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.

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