylon was 1.42, and the adjacent wells containing water ranged from 0.35 to 0.10. A similar vapor effect was shown for the 0.3% dose of Cinnamon Ceylon, where after 48 h of incubation the absorbance of the well containing 0.3% Cinnamon Ceylon was 0.56, and the adjacent wells containing only water ranged from 0.14 to 0.04. These data demonstrate that significant amounts of Cinnamon Ceylon transferred to adjacent wells during the incubation period.

To determine the highest dose of Cinnamon Ceylon that could be used and not cause a vapor effect in the MTT assay using hESC and hPF, cross patterns of cells were plated, and Cinnamon Ceylon was added to the central well only (Fig. 1C–F). When 1% Cinnamon Ceylon was present in the central well, a vapor effect was observed in adjacent wells with both hESC and hPF (Fig. 1C and E). When the dose was reduced to 0.3% Cinnamon Ceylon, the vapor effect was eliminated (Fig. 1D and F). Therefore 0.3% defined the high end of the dose range that was subsequently tested.

3.2. Cytotoxicity of Cinnamon Ceylon when tested with hESC and hPF

Dose response curves for Cinnamon Ceylon are shown respectively for hESC and hPF in Fig. 2A and B. IC_{50}s were similar for the two cell types (0.044% for hESC and 0.039% for hPF) and estimated NOAELs were found to be 0.03% for hESC and 0.01% for hPF. The inserts in Fig. 2A and B show the negative control (C1 set at 100%) and the vapor effect control (C2) which is the well adjacent to highest dose. Inspection of these figures demonstrates that a vapor effect did not occur in these experiments since both cell types have C2 means greater than 85% (C2 for hESC = 88.3% ± 0.0207 and for hPF = 92.22% ± 16.33).

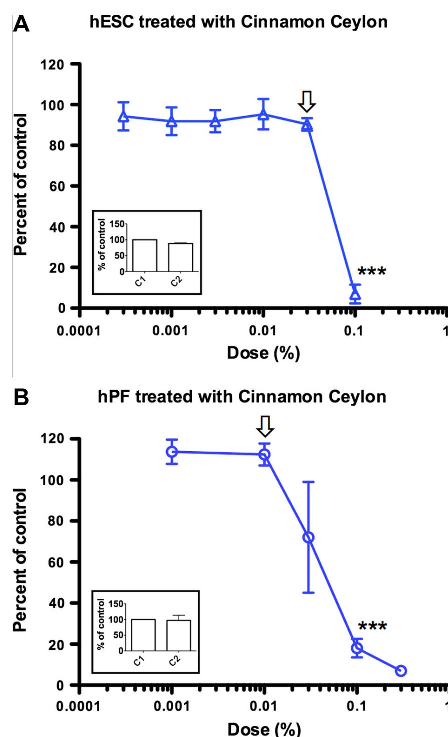


Fig. 2. Dose response curves for hESC and hPF exposed to Cinnamon Ceylon then evaluated using the MTT assay. Data are plotted as means and standard deviations for three experiments for both hESC (A) and hPF (B). NOAEL values are indicated by the open arrows. The insert graph for both (A) and (B) displays values for C1 (negative control) and C2, which is the vapor effect control, located next to the high dose of Cinnamon Ceylon. Asterisks indicate the lowest doses that are significantly different from the C1 control. *** = $p < 0.001$.

3.3. Dose response screen of eight cinnamon-flavored EC refill fluids

To determine if other cinnamon-flavored refill products were also cytotoxic, eight additional brands were purchased and subjected to an iterative screen. Each product was tested at successively decreasing high doses until no vapor effect was observed. The data in Fig. 3 are the dose response curves that were obtained for each product at a high dose that did not produce a vapor effect. In the initial screen, 1% was used as the highest dose, and all eight brands were tested with both cell types (Fig. 3A and B). Of these eight refill fluids, only #61 did not cause a vapor effect when tested with hESC at a high dose of 1%, and a useful dose response curve was obtained (Fig. 3A). For hPF, refill fluids #54, #61 and #65 did not cause a vapor effect at the 1% high dose (Fig. 3B). Sample #61 gave a partial dose response curve indicating low cytotoxicity, while #54 and #65 gave complete curves.

The remaining refill fluids were then rescreened at high dose of 0.1%. The products shown in Fig. 3C and D did not cause a vapor effect and varied in their cytotoxicity. Product #42 was not cytotoxic to either hESC or hPF at a high dose of 0.1%. Product #62 was the most cytotoxic in this group with the hESC being more sensitive than the hPF.

In the third screen, which was done at a high dose of 0.01%, products #53 and #58 did not cause a vapor effect for either hESC or hPF (Fig. 3E and F). Both products produced dose response curves with the hESC being more sensitive than the hPF. These data show that cinnamon-flavored EC refill fluids vary significantly in their cytotoxicity and that, in general, hESC were more sensitive to treatment than hPF.

3.4. Identification of cytotoxic chemicals in cinnamon-flavored refill fluids

Propylene glycol and vegetable glycerin, which are ingredients of EC fluids, were evaluated in our prior screen and were not cytotoxic (Bahl et al., 2012). GC–MS and HPLC analysis identified four additives in the sample of Cinnamon Ceylon and other cinnamon refill fluids. These were CAD, 2MOCA, dipropylene gly-

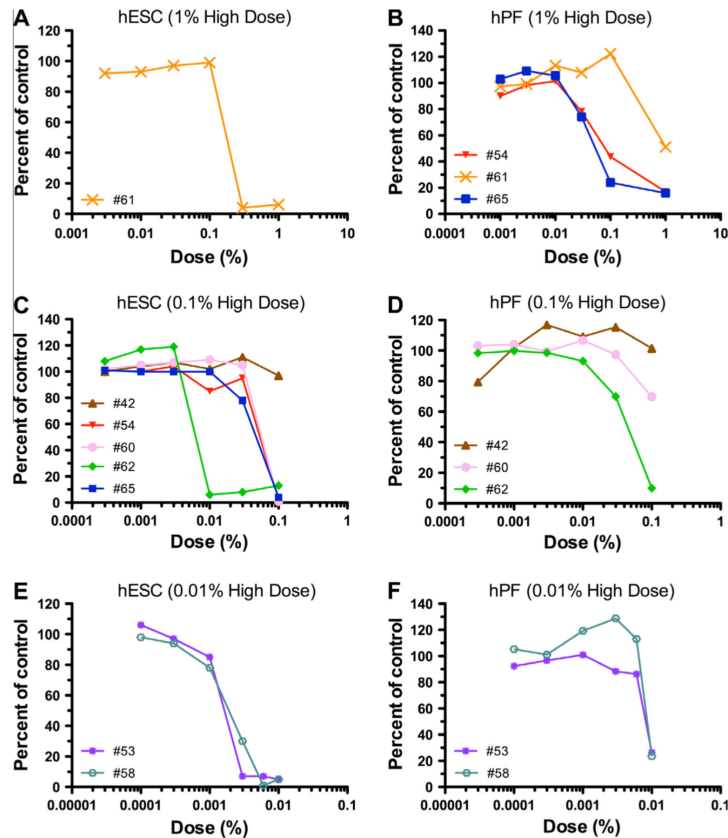


Fig. 3. Cytotoxicity screen of eight cinnamon-flavored refill fluids. Each product was screened iteratively until no vapor effect was observed. (A) hESC and (B) hPF treated with 1% as the highest dose. (C) hESC and (D) hPF treated with 0.1% as the highest dose. (E) hESC and (F) hPF treated with 0.01% as the highest dose. Each product was screened one time at successively decreasing high doses until a vapor effect was not observed, and the resulting curve was used to estimate relative toxicity of the different products.

col, and vanillin. Authentic standards of each chemical were purchased and tested for cytotoxicity with hESC and hPF using the MTT assay (Fig. 4). CAD and 2MOCA were the most cytotoxic of the four chemicals tested (Fig. 4A and B). Both chemicals produced similar IC_{50}s for both hESC and hPF. Dipropylene glycol and vanillin were the least cytotoxic of the four chemicals tested, and their IC_{50}s were higher than a user would likely experience (Fig. 4C and D). To confirm that cells were not surviving CAD treatment, micrographs of hESC (Fig. 4E–G) and hPF (Fig. 4H–J) are shown for the control, $IC_{50,}$ and the high dose of CAD. At the IC_{50} dose, most fields had fewer live cells and more dead cells than the controls (Fig. 4F and I). At the highest dose, most cells were dead in agreement with the MTT assay (Fig. 4G and J). The hierarchy of potency based on IC_{50}s for the hESC was CAD > 2MOCA >>> vanillin > dipropylene glycol and the hierarchy for the hPF was 2MOCA $\geqslant$ CAD >>> vanillin > dipropylene glycol. For CAD, 2MOCA and vanillin NOAEL values varied between the two cell types. For dipropylene glycol the estimated NOAEL was 7.45×10^{-3} M for hESC (for hPF, a reliable NOAEL could not be determined for dipropylene glycol).

3.5. HPLC analysis of cinnamon-flavored products

HPLC analysis was performed on 10 cinnamon-flavored refill fluids from various manufacturers (8 refill fluids from the iterative screen, Cinnamon Ceylon and a duplicate Tasty Puff Sinful Cinnamon) (Fig. 5). The duplicate bottles of Tasty Puff Sinful Cinnamon (#53 and 69) were identically labeled. 3D chromatograms were visually analyzed and arranged in order of increasing potency (Fig. 5). Each chemical was identified based on its elution time and peak shape. Average elution times for identified compounds are as follows: 2.7 min for methanol (this peak is present due to the pure methanol that was used to make solutions), 3.1 min for vanillin and/or an unidentified vanillin derivative, 4.4 min for CAD, 4.9 min for 2MOCA, and 7.0 min for nicotine (nicotine concentration was not quantified using this method).

The 3D chromatograms for all products were relatively simple and contained very few chemicals in comparison to combustible tobacco, which contains thousands of chemicals (EPA, 1992). The main chemicals in the refill fluids were nicotine plus the flavorings CAD, 2MOCA, and vanillin. Sample #61, which was the least cyto-

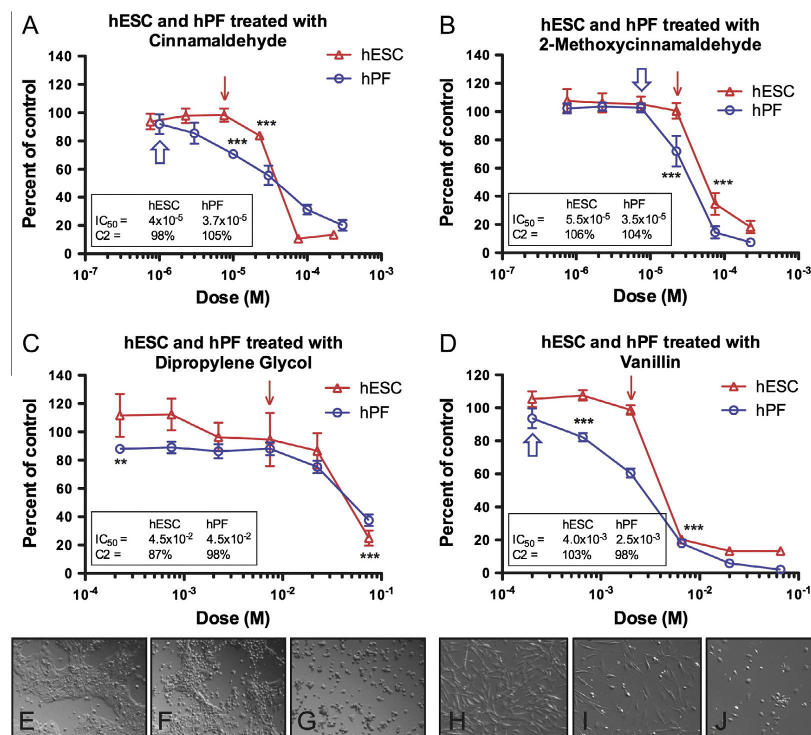


Fig. 4. Cytotoxic evaluation of four chemicals identified in cinnamon-flavored refill fluids. Dose response curves of hESC and hPF treated with (A) CAD, (B) 2MOCA, (C) dipropylene glycol, and (D) vanillin. Data are plotted as means and standard deviations of three experiments. IC_{50} = inhibitory concentration at 50%. NOAEL levels are indicated by solid (hESC) or open arrows (hPF). C2 = vapor effect control located next to the highest dose of chemical. Asterisks indicate the lowest doses that are significantly different from the C1 control. $^{**}p < 0.01$, $^{***}p < 0.001$. (E–G) Micrographs showing hESC from the control, IC_{50} and high dose of CAD. Colonies are well formed in the control, reduced in size in the IC_{50} dose, and absent from the high dose which has mainly dead cells. (H–J) Micrographs of hPF from the control, IC_{50}, and high dose of CAD. Many cells treated in the IC_{50} dose are dead and of the cells that have survived, they appear to be stressed. In the high dose, the hPF are mainly dead.

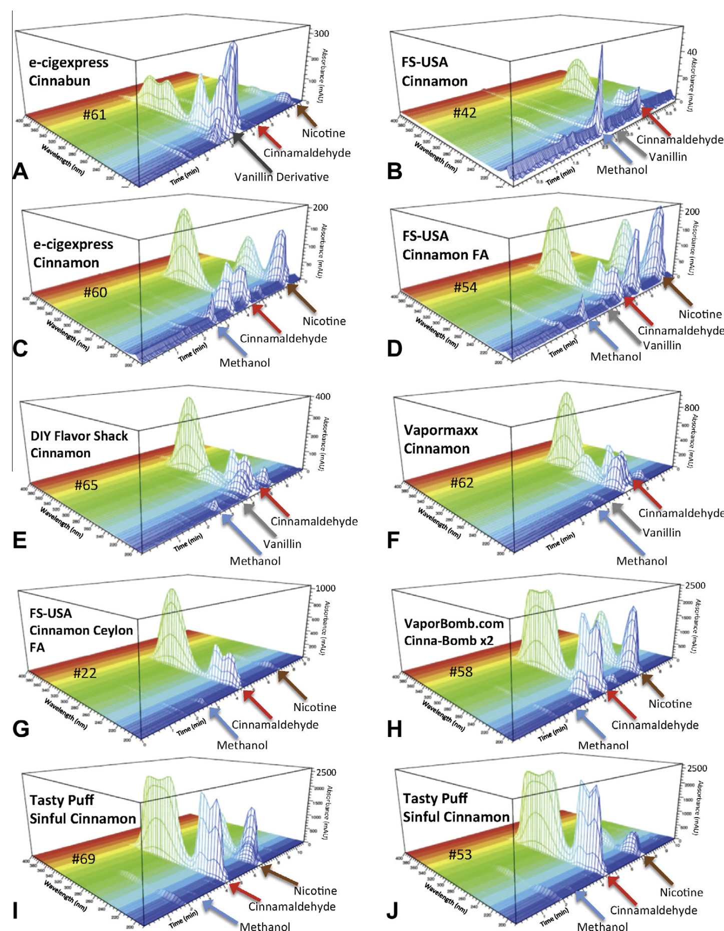


Fig. 5. 3D HPLC chromatograms for ten brands of cinnamon-flavored refill fluids. All products were flavored with CAD (A–J). Some products also contained vanillin (#42, 54, 65, 62), or a vanillin derivative (#61). All products except #42 (B), #65 (E) and #62 (F) contained nicotine. Some products also contained unidentified flavoring peaks (e.g. #60 and #54). The methanol peaks are due to solution preparation methods. For all chromatograms, the CAD peak obscures the 2MOCA peak. Peak height for CAD was highly variable ranging from about 30–2500 absorbance units. For #69 (I) and 53 (J), which are duplicate bottles of the same product, CAD concentration in both bottles was the same, while peak heights for nicotine are different between bottles.

toxic of the products tested (Fig. 3A and B), contained a CAD derivative (Fig. 5A), which had lower cytotoxicity than CAD or 2MOCA. None of the 3D chromatograms for cinnamon-flavored refill fluids had the same signatures, including the two duplicate bottles (#69 and 53) (Fig. 5I and J), which had identical amounts of CAD, but different amounts of 2MOCA and nicotine. For most products, CAD was the dominant peak, completely obscuring the 2MOCA peak and minimizing other flavoring peaks such as vanillin. To better visualize the smaller peaks, the 3D chromatograms of two samples were edited by rotating the image and truncating the *Y*-axis (absorbance), thereby revealing 2MOCA (Fig. 6A vs B) and vanillin (Fig. 6C vs D).

3.6. Concentrations of chemicals in cinnamon-flavored refill products

The concentrations of CAD, 2MOCA, and vanillin were quantified in each refill fluid product, and data were organized by increasing CAD concentration (Fig. 7A). The two bottles of Tasty Puff Sinful Cinnamon-flavored refill fluids (#69 and #53) contained identical amounts of CAD and were therefore arranged by 2MOCA concentration. One product (#65) contained all three chemicals, one (#58) contained only CAD, and the rest contained only two of the three compounds of interest at levels that could be quantified. The concentrations of each chemical varied by approximately

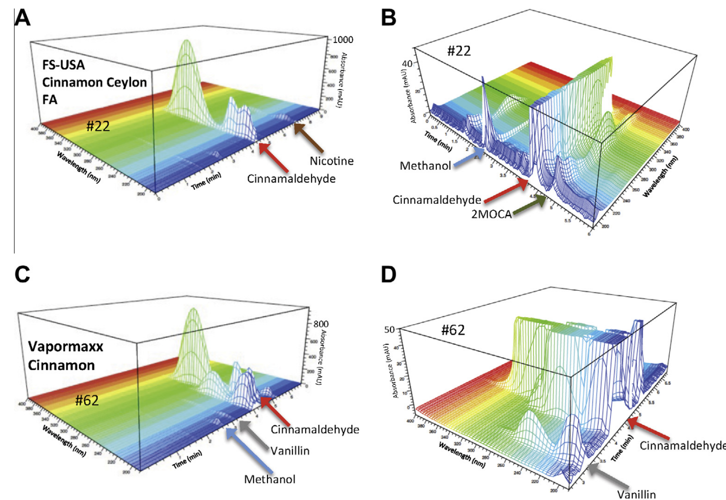


Fig. 6. 3D HPLC data with perspectives and the *Y*-axis (absorbance) adjusted to visualize vanillin and 2MOCA. (A): The original chromatogram of Freedom Smoke USA, Cinnamon Ceylon FlavourArt (#22). (B): Same 3D chromatogram of Freedom Smoke USA, Cinnamon Ceylon FlavourArt (#22) with the *Y*-axis shortened and the image rotated to visualize the 2MOCA peak. (C): The original chromatogram of Vapormaxx, Cinnamon (#62). (D): The same 3D chromatogram for Vapormaxx, Cinnamon (#62) with the *Y*-axis shortened and the image rotated to visualize the vanillin peak.

10–100 fold among products, e.g. CAD was about 100 times higher in #58, 69, and 53 than in #42.

For most samples and for each cell type, IC_{50} values were estimated using data from Fig. 3. IC_{50} values for both cells types were then compared to CAD concentrations in each product. As CAD concentration in the refill fluids increased, IC_{50} values decreased for both hESC and hPF (Fig. 7B and C).

Nicotine concentrations for 6 of the 8 refill fluids were reported on bottles by the manufacturer as: #61 (12 mg/ml), #60 (11 mg/ml), #65 (0 mg/ml), #22 (0 mg/ml), and #58 (0 mg/ml). There was no correlation between nicotine concentration and cytotoxicity.

4. Discussion

The rapid growth in worldwide sales of EC and their associated products make it important to understand their effects on human health (Etter et al., 2011; Hua et al., 2013; Williams et al., 2013). This study evaluated the volatility and cytotoxicity of 10 cinnamon-flavored EC refill fluids, compared their cytotoxicity using prenatal (hESC) and adult (hPF) models, and identified chemicals in these fluids that are causing cytotoxicity. Nicotine concentration did not correlate with cytotoxicity, in agreement with our prior study (Bahl et al., 2012). In general, the cinnamon-flavored refill fluids were cytotoxic with IC_{50} concentrations below 1% for hESC and hPF. It is possible that there were other cytotoxic chemicals in these fluids that our study did not identify.

Cinnamon-flavored refill fluids are highly volatile, and most produced vapor effects when tested in the MTT assay. Similar effects have been reported with other highly volatile chemicals in 96-well plate assays (Behar et al., 2012b; Blein et al., 1991). The highly volatile nature of the cinnamon-flavored refill fluids could result in inhalation exposure of users and bystanders during refilling or from fluid that has leaked onto the surface of the refill bottle.

The vapor effect caused by cinnamon-flavored refill fluids shifts the dose response curve to the left, thereby increasing the apparent cytotoxicity of the refill fluid. Iterative screening using decreasing high doses eliminated the vapor effect and allowed the relative potency of products to be compared. However, the IC_{50}s established in this study may underestimate toxicity due to the continual loss of volatile test chemical from the culture medium during exposure of cells.

In our original screen of EC refill fluids (Bahl et al., 2012), Cinnamon Ceylon was the most cytotoxic of the 36 products that were tested, and it was the only product that was cinnamon-flavored. In the current study, which focused on only cinnamon-flavored products, refill fluids varied significantly in their cytotoxicity, with Cinnamon Ceylon having an IC_{50} that was approximately midway in the overall range. In the prior study (Bahl et al., 2012) and current screen, a total of 45 EC refill fluids were tested. A comparison of IC_{50}s from both studies showed that for hPF, 5 of the 45 refill fluids fell into the highly cytotoxic category ($IC_{50} < 0.1\%$), and all 5 of these were cinnamon-flavored. For hESC, 18 of the 45 refill fluids were highly cytotoxic. Of these, 8 were cinnamon-flavored, and 4 of the 8 were the most cytotoxic of all products tested.

Our findings of cytotoxicity in cinnamon-flavored refill fluids are consistent with reports made by EC users in online forums. Cinnamon-flavored products have caused throat, mouth, and lung irritations and for some, the irritation ceased after switching to a different flavor (http://www.e-cigarette-forum.com/forum/health-safety-e-smoking/212870-do-you-vape-cinnamon-flavors-read.html, 2/19/13). Reports in online forums have advised against using cinnamon-flavored products (http://www.e-cigarette-forum.com/forum/new-members-forum/324775-greetings-toxicology.html, 2/19/13, http://www.e-cigarette-forum.com/forum/health-safety-e-smoking/212870-do-you-vape-cinnamon-flavors-read.html, 3/25/13), and one manufacturer, vaporbomb.com, reduced the amount of cinnamon flavoring in its product due to requests from users (http://www.vaporbomb.com/Cinna-Bomb-e-liquid-VBcinnabomb.htm?categoryId=-1, 3/25/13). Names of the

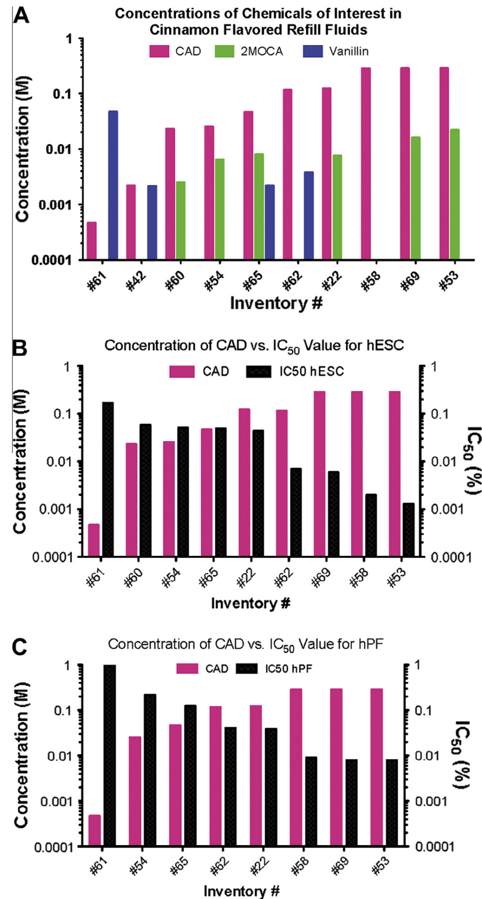


Fig. 7. Concentrations of chemicals that were identified in refill fluids. (A) Concentrations of CAD, 2MOCA, and vanillin in refill fluids as determined by HPLC. (B and C) Comparison of CAD concentration to IC_{50} values for hESC (B) and hPF (C). As CAD and 2MOCA concentrations increase, the IC_{50} values decrease.

products that were tested in this study are provided so that EC users are aware of those that produced the highest levels of cytotoxicity.

The sensitivity of hESC and hPF to cinnamon-flavored EC refill fluids was compared. hPF were chosen to model a differentiated adult cell from the lung, one of the first organs contacted by inhaled refill fluid vapor or EC aerosol. hESC model an early stage of post-implantation development (Nichols and Smith, 2009; Talbot and Lin, 2011) and can be used to gauge the effects that EC use by pregnant women could have on developing embryos. Generally, hESC were more sensitive to the cinnamon-flavored refill fluids than the hPF. These data correlate well with our previous study on EC refill fluids in which embryonic (hESC) and early postnatal (mNSC) cells were generally more sensitive to EC products than adult cells (hPF) (Bahl et al., 2012) and demonstrate the importance of testing more than one cell type when evaluating EC cytotoxicity. These observations are also consistent with the general finding that embryonic cells are more sensitive to environmental chemicals than adult cells (Grandjean et al., 2007). While further animal and clinical work is needed to determine what effects EC products have on developing embryos/fetuses, these data suggest that women should exercise caution when deciding whether to use EC products during pregnancy.

The chemicals in Cinnamon Ceylon were identified by GC–MS and HPLC, and authentic standards were tested for cytotoxicity. While dipropylene glycol and vanillin were cytotoxic only at high doses, CAD and 2MOCA were cytotoxic at doses found in the refill fluids. In other studies, dipropylene glycol and vanillin have been reported to have relatively low toxicity (Cosmetic Ingredient Review, 1985; Ho et al., 2011), as was observed in our study. It is possible that other potentially cytotoxic chemicals, such as metals, were present but not identified in this study. CAD is derived from the essential oil of cinnamon bark and is a highly bioactive compound serving many purposes (Jayaprakasha and Rao, 2011). It has been used as an anticancer agent (Nagle et al., 2012), an insecticide (Cheng et al., 2009), a fungicide (Bang et al., 2000; Shreaz et al., 2011), and a bactericide (Nostro et al., 2012). It is also used commercially as an additive in many foods and in fragrances (Cocchiara et al., 2005). The dental literature has reports of adverse reactions to CAD, and one case report links heavy use of cinnamon-flavored gum to the development of squamous cell carcinoma on the tongue (Westra et al., 1998). At IC_{50} values similar to those found in our study, CAD and 2MOCA are inhibitors of NF-κB, a transcription factor involved in immunity as well as inflammatory responses and developmental processes (Reddy et al., 2004). In addition, 2MOCA and CAD up-regulate apoptosis in cancerous cell lines, and CAD has strong toxic effects in other mammalian cell types (Mereto et al., 1994; Stammati et al., 1999; Unlu et al., 2010; Zhang et al., 2010). In the current study, hESC were sensitive to low concentrations of CAD and 2MOCA, suggesting that pregnant women should be cautious using these products.

Evaluation of HPLC 3D chromatograms showed that each refill fluid possesses a unique chromatographic signature, including the duplicate bottles of Tasty Puff Sinful Cinnamon (#69 and #53). Although the duplicates were labeled identically and contained the same amount of CAD, they varied in their 2MOCA and nicotine content. Qualitative evaluation of the chromatogram (Fig. 5I and J) shows that #69 has higher nicotine content than #53. Also noteworthy, refill fluids #22 (Cinnamon Ceylon) and #58 Vaporbomb.com Cinna-Bomb 2× were labeled zero nicotine, but nicotine was indeed identified by HPLC as a component in these products. These data demonstrate inaccuracies in labeling with respect to nicotine concentrations, as has been previously reported (Trehy et al., 2011; Goniewicz et al., 2012) and observed in our unpublished data. These labeling inaccuracies also extend to EC components other than nicotine. For example, cartridge fluid from one EC product that was advertised to contain tadalafil, the active ingredient in Cialis, instead contained an inactive isoform of the drug (Hadwiger et al., 2010).

The HPLC analysis further shows that cinnamon-flavored refill fluids vary significantly with respect to the chemicals that are used to create cinnamon flavor. In most products, CAD was the dominant flavorant with 2MOCA and vanillin sometimes being present in lesser amounts. All of the products had different amounts of these chemicals demonstrating that users cannot assume to know their content even when purchasing duplicate bottles. Products having the highest concentrations of CAD were identified as the most cytotoxic. 2MOCA would not be a useful substitute for creating cinnamon flavor as it was also highly cytotoxic when tested individually as an authentic standard. If manufacturers wish to

add CAD as the primary flavorant in cinnamon-flavored refill fluids, it would be advisable to use a non-cytotoxic dose. However, the NOAEL dose for CAD may vary among different cell types. Moreover, the MTT assay is based on mitochondrial activity and further evaluations will be required to fully understand other cell processes/components that could be adversely affected by CAD.

The results of this study could lead to improvements in EC manufacturing and flavor choice for users. CAD was highly cytotoxic in EC products that are currently marketed on the Internet. This flavor may need additional regulation to ensure cytotoxic chemicals such as CAD are either not used in EC products or are maintained at doses that are non-cytotoxic or otherwise damaging to cells. An alternative would be to substitute other flavorants that produce a cinnamon-like flavor, but have low cytotoxicity. Correlating a particular flavoring with high cytotoxicity will be an integral part of improving EC safety for users and will help inform companies and regulatory agencies about chemicals and flavors that are hazardous.

Conflict of Interest

The authors have no conflict of interest to declare.

Acknowledgments

This work was supported by the Tobacco-Related Disease Research Program of California (TRDRP XT-0118), and the California Institute of Regenerative Medicine (NE-A0005A-1E). Rachel Behar and Barbara Davis were supported by Cornelius Hopper Fellowships from the Tobacco-Related Disease Research Program of California and Dean's Fellowships. Dr. Sabrina Lin was supported by a Tobacco-Related Disease Research Program of California postdoctoral Fellowship. We thank Michael Dang and Jisoo Kim for assisting with the HPLC analysis, and Monique Williams for her comments on the manuscript. We would also like to thank the University of California, Riverside Analytical Chemical Instrumentation Facility and the Stem Cell Core for their assistance.

References

Ayers, J.W., Ribisl, K.M., Brownstein, J.S., 2011. Tracking the rise in popularity of electronic nicotine delivery systems (electronic cigarettes) using search query surveillance. Am. J. Prev. Med. 40, 448–453.
Bahl, V., Lin, S., Xu, N., Davis, B., Wang, Y.H., Talbot, P., 2012. Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models. Reprod. Toxicol. 34, 529–537.
Bang, K.H., Lee, D.W., Park, H.M., Rhee, Y.H., 2000. Inhibition of fungal cell wall synthesizing enzymes by trans-cinnamaldehyde. Biosci. Biotech. Bioch. 64, 1061–1063.
Behar, R.Z., Bahl, V., Wang, Y., Lin, S., Xu, N., Davis, B., Talbot, P., 2012a. A method for rapid dose-response screening of environmental chemicals using human embryonic stem cells. J. Pharmacol. Toxicol. Methods 66, 238–245.
Behar, R.Z., Bahl, V., Wang, Y., Weng, J.-H., Lin, S., Talbot, P., 2012b. Adapting the MTT cytotoxicity assay for use with mouse neuronal stem cells and human Embryonic stem cells. In: Current Protocols in Stem Cell Biology, Unit 1C 13. John Wiley & Sons, Inc., Hoboken, NJ, 23:1C.13.1–1C.13.21.
Blein, O., Ronot, X., Adolphe, M., 1991. Cross contamination associated with the use of multiwell culture plates for cytotoxicity assessment of volatile chemicals. Cytotechnology 6, 79–82.
Cheng, S.S., Liu, J.Y., Huang, C.G., Hsui, Y.R., Chen, W.J., Chang, S.T., 2009. Insecticidal activities of leaf essential oils from Cinnamomum osmophloeum against three mosquito species. Bioresour. Technol. 100, 457–464.
Cocchiara, J., Letizia, C.S., Lalko, J., Lapczynski, A., Api, A.M., 2005. Fragrance material review on cinnamaldehyde. Food. Chem. Toxicol. 43, 867–923.
Cosmetic Ingredient Review, 1985. Final report on the safety assessment of butylene glycol, hexylene glycol, ethoxydiglycol, and dipropylene glycol. Int. J. Toxicol. 4, 223–248.
EPA,1992. EPA Report/600/6-90/006F: Respiratory health effects of passive smoking: lung cancer and other disorders, Washington, DC.
Etter, J.F., 2010. Electronic cigarettes: a survey of users. BMC. Public. Health 10, 231.
Etter, J.F., Bullen, C., 2011. Electronic cigarette: users profile, utilization, satisfaction and perceived efficacy. Addiction 106, 2017–2028.
Etter, J.-F., Bullen, C., Flouris, A.D., Laugesen, M., Eissenberg, T., 2011. Electronic nicotine delivery systems: a research agenda. Tob. Control 20, 243–248.
Goniewicz, M.L., Kuma, T., Gawron, M., Knysak, J., Kosmider, L., 2012. Nicotine levels in electronic cigarettes. Nicotine. Tob. Res. 15, 158–166.
Goniewicz, M.L., Lingas, E.O., Hajek, P., 2013. Patterns of electronic cigarette use and user beliefs about their safety and benefits: An Internet survey. Drug. Alcohol. Rev. 32, 133–140.
Grandjean, P., Bellinger, D., Bergman, A., Cordier, S., et al., 2007. The faroes statement: human health effects of developmental exposure to chemicals in our environment. Basic Clin. Pharmacol. 102, 73–75.
Hadwiger, M.E., Trehy, M.L., Ye, W., Moore, T., Allgire, J., Westenberger, B., 2010. Identification of amino-tadalafil and rimonabant in electronic cigarette products using high pressure liquid chromatography with diode array and tandem mass spectrometric detection. J. Chromatogr. A 1217, 7547–7555.
Hallgren, O., Nihlberg, K., Dahlback, M., Bjermer, L., Eriksson, L.T., Erjefalt, J.S., Lofdahl, C.G., Westergren-Thorsson, G., 2010. Altered fibroblast proteoglycan production in COPD. Respir. Res. 11, 55.
Ho, K., Yazan, L.S., Ismail, N., Ismail, M., 2011. Toxicology study of vanillin on rats via oral and intra-peritoneal administration. Food. Chem. Toxicol. 49, 25–30.
Hua, M., Alfi, M., Talbot, P., 2013. Health-related effects reported by electronic cigarette users in online forums. J. Med. Internet. Res. 15, e59.
Jayaprakasha, G.K., Rao, L.J., 2011. Chemistry, biogenesis, and biological activities of Cinnamomum zeylanicum. Crc. Cr. Rev. Food Sci. 51, 547–562.
Kitamura, H., Cambier, S., Somanath, S., Barker, T., Minagawa, S., Markovics, J., Goodsell, A., Publicover, J., Reichardt, L., Jablons, D., et al., 2011. Mouse and human lung fibroblasts regulate dendritic cell trafficking, airway inflammation, and fibrosis through integrin v8-mediated activation of TGF-. J. Clin. Invest. 121, 2863–2875.
Laugesen, M., 2008. Safety report on the Ruyan e-cigarette cartridge and inhaled aerosol. Christchurch, New Zealand: Health New Zealand. <http://www.healthnz.co.nz/RuyanCartridgeReport30-Oct-08.pdf>.
Lin, S., Talbot, P., 2011. Methods for culturing mouse and human embryonic stem cells. Methods. Mol. Biol. 690, 31–56.
McCauley, L., Markin, C., Hosmer, D., 2012. An unexpected consequence of electronic cigarette use. Chest 141, 1110–1113.
McQueen, A., Tower, S., Sumner, W., 2011. interviews with "vapers": implications for future research with electronic cigarettes. Nicotine. Tob. Res. 13, 860–867.
Mereto, E., Brambilla-Campart, G., Ghia, M., Martelli, A., Brambilla, G., 1994. Cinnamaldehyde-induced micronuclei in rodent liver. Mutat. Res. 322, 1–8.
Nagle, A.A., Gan, F.F., Jones, G., So, C.L., Wells, G., Chew, E.H., 2012. Induction of tumor cell death through targeting tubulin and evoking dysregulation of cell cycle regulatory proteins by multifunctional cinnamaldehydes. PLoS ONE 7, e50125.
Nichols, J., Smith, A., 2009. Naive and primed pluripotent states. Cell Stem Cell 4, 487–492.
Nostro, A., Scaffaro, R., D'Arrigo, M., Botta, L., Filocamo, A., Marino, A., Bisignano, G., 2012. Study on carvacrol and cinnamaldehyde polymeric films: mechanical properties, release kinetics and antibacterial and antibiofilm activities. Appl. Microbiol. Biot. 96, 1029–1038.
Reddy, A.M., Seo, J.H., Ryu, S.Y., Kim, Y.S., Kim, Y.S., Min, K.R., Kim, Y., 2004. Cinnamaldehyde and 2-methoxycinnamaldehyde as NF-kappaB inhibitors from Cinnamomum cassia. Planta. Med. 70, 823–827.
Selman, M., Pardo, A., 2002. Idiopathic pulmonary fibrosis: an epithelial/fibroblastic cross-talk disorder. Respir. Res. 3, 3.
Shreaz, S., Sheikh, R.A., Bhatia, R., Neelofar, K., Imran, S., Hashmi, A.A., Manzoor, N., Basir, S.F., Khan, L.A., 2011. Antifungal activity of alpha-methyl trans cinnamaldehyde, its ligand and metal complexes: promising growth and ergosterol inhibitors. Biometals 24, 923–933.
Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.L., von Wright, A., 1999. Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food. Chem. Toxicol. 37, 813–823.
Talbot, P., Lin, S., 2011. Mouse and human embryonic stem cells: can they improve human health by preventing disease? Curr. Top. Med. Chem. 11, 1638–1652.
Togo, S., Holz, O., Liu, X., Sugiura, H., Kamio, K., Wang, X., Kawasaki, S., Ahn, Y., Fredriksson, K., Skold, C.M., et al., 2008. Lung fibroblast repair functions in patients with chronic obstructive pulmonary disease are altered by multiple mechanisms. Am. J. Respir. Crit. Care. Med. 178, 248–260.
Trehy, M.L., Ye, W., Hadwiger, M.E., Moore, T.W., Allgire, J.F., Woodruff, J.T., Ahadi, S.S., Black, J.C., Westenberger, B.J., 2011. Analysis of electronic cigarette cartridges, refill solutions, and smoke for nicotine and nicotine related impurities. J. Liq. Chromatogr. R. T. 34, 1442–1458.
Trtchounian, A., Willams, M., Talbot, P., 2010. Conventional and electronic cigarettes (e-cigarettes) have different smoking properties. Nicotine. Tob. Res. 12, 905–912.
Unlu, M., Ergene, E., Unlu, G.V., Zeytinoglu, H.S., Vural, N., 2010. Composition, antimicrobial activity and *in vitro* cytotoxicity of essential oil from Cinnamomum zeylanicum blume (Lauraceae). Food. Chem. Toxicol. 48, 3274–3280.
Vardavas, C.I., Anagnostopoulos, N., Kougias, M., Evangelopoulou, V., Connolly, G.N., Behrakis, P.K., 2012. Short-term pulmonary effects of using an electronic cigarette: impact on respiratory flow resistance, impedance, and exhaled nitric oxide. Chest 141, 1400–1406.
Westenberger, B.J., 2009. Evaluation of e-cigarettes. St. Louis, MO: Food and Drug Administration. Center for Drug Evalation and Research. <http://www.fda.gov/downloads/Drugs/ScienceResearch/UCM173250.pdf>.

Westra, W.H., McMurray, J.S., Califano, J., Flint, P.W., Corio, R.L., 1998. Squamous cell carcinoma of the tongue associated with cinnamon gum use: a case report. Head. Neck. 20, 430–433.

Williams, M., Talbot, P., 2011. Variability among electronic cigarettes in the pressure drop, airflow rate, and aerosol production. Nicotine. Tob. Res. 13, 1276–1283.

Williams, M., Villarreal, A., Bozhilov, K., Lin, S., Talbot, P., 2013. Metal and silicate particles including nanoparticles are present in electronic cigarette cartomizer fluid and aerosol. PLoS ONE 8, e57987.

Zhang, J.H., Liu, L.Q., He, Y.L., Kong, W.J., Huang, S.A., 2010. Cytotoxic effect of trans-cinnamaldehyde on human leukemia K562 cells. Acta. Pharm. Sinic. 31, 861–866.

APPENDIX C

Comparison of the Performance of Cartomizer Style Electronic Cigarettes from Major Tobacco and Independent Manufacturers.

Monique Williams, Amanda Villarreal, Barbara Davis, Prue Talbot

PLoS ONE 2016;11:e0149251. doi:10.1371/journal.pone.0149251

PLOS | ONE

RESEARCH ARTICLE

Comparison of the Performance of Cartomizer Style Electronic Cigarettes from Major Tobacco and Independent Manufacturers

Monique Williams, Amanda Villarreal, Barbara Davis, Prue Talbot*

Department of Cell Biology and Neuroscience, University of California Riverside, Riverside, California, United States of America

* talbot@ucr.edu

OPEN ACCESS

Citation: Williams M, Villarreal A, Davis B, Talbot P (2016) Comparison of the Performance of Cartomizer Style Electronic Cigarettes from Major Tobacco and Independent Manufacturers. PLoS ONE 11(2): e0149251. doi:10.1371/journal.pone.0149251

Editor: Yunchao Su, Georgia Regents University, UNITED STATES

Received: November 17, 2015

Accepted: January 28, 2016

Published: February 18, 2016

Copyright: © 2016 Williams et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Support was provided by the National Institutes of Health Grant #R01DA036493. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Objective

This study compared the performance of 12 brands of cartomizer style electronic cigarettes (EC) using different puffing protocols and measured the concentrations of nicotine in each product.

Methods

Air flow rate, pressure drop, and aerosol absorbance were measured using two different protocols, first 10 puffs and a modified smoke-out protocol.

Results

First 10 puff protocol: The air flow rate required to produce aerosol ranged between brands from 4–21 mL/s. Pressure drop was relatively stable within a brand but ranged between brands from 14–71 mmH_2O and was much lower than the earlier classic 3-piece models. Absorbance, a measure of aerosol density, was relatively consistent between puffs, but varied between brands. With the modified smoke-out protocol, most brands were puffed until 300 puffs. The pressure drop was relatively stable for all brands except three. Absorbance of the aerosol decreased as the number of puffs increased. Although there was some uniformity in performance within some brands, there was large variation between brands. The labeled and measured nicotine concentrations were within 10% of each other in only 1 out of 10 brands.

Conclusions

Over 10 puffs, the cartomizers all perform similarly within a brand but varied between brands. In smoke-out trials, most brands lasted at least 300 puffs, and performed similarly within brands with respect to pressure drop and absorbance. For five brands, products purchased at different times performed differently. These data show some improvement in

performance during evolution of these products, but nevertheless indicate problems with quality control in manufacture.

Introduction

The original electronic cigarettes (EC) were three piece models, which had a separate battery, atomizing unit, and a cartridge for holding a fluid that usually contained propylene glycol and/or glycerol, flavoring chemicals, and nicotine [1–3]. In 2009, manufacturers combined the atomizer and cartridge into a single replaceable unit called a cartomizer. Cartomizer style ECs, which are currently the dominant marketed model in the USA, are readily available in supermarkets, drug stores, convenience stores, gas stations, and on the Internet. Cartomizers come in different flavors (e.g., tobacco, menthol, and coffee) and nicotine concentrations ranging from 0–36 mg/mL [4]. Major tobacco companies entered the EC market with cartomizers style EC in 2013. Many users refill them when the fluid runs low, and there are 1000s of refill fluids present on the market [4–7]. Since EC do not burn tobacco and contain fewer chemicals than conventional cigarettes, they are sometimes considered safer by advocates and consumers [8,9]. However, there are relatively few studies evaluating the health effects caused by EC use [10–12], and there is concern that some components in EC aerosol may be harmful [6,13–15].

In an earlier study, we compared the performance of the classic and cartomizer style EC [16]. The two cartomizer brands, Smoking Everywhere Platinum and Crown 7 Imperial, behaved similarly within brands, but varied between brands [16]. Crown 7 Imperial cartomizers were able to produce aerosol for 400 ± 10 puffs, in contrast to Smoking Everywhere Platinum which lasted 160 ± 66 puffs [16]. As was seen in the classic models, as the cartomizer EC were puffed, there was an increase in pressure drop and a decrease in absorbance [2,16]. This variability within and between non-disposable EC brands has been seen in the concentration of nicotine delivered to the consumer [17].

The purpose of the current study was to compare the performance of a broad range of cartomizer style EC from major tobacco and independent manufactures. Both short and long term puffing protocols were used to examine performance. The concentrations of nicotine in cartomizer style EC was also determined and compared to label values.

Materials and Methods

Electronic Cigarette Selection

All EC were second generation cartomizer style models that were selected based on consumer reviews (Table 1). Brands selected for this study were: Smoking Everywhere Platinum (Smoking Everywhere, Sunrise, FL), Crown 7 Imperial Hydro (Crown Seven Shop, Scottsdale, AZ), NJOY NPRO 2N1 (Sottera Inc., Scottsdale, AZ), Safe Cig (The Safe Cig LLC, Los Angeles, CA), Liberty Stix Eagle (Liberty Stix, LLC, Cleveland, OH), Smoke 51 (Vapor Corp, Miami, FL), South Beach Smoke (South Beach Java LP, Wood Dale, IL), V2 Cigs (VMR Products LLC.), BluCig (Lorillard Inc., Greensboro, NC), Green Smoke (Green Smoke LLC, Richmond, VA), Mark Ten (Nu Mark LLC, Miami, FL) and Vuse (RJ Reynolds Vapor, Winston-Salem, NC) (Fig 1, Table 1).

Greensmoke, BluCig, SafeCig, and South Beach Smoke were among the leading brands chosen by consumers (Table 1). V2 Cig was selected because the composition of the EC fluid was provided, and it was highly rated by consumers. Smoke 51 was a brand that was not highly rated. NJOY NPRO and Liberty Stix Eagle were chosen because we had evaluated the classic

Table 1. Performance Properties of Electronic Cigarettes.

Brand	Nicotine conc (mg)	Meas. conc (mg/mL)	% change	Avg Air Flow Rate (mL/s)			Avg Pressure Drop (mmH_2O)			Avg Absorbance			Avg # Puffs	Consumer Rating
				1st 10	1st 10 —SO	Smoke Out	1st 10	1st 10— SO	Smoke Out	1st 10	1st 10— SO	Smoke out		
SE Platinum[a,b]	0	ND	NA	21 ± 0	20 ± 1	21 ± 1	68 ± 9	66 ± 8	79 ± 5	0.27 ± 0.15	0.26 ± 0.10	0.16 ± 0.05	160 ± 66	Highly Rated[c]
C7 Imperial[a]	High	13.54 ± 0.05	NA	9 ± 2	9± 2	15 ± 1	29 ± 13	20 ± 8	62 ± 8	0.24 ± 0.13	0.37 ± 0.07	0.21 ± 0.06	400 ± 10	Not Rated
NJOY NPRO	18	12.00 ± 0.12	-33.35	16 ± 2	8 ± 5	11 ± 4	71 ± 26	101 ± 125	106 ± 109	0.67 ± 0.32	0.41 ± 0.09	0.66 ± 0.13	300 ± 0	Highly Rated
SafeCig[b]	24	18.19 ± 0.16	-24.22	13 ± 0	14 ± 1	15 ± 2	68 ± 5	80 ± 11	93 ± 3	0.67 ± 0.09	0.72 ± 0.67	0.58 ± 0.24	300 ± 0	Highly Rated
LS Eagle	High	16.34 ± 0.13	NA	12 ± 2	9 ± 2	12 ± 4	59 ± 10	39 ± 15	68 ± 10	0.22 ± 0.12	0.36 ± 0.35	0.13 ± 0.07	172 ± 67	Not Rated
Smoke 51	16	25.72 ± 1.21	+60.75	4 ± 0	4 ± 0	4 ± 0	67 ± 24	85 ± 17	92 ± 15	0.39 ± 0.22	0.62 ± 0.13	0.36 ± 0.09	294 ± 11	Poorly Rated
SB Smoke	16	12.06 ± 0.20	-24.64	16 ± 1	14 ± 1	14 ± 1	45 ± 5	35 ± 5	42 ± 3	0.74 ± 0.31	0.70 ± 0.36	0.75 ± 0.23	300 ± 0	Top Five
V2 Cigs	18	15.61 ± 0.14	-13.28	12 ± 2	13 ± 0	13 ± 0	37 ± 8	45 ± 17	49 ± 15	0.86 ± 0.38	0.93 ± 0.08	0.87 ± 0.12	300 ± 0	Number One
BluCig	13–16	16.32 ± 0.24	+1.98	5 ± 2	9 ± 3	9 ± 3	14 ± 6	22 ± 9	22 ± 9	0.28 ± 0.29	0.63 ± 0.13	0.54 ± 0.15	300 ± 0	Highly Rated
Greensmoke	18	11.06 ± 0.17	-38.57	10 ± 0	9 ± 2	11 ± 2	64 ± 4	45 ± 11	74 ± 22	0.12 ± 0.09	0.56 ± 0.61	0.43 ± 0.34	233 ± 115	Top Five
Mark 10	2.5% weight	NA	NA	6 ± 3	7 ± 3	7 ± 3	23 ± 3	30 ± 22	33 ± 20	0.97 ± 0.05	0.79 ± 0.18	0.64 ± 0.10	268 ± 29	Major Tobacco
Vuse	4.8% weight	NA	NA	12 ± 2	12 ± 2	12 ± 2	49 ± 3	52 ± 4	55 ± 5	0.95 ± 0.24	0.94 ± 0.09	0.86 ± 0.05	214 ± 22	Major Tobacco

Abbreviations: 1st 10, average results from First 10 Puff protocol, 1st 10 –SO, average results from first 10 puffs during the smoke out, Smoke Out, average results from Modified Smoke-Out protocol, ND; Not Detected, NA; Not Applicable

[a] Products from Williams, Talbot 2011 NTR

[b] Products are available via third party vendors

[c] Product was the number one selling brand at time of 2011 study

doi:10.1371/journal.pone.0149251.t001

Fig 1. Cartomizer style EC used in this study. From Left to right: Smoking Everywhere Platinum (SEP), Crown 7 Imperial (C7I), NJOY NPRO (NJOY), SafeCig (SC), Liberty Stix Eagle (LSE), Smoke 51 (S51), South Beach Smoke (SBS), V2 Cigs (VC), BluCig (BC), Greensmoke (GS), Mark Ten (M10) and Vuse.

doi:10.1371/journal.pone.0149251.g001

three piece counterparts in our prior studies [2]. Mark Ten and Vuse were chosen because they are produced by major tobacco companies. Upon receipt, all EC were inventoried and stored at room temperature until tested.

Cartomizer Dissection and Fluid Separation

Fresh unused cartomizers were dissected to separate the fibers from the atomizing unit, as described previously [13]. The white plug in the end of the mouthpiece was removed to reveal the fibers surrounding the atomizing unit. The inner and outer fibers were centrifuged in MinElute Spin columns (Qiagen, Valencia, CA) at 14,000 revolutions/minute for 4–6 minutes to separate the fluid from the fibers [13].

HPLC Analysis of Cartomizer Fluids

Samples of EC fluid were evaluated using a Hewlett Packard Series 1100 HPLC equipped with a 200 × 4.6 mm Thermo Scientific Hypersil ODS C18 column with a particle size of 5 μm. The 5% stock solutions of cartomizer fluid were made by dilution in a non-buffered mobile phase consisting of 77% water/ 23% acetonitrile (Fisher Scientific, Fair Lawn, NJ). Stock solutions were then diluted further in non-buffered mobile phase to the working concentration of 0.5%. The diode array detector signal was set to 260 nm with a bandwidth of 40 nm with a reference signal of 380 nm and bandwidth of 10 nm at a temperature of 35°C and a 0.8 ml/min flow rate. The mobile phase consisted of HPLC-grade chemicals (Fisher Scientific, Fair Lawn, NJ) in the following make up: 76.9% water, 23% acetonitrile, and 0.1% triethylamine; the pH of the mobile phase was adjusted daily to 7.6 using phosphoric acid (Fisher Scientific, Fair Lawn, NJ) and sodium hydroxide (EM Scientific, Gibbstown, NJ). The injection volume for all samples was 5 μl. The nicotine limit of quantification for this method was 10 μg/ml with a limit of detection of 50 ng/ml. The values reported are the means and standard deviations of the five runs. Full method details, including method validation, were published previously [5,7].

Smoking Machine Set-Up

Experiments were done using a smoking machine that was connected through Tygon tubing to a water manometer, which is in turn was connected through Tygon tubing to a peristaltic pump [2,16,18–20]. EC puffs lasted 4.3 seconds and were taken every minute [21]. All cigarettes were smoked at the lowest airflow rate that produced a robust puff of aerosol. During each puff, pressure drop was measured using a water manometer. The aerosol was captured in a test tube every 10 puffs, and absorbance was measured in a spectrophotometer at a 420-nanometer reading [2,16]. Additionally, the airflow rate was calculated using the pump speed and a conversion factor provided by the pump manufacturer (Barnant Company, Barrington, IL).

Performance Characteristics Experiments

First 10 Puff Protocol. Each EC was puffed 10 times with puffs spaced 1 minute apart. Pressure drop and air flow rate were recorded for each puff. Aerosol density was recorded for every other puff. Experiments were performed three times using a different EC cartomizers each time as described previously [2,16].

Smoke-out Protocol. To determine how air flow rate, pressure drop, and aerosol absorbance change during prolonged use, EC were puffed once every minute until cartridges were exhausted (pump speed reached its maximum and/or three consecutive puffs had aerosol densities below 0.05 absorbance units) or until 300 puffs were reached. Pressure drop and air flow rate were recorded for every puff, and aerosol absorbance was recorded every tenth puff. Air flow rate was increased by increasing pump speed by one interval on the pump dial whenever aerosol density dropped below 0.05 absorbance units or until pump speed reached its maximum air flow rate (24 mL/s) [2,16]. Three experiments were performed with each brand of EC. Each experiment used a different cartomizer. All cartomizers were fresh and had not been used previously by us. The lowest pump speed that produced robust aerosol was used for each brand. The pump was activated manually every minute, and pump speed was turned to zero between puffs to further resemble an active smoker. Pump speed was only increased if EC stopped producing aerosol.

Results

Appearance of EC

Cartomizer style EC come in different shapes, colors, and sizes. The 12 brands of cartomizer style EC that were used in this study are shown in Fig 1. Many manufacturers try to make their product resemble an actual cigarette (cig-a-like), although they are generally longer and heavier than conventional cigarettes. Most brands used in this study resembled conventional cigarettes.

Performance Testing of Cartomizer Style EC

First 10 puffs protocol. EC performance was compared among 12 brands for the first 10 puffs (Fig 2, Table 1). Pressure drop, which measures the leakiness of the EC to air during a puff, remained stable within a brand over the first 10 puffs, but varied between EC brands (Fig 2A). In contrast to the classic models of EC [2,16], most brands had pressure drops that were within the range of conventional cigarettes (~30–70 mm H_2O), except for BluCig, Mark Ten, and Crown 7 Imperial, which were below this range (Fig 2A).

During the first 10 puffs, all EC required a single airflow rate to produce aerosol, and this rate, which ranged from 4–21 mL/s, did not change for any brand during puffing (Fig 2A, Table 1). In Trtchounian et al 2010, all conventional cigarettes required an air flow rate of 7

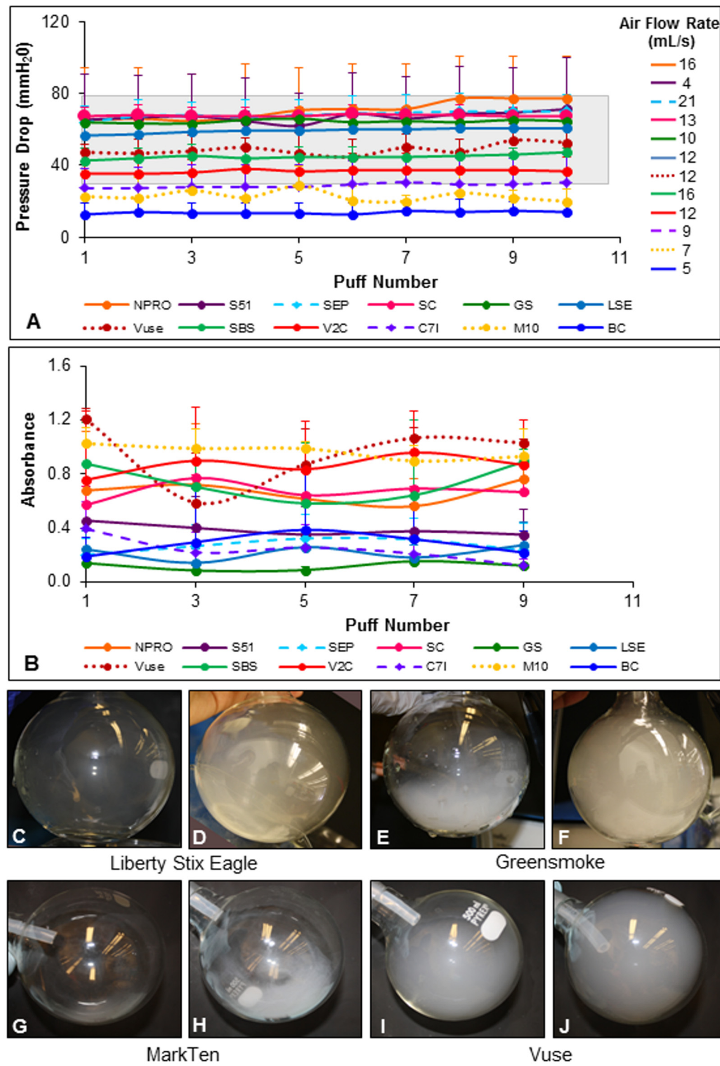


Fig 2. Pressure drop, airflow rate, and absorbance for the first 10 puffs. (A) Average pressure drop vs puff number for each brand. Air flow rates for each brand are listed in the legend on the right of the graph. The grey shaded box represents the pressure drop range for conventional cigarettes.[2,16] (B) Average absorbance vs puff number for EC over the first 9 puffs was similar within brands, but varied between brands. In A and B, each point is the mean ± standard deviation of three experiments. (C-J) Images of aerosol produced by two different cartomizers from Liberty Stix Eagle (C, D), Greensmoke (E, F). Mark Ten (G, H) and Vuse (I, J).

doi:10.1371/journal.pone.0149251.g002

mL/s to produce smoke. Unlike conventional cigarettes, all EC brands, except three (Mark Ten, Smoke 51 and BluCig), required higher air flow rates than conventional cigarettes (Fig 2A, Table 1).

Aerosol absorbance, which is related to density, was measured spectrophotometrically over the first 10 puffs (Fig 2). The aerosol density was relatively stable for the first 10 puffs within a brand, but varied among brands (Fig 2B). Vuse and Mark Ten had the highest average absorbances (0.95± 0.24 and 0.97 ± 0.05, respectively) and Greensmoke had the lowest (0.12 ± 0.09) (Fig 2B, Table 1). For some products, aerosol density varied between cartomizers within a brand as shown in Fig 2C–2J. Two Liberty Stix Eagle (Fig 2C and 2D), two Greensmoke (Fig 2E and 2F), two Mark Ten (Fig 2G and 2H) and two Vuse (Fig 2I and 2J) cartomizers produced aerosol with very different densities within each brand. This variation in aerosol density within brands could contribute to the high standard deviations in absorbance readings observed for some brands (Fig 2B).

Modified Smoke-out Protocol. EC pressure drop, air flow rate required for aerosol production, aerosol absorbance, and puff number were evaluated by puffing cartomizers until either aerosol was no longer produced or 300 puffs were taken (Fig 3, S1 Fig, and Table 1).

Pressure drop data for six brands of EC are shown in (Fig 3A, 3C, 3E, 3G, 3I and 3K), and data for four additional brands are in (S1A, S1C, S1E, and S1G Fig). For each brand, three different fresh cartomizers were compared. Within brands, three distinct patterns of data were observed: (1) all three cartomizers performed similarly or the same, (2) two cartomizers were similar, while the third performed differently, and (3) all three cartomizers performed differently. The first performance pattern (all similar) was observed for BluCig, Vuse, Safe Cig, Smoke 51, South Beach Smoke, and V2 Cigs (Figs 3A, 3C, S1A, S1C, S1E and S1G). While occasionally a few puffs varied within a trial, most puffs were similar for a given cartomizer throughout the entire smoke out (Fig 3A and 3C and S1A, S1C, S1E and S1G Fig, Table 1). The second pattern (two similar and one different cartomizer) was seen in NJOY NPRO and Greensmoke (Fig 3E and 3G). The pressure drop for NJOY trials 1 and 3 (red and blue lines) were very similar, while trial 2 (green line) differed (Fig 3E). In trial 2, the pressure drop decreased, then peaked at puff 30 (300 mmH_2O), decreased again, then peaked at puff 90 (300 mmH_20), then steadily decreased until puff 300 (Fig 3E). The Greensmoke cartomizers for trials 2 and 3 (green and blue lines in Fig 3G) performed similarly, while trial 1 (red line in Fig 3G) was clearly different. In Greensmoke trial 1 (red line in Fig 3G), the EC required repeated increases in air flow rate to produce aerosol, and this was accompanied by a corresponding increase in pressure drop. The pressure drop from trial 1 had steady increases starting at ~ puff 50 (Fig 3G). The third pattern was observed for Liberty Stix Eagle and Mark Ten in which the data from three trials were different from each other (Fig 3I and 3K). Each Liberty Stix Eagle cartomizer required increases in air flow rate in order to maintain aerosol production, and these increases were accompanied by corresponding increases in pressure drop (Fig 3I). In contrast for Mark Ten remained fairly constant through the smoke-out, but the three cartomizers had different pressure drops (Fig 3K)

The air flow rate was measured for every puff during the smoke out protocol and the initial airflow rates and any increases are indicated by arrows (Fig 3, S1 Fig). Smoke 51 and V2 Cigs used the same air flow rate (arrows) for all three trials (4 mL/s and 13 mL/s) for all 300 puffs (S1C and S1G Fig). For South Beach Smoke, two of the three cartomizers used a single air flow rate throughout the entire trial (15 mL/s), while the third cartomizer required an increase in the air flow rate to continue aerosol production (S1E Fig). For BluCig and Vuse and Mark Ten, all three cartomizers required a single air flow rate (arrows) throughout their trials, although the airflow rates varied within a brand (Fig 3A, 3C and 3K). For NJOY NPRO, each cartomizer used a different initial air flow rate (arrows), and each required increases throughout the 300 puffs (Fig 3E).

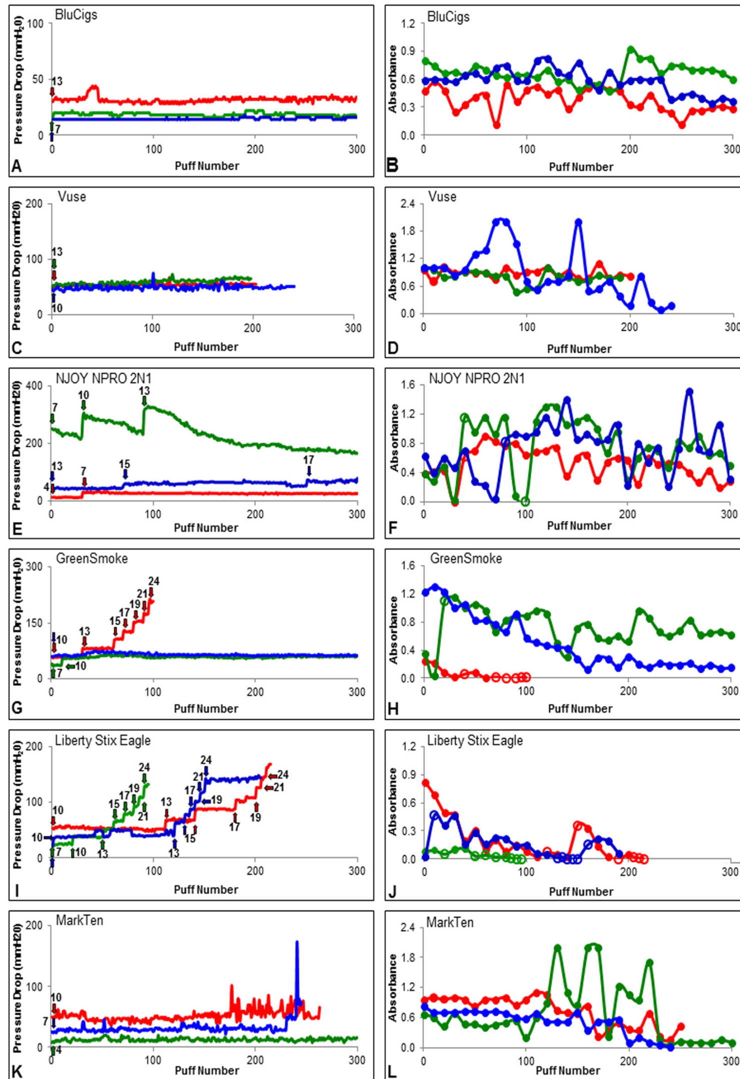


Fig 3. Results from the smoke-out protocol for six EC. In A, C, E, G, I, and K, pressure drop is plotted versus puff number for six brands. Arrows in A, C, E, G, I, and K indicate starting airflow rates (ml/s) and increases in airflow rate that were needed to continue aerosol production. In B, D, F, H, J, and L, absorbance is plotted versus puff number for the same six brands. Open circles indicate puffs where airflow rate was increased to maintain aerosol production. Data are shown for three different cartomizers for each brand. Trial 1 = red, trial 2 = green, and trial 3 = blue.

doi:10.1371/journal.pone.0149251.g003

For Greensmoke, the cartomizers in trials 2 and 3 required a single air flow rate (arrows) throughout the entire trial (10 mL/s), whereas the cartomizer in trial 1 required frequent increases in air flow rate to continue aerosol production (Fig 3G). The three cartomizers from Liberty Stix Eagle all required frequent increases in air flow rate to sustain aerosol production (Fig 3I).

The aerosol absorbance varied from puff to puff within brands as well as between brands (Fig 3, S1 Fig and Table 1). For South Beach Smoke, two trials were very similar, while the third trial had the same absorbance pattern, but produced less aerosol. The average yield for the three absorbance smoke-out trials was 0.75 ± 0.23 (S1F Fig, Table 1). Within the BluCig, Smoke 51, and V2 Cig groups, absorbance was similar for each trial with average absorbances of 0.54 ± 0.15, 0.36 ± 0.09, and 0.87 ± 0.12, respectively (Figs 3A, S1D and S1H, Table 1). For Greensmoke and SafeCig, absorbance decreased throughout the smoke-out (Fig 3G, S1B Fig). All three trials for NJOY NPRO produced significant amounts of aerosol, but the trials were not very similar (Fig 3F). All trials for Liberty Stix Eagle and one trial for Greensmoke did not produce a lot of aerosol, and thus required more frequent increases in air flow rate (Fig 3H and 3J). For SafeCig, the three trials all produced different amounts of aerosol in the beginning but towards the end of the smoke-out, the results were similar (S1B Fig). For both Mark Ten and Vuse, two cartomizers within groups produced similar aerosol, while the third was in each group was variable (Fig 3D and 3L).

All products except Vuse, Liberty Stix Eagle, and 2 of 3 Mark Ten cartomizers could be smoked up to 300 puffs (Fig 3D, 3J and 3L, Table 1). Greensmoke trial 1 (red line) stopped producing aerosol at puff 100, while the other two cartomizers produced 300 puffs (Fig 3H). The three trials for Liberty Stix Eagle did not last longer than 200 puffs (Fig 3J, Table 1).

The first 10 puffs from Fig 2 were compared to the first 10 puffs from the smoke-out (Fig 3 and S1 Fig) to determine how much variability there would be between two experiments done at different times with products purchased at different times (Table 1). Five of the brands (NJOY, Liberty Stix Eagle, Smoke 51, BluCig, and Greensmoke) produced quite different performance characteristics when comparing the data from the first 10 puff experiment to the first 10 puffs in the smoke-out experiment. As an example, comparisons for these two experiments for NJOY are: air flow rate: 16 and 8 ml/sec; pressure drop 71 and 101 mm H_2O; and 0.67 and 0.41 absorbance units.

Nicotine Concentrations in Cartomizer Style Brands. Nicotine concentrations were determined in the cartomizer fluid from each sample evaluated in the performance trials (Table 1). Of 10 brands analyzed, only BluCig had a measured nicotine concentration within 10% of the value given on the manufacturer's label. Most brands had less nicotine than the label indicated, and one brand (Smoke 51) had 60% more nicotine than indicated on the label.

Discussion

The performance characteristics of 12 brands of cartomizer style EC were compared using short and long puffing protocols, and nicotine concentrations on labels vs measured concentrations were compared for each product that contained nicotine. Although cartomizer style EC are designed similarly, performance characteristics, such as air flow rate, pressure drop, and aerosol density varied among brands, which is consistent with our previous data [2,16]. Some of the cartomizer products performed similarly within brands (e.g. BluCig, Smoke 51, and V2 Cigs), while others did not (e.g. NJOY NPRO, Greensmoke, and Liberty Stix Eagle). In addition, some products performed differently when purchased at different times.

Fig 4 summarizes and compares performance properties across four styles of EC, and S1 Table summarizes data for individual brands collected in this and our earlier studies. As mentioned earlier, pressure drop relates to the leakiness of the EC to air during a puff, and the

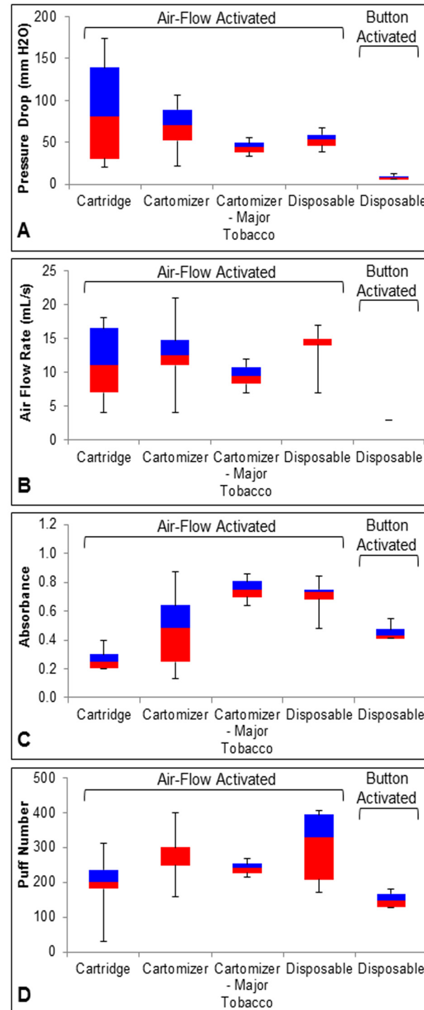


Fig 4. Comparison of performance properties across different styles of EC. Four performance properties, pressure drop (A), air flow rate (B), absorbance (C), and puff number (D), are summarized in box-whisker plots for cartridge models [2]; cartomizer models from our previous (16) and current study; disposable button-activated models [20]; disposable airflow activated models [20]. Each box shows the median, 75% percentile (blue), 25% percentile (red), and minimum and maximum values. The number of brands in each group was: six cartridge style, 10 cartomizer style, two major tobacco, five airflow-activated disposable brands, and four button-activated disposable brands.

doi:10.1371/journal.pone.0149251.g004

lower the pressure drop the easier it is to draw air into the EC and produce aerosol. For most cartomizers in the current study, pressure drop was relatively stable during prolonged use, unlike the first generation classic cartridge models which had variable pressure drops (Fig 4A) [2,16]. Pressure drop for cartomizer EC ranged from 30 mm H_2O to 100 mm H_2O (Fig 4A). Cartomizers (Vuse and Mark Ten) from two major tobacco companies as well as the two disposable styles (button activated and air flow activated) had relatively low and uniform pressure drops both between and within brands [20]. The button activated models were interesting in that they had lower pressure drops than any of the other styles. As these devices have evolved, it appears pressure drop has become more uniform within a style and pressure drop values have become similar to those of conventional cigarettes [2].

The various styles of EC required different air flow rates for aerosol production (Fig 4B). Cartridge models were highly variable in the air flow rate required for activation and also required frequent increases in air flow rate during the smoke-out protocol for continued aerosol production [2,16]. Cartomizer style EC were activated by a broad range of air flow rates, with most brands producing aerosol between 4–21 mL/s (Fig 4B) [2,16]. Air flow rate requirements for activation were very similar in the major tobacco group. Button-activated disposable models all used the same air flow rate for activation. All air flow activated models required between 14–17 mL/s, except for BluCigs which were activated by 7 mL/s [20]. The evolution of EC products towards lower air flow rates for activation may be a benefit for users.

In our previous performance studies, aerosol absorbance, which is a measure of aerosol density, was quite uniform within each group of EC [2,16,20]. In contrast, the aerosol absorbance for the cartomizer models in this study (excluding major tobacco) was variable and ranged from 0.13 to 0.87 average absorbance units/smoke-out (Table 1, Fig 4C). This range was greater than for any of the other groups (Fig 4). Within the cartomizer group, absorbance for each brand differed with some brands producing fairly uniform aerosol between cartomizers, while others did not. The cartomizers from the major tobacco companies and the air flow activated models produced aerosol with the highest densities. The variability in aerosol absorbance in the in some brands in the cartomizer group indicates a need for better quality control in the manufacturing of these devices.

While puff number in the major tobacco and button activated models were very uniform within groups, puff number varied in the other three categories (Fig 4D). Puff number was highest in the air activated style EC and lowest in the button activated. Cartridge style EC lasted for a wide range of puffs, as few as 25 to as many as 300 puffs. Except for Smoking Everywhere Platinum, Liberty Stix Eagle, Greensmoke, Vuse, and Mark Ten, cartomizer style EC lasted for 300 puffs or more, which is longer than the often advertised puff number (one cartomizer = about 1 package of conventional cigarettes or 200 puffs according to most/some/all advertisements) (Fig 4D). Vuse advertises that their brand will last about 200 puffs, and all units we tested lasted at least this long. Button-activated EC never lasted longer than 200 puffs. While the air-flow activated models varied in the number of puffs, most models lasted less than 300 puffs. None of the disposable brands lasted their advertised number of puffs, which could be attributed to the battery. In most cases, disposable units stopped producing aerosol because the battery, which is not rechargeable, died. It is not known how long the disposable units sit in warehouses and in shops before use, but most have probably lost some of their charge before purchase [20].

There were also discrepancies in the labeling of nicotine concentrations on EC packages, as reported previously for other EC products [7,22,23]. Only one brand, BluCig, met the American E-Liquid Manufacturing Standards Association (AEMSA) standard for nicotine labeling which requires that the measured and labeled concentration deviate by less than 10%. Most cartomizer brands contained less nicotine than the label on the cartons indicated, although one brand (Smoke 51) had 60% more than the labeled concentration. These labeling discrepancies

are in agreement with a recent study that measured the amount of nicotine in refill fluids and found that 35 out of 54 products had nicotine concentrations that deviated from the labeled concentration by more than 10% [7]. Two brands (Liberty Stix Eagle and Crown 7 Imperial) did not give a nicotine concentration, but ranked nicotine as low, medium, and high, or bold. Proper nicotine labeling is a public health concern. Some EC refill bottles without any label contained over 100 mg/ml of nicotine [7], and some do-it-yourself flavor products that are presumed to be nicotine free contained nicotine [24]. The variations in performance parameters and discrepancies in nicotine concentrations may help understand the variability in consumer puffing patterns and why EC users take more puffs, longer puffs, and more frequent puffs [21,25,26].

In summary, performance parameters were generally more consistent in cartomizer style EC than in the classic cartridge style, (except for aerosol absorbance which was most variable in the cartomizer group), indicating an improvement in performance with the evolution of these products. However, for 5 of the brands there was considerable variation in products purchased at different times. Of the four classes of EC that we have studied, major tobacco cartomizers and button-activated disposable brands were the most uniform for all performance parameters across and within brands; however, puff number for button-activated models was lower than advertised and lower than any of the other groups. For the cartomizer style EC in the 10 puff protocol, there was little variation within brands, but significant variation between brands. In the smoke-out protocol, most cartomizer brands had relatively stable pressure drop, air flow rate, and absorbance, while a few cartomizers behaved differently than others in their group. The highly variable aerosol absorbances observed in the cartomizer group, the variation in performance parameters within some brands in the cartomizer group, the low puff numbers achieved with disposable brands, and the variation in performance for some products purchased at different times indicate a need for better quality control in the manufacture and design of EC.

Supporting Information

S1 Fig. Results from the smoke-out protocol for four EC brands. (A, C, E, and G) Pressure drop is plotted versus puff number for SafeCig, Smoke 51, South Bach Smoke, and V2 Cigs. Arrows in A, C, E and G) indicate starting airflow rates (ml/s) and increases in airflow rate that were needed to continue aerosol production. (B, D, F and H) Absorbance is plotted versus puff number for the same brands. Open circles indicate puffs where airflow rate (pump speed) was increased to maintain aerosol production. Data are shown from three different cartomizers for each brand. Trial 1 = red, trial 2 = green, and trial 3 = blue.
(PDF)

S1 Table. Consolidation of Performance Parameters for all EC devices.
(DOCX)

Acknowledgments

We would like to thank An To, Ivana Villarreal, and Michael Dang for their assistance in the lab. We would also like to thank Esther Omaiye for her helpful suggestion on the manuscript and Laurie Graham for her help maintaining the smoking machine.

Author Contributions

Conceived and designed the experiments: MW PT. Performed the experiments: MW AV BD. Analyzed the data: MW AV BD PT. Contributed reagents/materials/analysis tools: PT. Wrote the paper: MW BD PT.

References

1. Grana R, Benowitz N, Glantz SA (2014) E-cigarettes: a scientific review. Circulation 129: 1972–1986. PMID: 24821826
2. Trtchounian A, Williams M, Talbot P (2010) Conventional and electronic cigarettes (e-cigarettes) have different smoking characteristics. Nicotine Tob Res 12: 905–912. doi: 10.1093/ntr/ntq114 PMID: 20644205
3. Trtchounian A, Talbot P (2011) Electronic nicotine delivery systems: is there a need for regulation? Tob Control 20: 47–52. doi: 10.1136/tc.2010.037259 PMID: 21139013
4. Zhu SH, Sun JY, Bonnevie E, Cummins SE, Gamst A, Yin L, et al. (2014) Four hundred and sixty brands of e-cigarettes and counting: implications for product regulation. Tob Control 23 Suppl 3: iii3–iii9. doi: 10.1136/tobaccocontrol-2014-051670 PMID: 24935895
5. Bahl V, Lin S, Xu N, Davis B, Wang Y, Talbot P (2012) Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models. Reprod Toxicol 34: 529–537. doi: 10.1016/j.reprotox.2012.08.001 PMID: 22989551
6. Behar RZ, Davis B, Wang Y, Bahl V, Lin S, Talbot P (2014) Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids. Toxicol In Vitro 28: 198–208. doi: 10.1016/j.tiv.2013.10.006 PMID: 24516877
7. Davis B, Dang M, Kim J, Talbot P (2015) Nicotine concentrations in electronic cigarette refill and Do-it-Yourself fluids. Nicotine Tob Res. 17(2):134–141. doi: 10.1093/ntr/ntu080 PMID: 24862971
8. Polosa R, Rodu B, Caponnetto P, Maglia M, Raciti C (2013) A fresh look at tobacco harm reduction: the case for the electronic cigarette. Harm Reduct J 10(1): 19. doi: 10.1186/1477-7517-10-19
9. Meikle J (2015) Vaping: e-cigarettes safer than smoking, says Public Health England. Guard. The Guardian. 19 August 2015. Available: http://www.theguardian.com/society/2015/aug/19/public-health-england-e-cigarettes-safer-than-smoking. Accessed August 19, 2015.
10. Pisinger C, Dossing M (2014) A systematic review of health effects of electronic cigarettes. Prev Med 69: 248–260. doi: 10.1016/j.ypmed.2014.10.009 PMID: 25456810
11. Glasser AM, Cobb CO, Teplitskaya L, Ganz O, Katz L, Rose SW, et al. (2015) Electronic nicotine delivery devices, and their impact on health and patterns of tobacco use: a systematic review protocol. BMJ Open 5: e007688–e007688. doi: 10.1136/bmjopen-2015-007688 PMID: 25926149
12. Hua M, Alfi M, Talbot P (2013) Health-related effects reported by electronic cigarette users in online forums. J Med Internet Res 15: e59. doi: 10.2196/jmir.2324 PMID: 23567935
13. Williams M, Villarreal A, Bozhilov K, Lin S, Talbot P (2013) Metal and silicate particles including nanoparticles are present in electronic cigarette cartomizer fluid and aerosol. PLoS One 8(3): e57987. doi: 10.1371/journal.pone.0057987 PMID: 23526962
14. Williams M, To A, Bozhilov K, Talbot P (2015) Strategies to reduce tin and other metals in electronic cigarette aerosol. PLoS One 10: e0138933. doi: 10.1371/journal.pone.0138933 PMID: 26406602
15. Jensen RP, Luo W, Pankow JF, Strongin RM, Peyton D (2015) Hidden formaldehyde in e-cigarette aerosols. N Engl J Med 372(4): 389–392.
16. Williams M, Talbot P (2011) Variability among electronic cigarettes in the pressure drop, airflow rate, and aerosol production. Nicotine Tob Res 13(12): 1276–1283. doi: 10.1093/ntr/ntr164 PMID: 21994335
17. Goniewicz ML, Kuma T, Gawron M, Knysak J, Kosmider L (2013) Nicotine levels in electronic cigarettes. Nicotine Tob Res 15: 158–166. doi: 10.1093/ntr/nts103 PMID: 22529223
18. Knoll M, Shaoulian R, Magers T, Talbot P (1995) Ciliary beat frequency of hamster oviducts is decreased in vitro by exposure to solutions of mainstream and sidestream cigarette smoke. Biol Reprod 53: 29–37. doi: 10.1095/biolreprod53.1.29 PMID: 7669854
19. Knoll M, Talbot P (1998) Cigarette smoke inhibits oocyte cumulus complex pick-up by the oviduct in vitro independent of ciliary beat frequency. Reprod Toxicol 12: 57–68. PMID: 9431573
20. Williams M, Ghai S, Talbot P (2014) Disposable Electronic Cigarettes and Electronic Hookahs: Evaluation of Performance. Nicotine Tob Res 17(2):201–208. doi: 10.1093/ntr/ntu118 PMID: 25104117
21. Hua M, Yip H, Talbot P (2013) Mining data on usage of electronic nicotine delivery systems (ENDS) from YouTube videos. Tob Control 22: 103–106. doi: 10.1136/tobaccocontrol-2011-050226 PMID: 22116832
22. Cameron JM, Howell DN, White JR, Andrenyak DM, Layton ME, Roll JM. (2014) Variable and potentially fatal amounts of nicotine in e-cigarette nicotine solutions. Tob Control 23: 77–78. doi: 10.1136/tobaccocontrol-2012-050604 PMID: 23407110

23. Trehy ML, Ye W, Hadwiger ME, Moore TW, Allgire JF, Woodruff JT, et al. (2011) Analysis of electronic cigarette cartridges, refill solutions, and smoke for nicotine and icotine related impurities. J Liq Chromatogr Relat Technol 34: 1442–1458. doi: 10.1080/10826076.2011.572213

24. Davis B, Razo A, Nothnagel E, Chen M, Talbot P (2015) Unexpected nicotine in Do-it-Yourself electronic cigarette flavourings. Tob Control 0: 1–2 doi: 10.1136/tobaccocontrol-2015-052468

25. Behar R, Hua M, Talbot P (2015) Puffing topography and nicotine intake of electronic cigarette users. PLoS One 0(2): e0117222. doi: 10.1371/journal.pone.0117222

26. Robinson RJ, Hensel EC, Morabito PN, Roundtree KA (2015) Electronic cigarette topography in the natural environment. PLoS One 10: e0129296. doi: 10.1371/journal.pone.0129296 PMID: 26053075

APPENDIX D

Counterfeit Electronic Cigarette Products with Mislabeled Nicotine Concentrations

Esther Omaiye, Iliana Cordova, Barbara Davis, Prue Talbot

Tobacco Reg Sci 2017;3:347–57. doi:10.18001/TRS.3.3.10

Counterfeit Electronic Cigarette Products with Mislabeled Nicotine Concentrations

Esther E. Omaiye, MS, BS
Iliana Cordova, BS
Barbara Davis, BS
Prue Talbot, PhD, MA

Objectives: We compared nicotine concentrations in one brand of refill fluids that were purchased in 4 countries and labeled 0 mg of nicotine/mL. We then identified counterfeit e-cigarette products from these countries. **Methods:** Overall, 125 e-cigarette refill fluids were purchased in Nigeria, the United States (US), England, and China. Nicotine concentrations were measured using high performance liquid chromatography and compared to labeled concentrations. Refill fluids were examined to identify physical differences and grouped into authentic and counterfeit products. **Results:** Whereas nicotine was in 51.7% (15/29) of the Nigerian, 3.7% (1/27) of the Chinese and 1.6% (1/61) of the American refill fluids (range = 0.4 - 20.4 mg/mL), 8 British products did not contain nicotine. Products from China, the US, and Nigeria with trace amounts of nicotine (0.4 to 0.6 mg /mL) were authentic; however, all products from Nigeria with more than 3.7 mg/mL were counterfeit. **Conclusions:** We introduce 2 novel issues in the e-cigarette industry, the production of counterfeit refill fluids under a brandjacked label and inclusion of nicotine in 81.3% of the counterfeit products labeled 0 mg/mL. This study emphasizes the need for better control and monitoring of nicotine containing products and sales outlets.

Key words: electronic cigarettes; counterfeit tobacco-related products; nicotine; addiction; toxicity; public health; tobacco regulation
***Tob Regul Sci.*™** 2017;3(3):347-357
DOI: https://doi.org/10.18001/TRS.3.3.10

Nicotine is readily available in electronic cigarette (e-cigarette) refill fluids that are sold worldwide. Nicotine concentrations in these products range from 0 to over 100 mg/mL, and product concentration labels are often inaccurate.[1–3] Even do-it-yourself flavoring products used to create these fluids sometimes contain nicotine.[4] The widespread distribution and use of nicotine-containing e-cigarette products presents a new public health problem that could increase nicotine addiction, cause poisoning, and lead to other unwanted health effects.[5–12]

In this study we evaluated e-cigarette refill fluids produced by one manufacturer and sold worldwide. Specifically, we quantified nicotine in products labeled 0 mg/mL, evaluated products to determine authenticity, and identified counterfeit zero nicotine refill fluids that contained nicotine.

METHODS

Sample Collection and Assessment

Between March 2015 and May 2016, 125 of LiQua e-cigarette refill fluids (Ritchy Group Limited) were purchased in Nigeria (29 refill fluids, 7 flavors, purchased over-the-counter in an Abuja department store and at an online store in Lagos), the United States (61 refill fluids, 50 flavors, purchased over the Internet from Kansas and California), England (8 refill fluids, in 8 flavors, purchased over the Internet from Northamptonshire), and China (27

Esther E. Omaiye, Graduate Student, Environmental Toxicology Graduate Program, University of California, Riverside, CA. Iliana Cordova, Undergraduate Student, Department of Cell Biology and Neuroscience, University of California, Riverside, CA. Barbara Davis, Graduate Student, Department of Cell Biology and Neuroscience, University of California, Riverside, CA. Prue Talbot, Professor, Department of Cell Biology and Neuroscience, University of California Riverside, CA.
Correspondence Dr Talbot; talbot@ucr.edu

refill fluids, 25 flavors, purchased over the Internet from Xiamen and Guangdong). These countries were chosen to represent different: (1) global regions; (2) levels of economic development; and (3) levels of consumer product regulation and quality. Labeled nicotine concentration for all 125 products was "0 mg/mL," which was interpreted as zero nicotine. Ritchy Group Limited is a Russian company with production plants in China and Italy and contact centers in Moscow, Kansas, the Czech Republic, and China that distributes products to over 85 countries (www.ritchy.com). Ritchy was chosen because of its broad global distribution of refill fluids, thereby enabling comparison of products purchased in the 4 countries. When possible, products with the same flavors were purchased in multiple countries.

Each product was assigned an inventory number, photographed, and stored at 4°C. All products were received sealed and undamaged, and were analyzed within one month of receipt. All products came in individual boxes, except those from Guangdong (China). Coloration of each fluid was compared visually.

Nicotine Concentration Quantification

We used high-performance liquid chromatography (HPLC) to quantify nicotine in each sample using a method described previously.[3] The limit of quantification for nicotine was 10 μg/ml with a limit of detection of 50 ng/ml.

Authentication of E-cigarette Refill Fluids

We defined counterfeit products as ones not manufactured by Ritchy but sold under the Ritchy label. We used the Quick Response (QR) barcode, European Article Number (EAN) barcode, and guidelines from consumer websites to determine if refill fluids were authentic or counterfeit.[13,14] We examined products for the presence of QR codes as recommended by personnel at Ritchy. QR codes on refill fluids have 5 sets of 4-digit numbers printed on white stickers located on the bottom or the caps of refill fluid boxes or bottles. These codes were either inputted or scanned into the verification section (www.ritchy.com/check) on the Ritchy website, which recognizes numbers that belong to authentic Ritchy products and distinguishes ones not generated by Ritchy.

We also accessed the globally used 13-digit EAN barcode, which identifies items for sale at retail establishments, for authentication. This barcode consists of: (1) the GS1 prefix which identifies the country where the product was manufactured or the member organization to which the manufacturer is registered; (2) the unique manufacturer's identification code assigned by the GS1 office; (3) the item or product code which is selected by the manufacturer; and (4) the check digit which proves that the manufacturer has thoroughly inspected the item. EAN barcodes appeared on stickers pasted or printed directly on each refill fluid box or bottle and were scanned using ICONIT software. The user is directed to an Internet site that: (1) identifies the product as a Ritchy product; (2) fails to identify the product; or (3) identifies an incorrect Ritchy product indicating the barcode had been hijacked. A second line of EAN identification was performed using a government supported online database (www.gepir.gs1.org) that provides information on the company, products, and illegal EAN numbers.

In addition, we used guidelines from e-cigarette websites and forums to identify counterfeit LiQua products.[13,14] These criteria included the quality of printing on boxes and bottles (which is inferior on counterfeit products), the appearance of identical product images on the Ritchy website, and the packaging of the product in a box at the time of receipt, which is characteristic of authentic Ritchy products. "Product Name on Database" was not available for the LiQua Q and LiQua HP products and some premium LiQua flavors.

RESULTS

Nicotine Concentrations in Zero Nicotine Products

Nicotine was quantified in 125 LiQua e-cigarette refill fluids labeled 0 mg (Table 1, Figures 1A-D, and Supplementary Table 1). No nicotine was found in 108 samples (Table 1 and Supplementary Table 1). Figures 1A and 1B show Nigerian products that contained nicotine peaks as indicated by the red arrow at 8 min in Bright Tobacco flavor (A) and Menthol flavor (B). Figures C and D show that the same flavors purchased in the United States (US) contained no nicotine, as indicated by the black arrows. Samples of Two-Apples from the US, China, and Lagos contained trace

Table 1
Counterfeit and Suspected Counterfeit E-cigarette Refill Fluids[a]

#	Co[b]	Flavor	[Q] (mg/mL)[c]	Coloration[d]	QR[e]	EAN[f]	Mfr. Name[g]	Product Name on Database
1	NG-A	MB	20.4 ± 0.3	Coral	NC	IC	RGHK	Variety (0mg)
2	NG-A	MB	12.3 ± 0.2	Orange	NC	IC	RGHK	Variety (0mg)
3	NG-A	MB	12.4 ± 0.2	Orange	NC	IC	RGHK	Variety (0mg)
4	NG-A	MB	12.3 ± 0.1	Coral	NC	IC	RGHK	Variety (0mg)
5	NG-A	MB	14.9 ± 0.4	Deep Orange	NC	IC	RGHK	Variety (0mg)
6	NG-A	MB	15.5 ± 0.4	Deep Orange	NC	IC	RGHK	Variety (0mg)
7	NG-A	MB	13.6 ± 0.6	Deep Orange	NC	IC	RGHK	Variety (0mg)
8	NG-A	Bright Tob.	13.6 ± 0.2	Coral	NC	IC	RGHK	Energy Drink (18mg)
9	NG-A	Bright Tob.	12.9 ± 0.5	Light Orange	NC	IC	RGHK	Energy Drink (18mg)
10	NG-A	Menthol	9.2 ± 0.0	Clear (translucent)	NC	IC	RC: 13	Illegal Number
11	NG-A	Menthol	3.7 ± 0.0	Clear	NC	IC	RC: 13	Illegal Number
12	NG-A	Menthol	4.2 ± 0.1	Clear (translucent)	NC	IC	RC: 13	Illegal Number
13	NG-A	Menthol	4.1 ± 0.0	Clear (translucent)	NC	IC	RC: 13	Illegal Number
14	NG-A	Watermelon	ND	Clear	NC	IC	SLHK	No record found
15	NG-A	Watermelon	ND	Clear	NC	IC	SLHK	No record found
16	NG-A	Watermelon	ND	Clear	NC	IC	SLHK	No record found
17	CN-G	Two Apples	0.4 ± 0.0	Yellow	NB	NB	N/A	N/A
18	CN-G	Cola	ND	Clear	NB	NB	N/A	N/A
29	CN-G	Peach	ND	Clear	NB	NB	N/A	N/A
20	CN-G	Licorice	ND	Clear w/ YT	NB	NB	N/A	N/A
21	CN-G	Brownie	ND	Clear	NB	NB	N/A	N/A
22	CN-G	Berry Mix	ND	Clear	NB	NB	N/A	N/A
23	CN-G	Cheesecake	ND	Clear	NB	NB	N/A	N/A

(continued on next page

amounts of nicotine (range = 0.4 to 0.6 mg/mL), probably due to contamination or carry over during manufacturing. In contrast, all LiQua Bright Tobacco, MB, and Menthol flavors purchased in Abuja (N = 13), contained 3.7 - 20.4 mg/mL of nicotine (Table 1). Nicotine concentrations varied within the same flavor purchased at separate times, eg, the first set of MB fluids contained 20.4 mg/mL of nicotine (product #1, Table 1), whereas the second (products #2 - #4) and third (products #5 - #7) sets contained 12.3 and 14.6 mg/mL, respectively.

Physical Properties of E-cigarette Refill Fluids

Within LiQua flavor groups, color varied by country, eg, Bright Tobacco purchased in Abuja was coral to light orange, but clear in other countries (Table 1, Supplementary Table 1, and Figure 1E). The color of LiQua MB flavors purchased in Abuja at separate times varied from coral to orange (Figure 1F). This color variation in counterfeit products is suggestive of inconsistencies during manufacture. Watermelon-flavored products purchased in Abuja were clear and identical to those purchased in the US (Table 1; Supplementary Table 1).

 DOI: https://doi.org/10.18001/TRS.3.3.10

Table 1 (continued)
Counterfeit and Suspected Counterfeit E-cigarette Refill Fluids[a]

#	Co[b]	Flavor	[Q] (mg/mL)[c]	Coloration[d]	QR[e]	EAN[f]	Mfr. Name[g]	Product Name on Database
24	CN-G	Ry4 Tob.	ND	Pale yellow	NB	NB	N/A	N/A
25	CN-G	Bright Tob.	ND	Pale yellow	NB	NB	N/A	N/A
26	CN-G	Virginia Tob.	ND	Clear w/ YT	NB	NB	N/A	N/A
27	CN-G	Traditional Tob.	ND	Pale yellow	NB	NB	N/A	N/A
28	CN-G	Mild Kretek Tob.	ND	Clear w/ YT	NB	NB	N/A	N/A
29	CN-G	Red Oriental Tob.	ND	Clear w/ YT	NB	NB	N/A	N/A
30	CN-G	Golden Oriental Tob.	ND	Clear w/ YT	NB	NB	N/A	N/A
31	CN-G	American Blend Tob.	ND	Clear	NB	NB	N/A	N/A
32	CN-G	Goldenrod Oriental Tob.	ND	Clear w/ YT	NB	NB	N/A	N/A
33	CN-G	Vermillion Oriental Tob.	ND	Yellow-Orange	NB	NB	N/A	N/A

Note.
a #1 – 16 were verified to be counterfeit using all criteria. Packaging for #17-33 was not available and these were suspected of being counterfeit. Supplementary Table 1 contains all authentic products
b Co =Country and location of product purchase (NG-A = Nigeria, Abuja; CN-G = China, Guangdong)
c [Q] = Quantified nicotine concentration (± standard deviation) using HPLC (ND = Not Detected)
d Coloration = Color of the refill fluids (YT = yellow tint;)
e QR = Availability and verification of manufacturer's Quick Response Code (C = Correct code = Verified; NC/NB = No Code/No Box = Unverified)
f EAN = Availability and verification of company and product information using the European Article Number barcode (IC = Incorrect; NB = None)
g Mfr. Name = Name of manufacturer to which product EAN barcode is linked; RGHK = Ritchy Group Ltd HK; SLHK = Spoilt Ltd HK; RC:13 = Illegal/None; N/A = Not Available

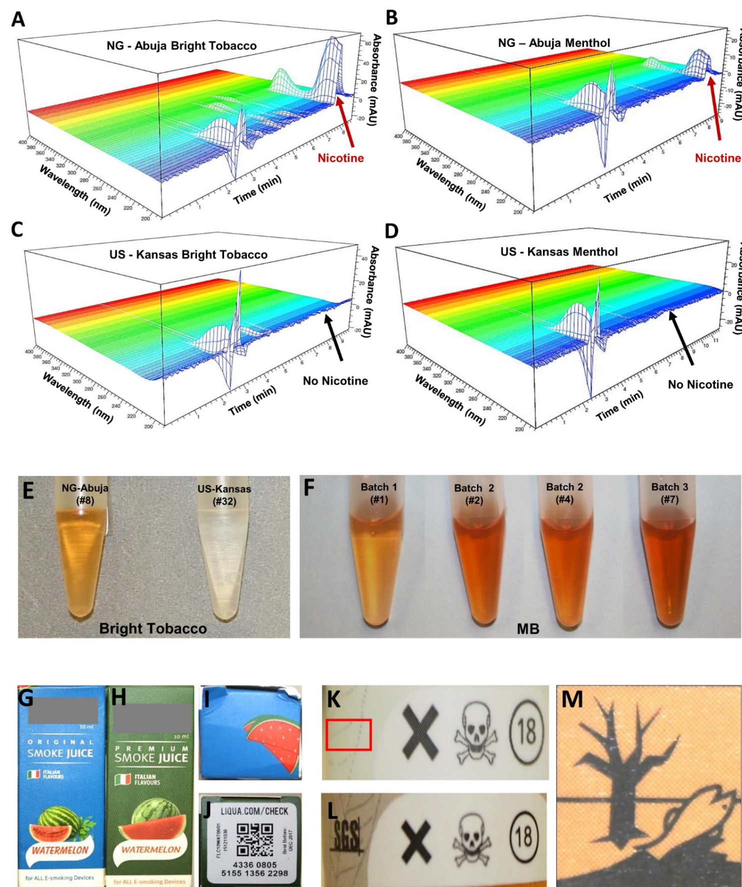
Labeling on Abuja products was fuzzy and of inferior quality compared to products from other countries which were of superior quality. Watermelon-flavored fluids from Abuja were in blue boxes without a QR code for authentication (Figures 1G and 1I), but the Kansas sample was in a green box with a QR code (Figures 1H and 1J) and was identical to the image on the Ritchy website. Bright Tobacco labels from Abuja were printed on a tan background, but labels from other countries were on white backgrounds that were identical to images on the Ritchy website. The MB flavor had no semblance to product images on www.ritchy.com but existed only on websites discussing "Fake LiQua e-juices." Samples from Guangdong, China were not received with boxes; therefore, the semblance and quality of packaging could not be evaluated.

All products from Abuja had identical lot/batch numbers unlike products from other countries, which had different lot/batch numbers for each sample. Only the "variety pack of ten," purchased from Santa Clara (California) and Xiamen had the same production lot/batch numbers on the fluids as well as on the variety pack box.

Identification of Counterfeit Products

We examined refill fluids to determine if they were counterfeit (Table 1) or authentic (Supple-

Figure 1
Comparison of 0 mg E-cigarette Refill Fluids and Identification of Counterfeit Products



Note.
(A-D) Three-dimensional HPLC chromatograms showing presence or absence of nicotine in e-cigarette products labeled 0 mg of nicotine. X axis = time (minutes), Y-axis = absorbance (mAU), and Z-axis = wavelength (nm).
(E-F) These are the color variations between identical refill fluids for Bright Tobacco Nigeria versus USA (E) and MB flavors (F).
(G-J) Differences in packaging between Watermelon from Nigeria (G) without a QR code (I) and USA (H) with a QR code for authentication (J).
(K -L) Warning labels and certification logos on Bright Tobacco refill fluid boxes purchased in the USA (K) without the SGS logo (red box) and in Nigeria (L) with the SGS logo.
The ecotoxic symbol (M) was present on only the counterfeit LiQua refill fluids.

mentary Table 1) using the QR code, EAN barcode, and differences in physical properties of the products. Using QR codes, we verified that products from the US (except for one), England, Lagos, and China (Xiamen) were authentic. Products from Abuja had no QR codes on their boxes and products from Guangdong (China) were received without boxes, and therefore, their authenticity could not be verified (Table 1).

We obtained additional information on counterfeit products using the EAN barcode (Table 1). Counterfeit Nigerian products were registered to: (1) Ritchy Group LTD but were linked to the incorrect product, eg, the 10 ml Bright Tobacco code identified it as a 30 ml Energy Drink; (2) Spoilt LTD, a different company, identified by the barcode as an "illegal number" (eg, watermelon); or (3) no company, meaning matching documents were unavailable (eg, menthol flavors) and it could not be verified (Table 1). All flavors from other locations had barcodes and were identical to flavors found on www.ritchy.com.

Labeling and Warning Symbols

All boxes had a skull and crossbones, over 18, and X (harmful) symbols (Figures 1K and 1L); however, only the counterfeit samples had the Société Générale de Surveillance (SGS) insignia and the ecotoxic symbol (Figures 1L and 1M). SGS is a worldwide organization that inspects, verifies, tests, and certifies that imported goods have been checked and meet quality control standards (www.sgs.com). Similar health warnings were reported on the bottles or boxes of all refill fluids.[15] Only LiQua HP flavors stated that a user should "contact a poison center or seek medical assistance if you feel ill after use."

Association between Nicotine and Counterfeit Refill Fluids

We used the above criteria to determine that 16/125 refill fluids labeled 0 mg were counterfeit products sold under a brandjacked label. About 81% (13/16) of the counterfeit products contained nicotine (3.7 – 20.4 mg/mL). The 3 counterfeit flavors with nicotine were MB, Menthol, and Bright Tobacco. Approximately 19% (3/16) of watermelon flavored LiQua, purchased in Abuja, were also counterfeit but did not contain nicotine.

DISCUSSION

We introduce novel issues in tobacco control and global health – the production of counterfeit e-cigarette refill fluids and the inclusion of nicotine in counterfeit products labeled 0 mg. The identification of nicotine in e-cigarette products that should be nicotine-free is a health concern for several reasons. First, zero-nicotine users with access to counterfeit products could develop an unwanted addiction that may be difficult to break. Secondly, a growing number of pregnant women use nicotine-free refill fluids[16] and could unwittingly expose their fetuses/newborns to a neuroteratogen.[5] Thirdly, refill fluids containing nicotine have caused numerous poisonings, often in children;[11,17] this potential danger is not apparent from the mislabeled counterfeit products. Finally, some e-cigarette users gradually decrease nicotine use with e-cigarettes.[18] If these users purchase counterfeit products containing nicotine, they may be unsuccessful in weaning themselves off nicotine.

Refill fluid users can identify counterfeit products using the criteria presented in this paper. Counterfeit fluids purchased in Abuja were ₦500.00 NGN in contrast to authentic products purchased from recommended LiQua distributors in Lagos for ₦1500.00 NGN. Although counterfeit products with nicotine were only purchased in Abuja, these products are readily distributable to other countries, and we had no difficulty bringing them into the USA. In addition, the counterfeit products varied in color within flavors, suggesting inconsistencies in their manufacture.

Unlike earlier generations,[19] the authentic products in this study were generally labeled with safety warnings and reasonably accurate nicotine concentrations. LiQua Q flavors purchased in California carried the Proposition 65 warning stating the product contains substances that may cause cancer or produce reproductive/developmental problems.[20] However, only LiQua HP flavors contained warnings such as not recommended for non-smokers, contact with skin maybe toxic, keep out of reach of children and pets, and contact a poison center if you feel ill after use. The SGS logo implies products have undergone supervision and quality control from acquisition of raw materials through manufacturing to final production and distribution. Users of refill fluids should be skeptical of this logo as it appeared only on counterfeit products.

Counterfeit products have been problematic in the conventional tobacco cigarette industry.[21] Our study demonstrates that the problem of counterfeit products extends to the e-cigarette retail market. However, because our study is limited to products from one company and 4 countries, future studies are needed to determine the breadth of counterfeit e-cigarette sales.

Conclusions

This is the first report that counterfeit e-cigarette products with inaccurate nicotine labeling and invalid quality control certification logos are being produced under a brandjacked label. Users of these products would be exposed to nicotine without their knowledge, which could lead to unwanted nicotine-induced health effects, as recently summarized by the US Surgeon General.[12] In addition, the counterfeit products varied in color within flavors, suggesting inconsistencies in their manufacture. These data will be useful in establishing regulatory policies for e-cigarettes.

IMPLICATIONS FOR TOBACCO REGULATION

We introduce a new issue in the emerging e-cigarette industry, the inclusion of nicotine in counterfeit products labeled 0 mg/mL. Nicotine also has been reported in some DIY e-cigarette flavor products that should be nicotine-free.[4] Mislabeled counterfeit and DIY e-cigarette products containing nicotine are a public health concern that could be addressed by agencies involved in the regulation of tobacco products. These findings emphasize the need for education of e-cigarette users to the existence of zero-nicotine products that contain nicotine and for identification and confiscation of counterfeit products.

Conflict of Interest Statement

No conflicts of interest are reported by any of the authors.

Acknowledgments

Grant number R21DA037365 to PT from the National Institutes of Health (NIH) and the FDA Center for Tobacco Products supported this research. EO was supported in part by California Institute for Regenerative Medicine Bridges award grant number TB01-0777 and BD was supported by a National Science Foundation Predoctoral Fellowship. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH, the FDA, or other granting agencies. The authors thank Rachel Behar for her helpful comments on the manuscript and Paul Gerald Iyaji and Baba Onotu for making the Nigeria products available for analysis. Use of trade names is only for information purpose and does not imply endorsement.

References

1. Trehy ML, Ye W, Hadwiger ME, et al. Analysis of electronic cigarette cartridges, refill solutions, and smoke for nicotine and nicotine related impurities. *Journal of Liquid Chromatography & Related Technologies*. 2011;34(14):1442–1458.
2. Cameron JM, Howell DN, White JR, et al. Variable and potentially fatal amounts of nicotine in e-cigarette nicotine solutions. *Tob Control*. 2013;23(1):77–78.
3. Davis B, Dang M, Kim J, Talbot P. Nicotine concentrations in electronic cigarette refill and do-it-yourself fluids. *Nicotine Tob Res*. 2014;17(2):134–141.
4. Davis B, Razo A, Nothnagel E, et al. Unexpected nicotine in do-it-yourself electronic cigarette flavourings. *Tob Control*. 2015;25(e1):e67–e68.
5. Dwyer JB, McQuown SC, Leslie FM. The dynamic effects of nicotine on the developing brain. *Pharmacol Ther*. 2009;122(2):125–139.
6. Benowitz NL. Pharmacology of nicotine: addiction, smoking-induced disease, and therapeutics. *Annu Rev Pharmacol Toxicol*. 2009;49(1):57–71.
7. Cantrell FL. Adverse effects of e-cigarette exposures. *J Community Health*. 2013;39(3):614–616.
8. Kim JW, Baum CR. Liquid nicotine toxicity. *Pediatr Emerg Care*. 2015;31(7):517–521.
9. Winer S. Police investigating toddler's death from nicotine overdose. Available at: http://timesofisrael.com/police-investigating-toddler-death-from-nicotine-overdose. Accessed January 27, 2017.
10. Mohney G. First child's death from liquid nicotine as "Vaping" grows. Available at: http://abcnews.go.com/Health/childs-death-liquid-nicotine-reported-vaping-gains-popularity/story?id=27563788. Accessed January 27, 2017.
11. Hua M, Talbot P. Potential health effects of electronic cigarettes: a systematic review of case reports. *Prev Med Rep*. 2016;4:169–178.
12. US Department of Health and Human Services (DHHS), Office of the Surgeon General. *Facing Addiction in America: The Surgeon General's Report on Alcohol, Drugs, and Health*. Washington, DC: DHHS; 2016.
13. How to Spot Fake LiQua Juice. Available at: https://plainvape.wordpress.com/2013/11/10/how-to-spot-fake-liqua-juice/. Accessed January 27, 2017.
14. I doubt this LiQua e-liquid is Original: FastTech

forums. Available at: https://www.fasttech.com/forums/1297106/t/1127136/i-doubt-this-liqua-e-liquid-is-original. Accessed January 27, 2017.

15. Kim S, Goniewicz M, Yu S, et al. Variations in label information and nicotine levels in electronic cigarette refill liquids in South Korea: regulation challenges. *Intl J Environ Res Public Health*. 2015;12(5):4859–4868.
16. Mark KS, Farquhar B, Chisolm MS, et al. Knowledge, attitudes, and practice of electronic cigarette use among pregnant women. *J Addict Med*. 2015;9(4):266–272.
17. American Association of Poison Control Centers. E-cigarettes and liquid nicotine data. Available at: https://aapcc.s3.amazonaws.com/files/library/E-cig__Nicotine_Web_Data_through_12.2016.pdf. Accessed January 27, 2017.
18. McQueen A, Tower S, Sumner W. Interviews with "Vapers": implications for future research with electronic cigarettes. *Nicotine Tob Res.* 2011;13(9):860–867.
19. Trtchounian A, Talbot P. Electronic nicotine delivery systems: is there a need for regulation? *Tob Control*. 2010;20(1):47–52.
20. Office of Environmental Health Hazard Assessment. State of California Environmental Protection Agency Office of Environmental Health Hazard Assessment Safe Drinking Water and Toxic Enforcement Act of 1986. Chemicals Known to the State to Cause Cancer or Reproductive Toxicity. 2016. Available at: http://oehha.ca.gov/media/downloads/proposition-65//p65single10212016.pdf. Accessed January 27, 2017.
21. National Research Council and Institute of Medicine. *Understanding the U.S. Illicit Tobacco Market: Characteristics, Policy Context, and Lessons from International Experiences.* Washington, DC: The National Academies Press;

2015.

Supplementary Table 1
Authentic and Suspected Authentic E-cigarette Refill Fluids[a]

#	Co[b]	Flavor	[Q] (mg/mL)[c]	Coloration[d]	QR[e]	EAN[f]	Mfr. Name[g]	Product Name on Database
1	NG-L	Two Apples	0.4 ± 0.1	Yellow	C	C	RGHK	No record found
2	NG-L	Two Apples	0.6 ± 0.1	Light yellow	C	C	RGHK	No record found
3	NG-L	Strawberry	ND	Clear	C	C	RGHK	Strawberry(0mg)
4	NG-L	Menthol	ND	Clear w/ GT	C	C	RGHK	Menthol(0mg)
5	NG-L	Menthol	ND	Clear w/ GT	C	C	RGHK	Menthol(0mg)
6	NG-L	Menthol	ND	Clear w/ GT	C	C	RGHK	Menthol(0mg)
7	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
8	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
9	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
10	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
11	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
12	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
13	NG-L	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
14	US-K	Two Apples	0.5 ± 0.0	Yellow	C	C	RGHK	No record found
15	US-K	Two Mints	ND	Clear	C	C	RGHK	No record found
16	US-K	Peach	ND	Clear	C	C	RGHK	No record found
17	US-K	Ry4 Tob.	ND	Pale yellow	C	C	RGHK	No record found
18	US-K	Red Oriental Tob.	ND	Light yellow	C	C	RGHK	No record found
19	US-K	Licorice	ND	Clear w/ BT	C	C	RGHK	No record found
20	US-K	Menthol	ND	Clear w/ YT	C	C	RGHK	Menthol(0mg)
21	US-K	Mints	ND	Clear	C	C	RGHK	Mints(0mg)
22	US-K	Apple	ND	Clear	C	C	RGHK	Apple(0mg)
23	US-K	Citrus Mix	ND	Clear	C	C	RGHK	Citrus Mix(0mg)
24	US-K	Watermelon	ND	Clear	C	C	RGHK	Watermelon(0mg)
25	US-K	Watermelon	ND	Clear	C	C	RGHK	Watermelon(0mg)
25	US-K	Banana	ND	Clear	C	C	RGHK	Banana(0mg)
27	US-K	Berry Mix	ND	Clear	C	C	RGHK	Berry Mix(0mg)
28	US-K	Blueberry	ND	Clear	C	C	RGHK	Blueberry(0mg)
29	US-K	Coffee	ND	Clear	C	C	RGHK	Coffee(0mg)
30	US-K	Cola	ND	Clear	C	C	RGHK	Cola(0mg)
31	US-K	Energy Drink	ND	Clear w/ YT	C	C	RGHK	Energy Drink(0mg)
32	US-K	Bright Tobacco	ND	Clear	C	C	RGHK	Bright Tob(0mg)
33	US-K	Traditional Tob.	ND	Champagne	C	C	RGHK	Traditional Tob(0mg)
34	US-K	French Pipe Tob.	ND	Clear w/ YT	C	C	RGHK	French PipeT(0mg)
35	US-K	American Blend Tob.	ND	Clear w/ YT	C	C	RGHK	Amer Blend(0mg)
36	US-K	Vanilla	ND	Clear	C	C	RGHK	Vanilla(0mg)
37	US-K	Chocolate	ND	Yellow	C	C	RGHK	Chocolate(0mg)
38	US-K	HP Overdrive	ND	Dark Brown	C	C	RGHK	No record found
39	US-K	HP Fruity Velocity	ND	Clear	C	C	RGHK	No record found

(continued on next page)

Supplementary Table 1 (continued)
Authentic and Suspected Authentic E-cigarette Refill Fluids[a]

#	Co[b]	Flavor	[Q] (mg/mL)[c]	Coloration[d]	QR[e]	EAN[f]	Mfr. Name[g]	Product Name on Database
40	US-K	HP summer Drift	ND	Clear w/ YT	C	C	RGHK	No record found
41	US-K	HP Sweet Accelerator	ND	Clear w/ YT	C	C	RGHK	No record found
42	US-K	Q Apple	ND	Clear	C	C	RGHK	No record found
43	US-K	Q Peach	ND	Clear	C	C	RGHK	No record found
44	US-K	Q Menthol	ND	Clear	C	C	RGHK	No record found
45	US-K	Q Berry Mix	ND	Clear	C	C	RGHK	No record found
46	US-K	Q Pina Colada	ND	Clear	C	C	RGHK	No record found
47	US-K	Q Cherribakki	ND	Clear w/ YT	C	C	RGHK	No record found
48	US-K	Q The Moment	ND	Clear	C	C	RGHK	No record found
49	US-K	Q Havana Libre	ND	Clear	C	C	RGHK	No record found
50	US-K	Q Blackberry Jack	ND	Clear	C	C	RGHK	No record found
51	US-K	Q Double Bubble	ND	Clear	C	C	RGHK	No record found
52	US-K	Q Fragola Fresca	ND	Clear	C	C	RGHK	No record found
53	US-K	Q American Blend	ND	Clear w/ YT	C	C	RGHK	No record found
54	US-K	Q Honeydew Drop	ND	Clear w/ YT	C	C	RGHK	No record found
55	US-K	Q Golden Roanoke	ND	Clear w/ YT	C	C	RGHK	No record found
56	US-K	Q Piedmont Sunrise	ND	Clear	C	C	RGHK	No record found
57	US-K	Q Turkish Tob.	ND	Clear	C	C	RGHK	No record found
58	US-K	Q Traditional Tob.	ND	Champagne	C	C	RGHK	No record found
59	US-C	Citrus Mix	ND	Clear	C	C	RGHK	Citrus Mix(0mg)
60	US-C	Cherry	ND	Clear	C	C	RGHK	Cherry(0mg)
61	US-C	Menthol	ND	Clear w/ YT	C	C	RGHK	Menthol(0mg)
62	US-C	Apple	ND	Clear	C	C	RGHK	Apple(0mg)
63	US-C	Berry Mix	ND	Clear	C	C	RGHK	Berry Mix(0mg)
64	US-C	Grape	ND	Clear	C	C	RGHK	Grape(0mg)
65	US-C	Coffee	ND	Clear	C	C	RGHK	Coffee(0mg)
66	US-C	Strawberry	ND	Clear	C	C	RGHK	Strawberry(0mg)
67	US-C	Tiramisu	ND	Clear w/ YT	C	C	RGHK	Tiramisu(0mg)
68	US-C	Bright Tob.	ND	Clear	C	C	RGHK	Bright Tob(0mg)
69	US-C	Peach	ND	Clear w/ YT	C	C	RGHK	No record found
70	US-C	Cuban Cigar Tob.	ND	Clear	C	C	RGHK	No record found
71	US-C	Q Apple	ND	Clear	C	C	RGHK	No record found
72	US-C	Q Peach	ND	Clear	C	C	RGHK	No record found
73	US-C	Q Menthol	ND	Clear	C	C	RGHK	No record found
74	GB-N	Bright Tob.	ND	Clear	C	C	RGHK	Bright Tob(0mg)
75	GB-N	Menthol	ND	Clear w/ BT	C	C	RGHK	Menthol(0mg)
76	GB-N	Apple	ND	Clear	C	C	RGHK	Apple(0mg)
77	GB-N	Berry Mix	ND	Clear	C	C	RGHK	Berry Mix(0mg)
78	GB-N	Peach	ND	Clear	C	C	RGHK	No record found

(continued on next page)

Supplementary Table 1 (continued)
Authentic and Suspected Authentic E-cigarette Refill Fluids[a]

#	Co[b]	Flavor	[Q] (mg/mL)[c]	Coloration[d]	QR[e]	EAN[f]	Mfr. Name[g]	Product Name on Database
79	GB-N	Q Apple	ND	Clear	C	C	RGHK	No record found
80	GB-N	Q Peach	ND	Clear	C	C	RGHK	No record found
81	GB-N	Q Berry Mix	ND	Clear	C	C	RGHK	No record found
82	CN-X	Bright Tob.	ND	Clear	C	C	RGHK	Bright Tob(0mg))
83	CN-X	Menthol	ND	Clear	C	C	RGHK	Menthol(0mg)
84	CN-X	Apple	ND	Clear	C	C	RGHK	Apple(0mg)
85	CN-X	Citrus Mix	ND	Clear	C	C	RGHK	Citrus Mix(0mg)
86	CN-X	Cherry	ND	Clear	C	C	RGHK	Cherry(0mg)
87	CN-X	Berry Mix	ND	Clear	C	C	RGHK	Berry Mix(0mg)
88	CN-X	Strawberry	ND	Clear	C	C	RGHK	Strawberry(0mg)
89	CN-X	Grape	ND	Clear	C	C	RGHK	Grape(0mg)
90	CN-X	Coffee	ND	Clear	C	C	RGHK	Coffee(0mg)
91	CN-X	Tiramisu	ND	Clear w/ YT	C	C	RGHK	Tiramisu(0mg)
92	US-K	Turkish Tob.	ND	Clear	NC	C	RGHK	Turkish Tob. (0mg)

Note.
a #1 – 91 were verified to be authentic using all criteria. #92 could not be verified using the QR code
b Co =Country and location of product purchase (NG-L = Nigeria, Lagos; US-K = USA, Kansas; US-C = US, California; CN-X = China, Xiamen)
c [Q] = Quantified nicotine concentration (± standard deviation) using HPLC (ND = Not Detected)
d Coloration = Color of the refill fluids (YT = yellow tint; GT = green tint; BT = brown tint)
e QR = Availability and verification of manufacturer's Quick Response Code (C = Correct code = Verified; NC/NB = No Code/No Box = Unverified)
f EAN = Availability and verification of company and product information using the European Article Number barcode (IC = Incorrect; NB = None)
g Mfr. Name = Name of manufacturer to which product EAN barcode is linked; RGHK = Ritchy Group Ltd HK; SLHK = Spoilt Ltd HK; RC:13 = Illegal/None; N/A = Not Available