Apparent Lack of Cytotoxicity in Brands of e-Liquids Previously Reported As Being Cytotoxic

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Abstract

US sales of e-cigarettes (e-cigs) and e-liquids reportedly will exceed $1 billion in 2013. While there is much evidence to indicate that short-term use of e-cigarettes as a smoking cessation tool does not present undue health risks in healthy adults, little is known about health risks associated with long-term use of the product. To date there appears to have been only one report of *in vitro* toxicological assays that are commonly used to assess the toxicity of mainstream cigarette smoke being applied to the mainstream aerosol from e-cigs (Romagna *et al.*, 2013); but that research used puffing conditions now known to be inappropriate for e-cigs; and puffing of e-cigs is also known to give cytotoxic substances such as acrolein when dry puffs are taken (Farsalinos *et al.*, 2013). Thus, it makes sense to determine e-liquid cytotoxicity first. There has been one report (Bahl *et al.*, 2012) that described the use of the MTT *in vitro* assay with three different cell lines (hESC, mNSC, hPF) to estimate the cytotoxicity of e-liquids used to refill the reservoirs of e-cigs. While several e-liquids were reported to be cytotoxic, no cytotoxic compounds were identified in that report. For this research, four of the same brand-styles of flavors that were reported as cytotoxic by Bahl were obtained. An additional brand-style of e-liquid was obtained from another manufacturer. All five samples were assayed with the Neutral Red Uptake (NRU) cytotoxicity assays with CHO cells (assays were purchased from Labstat International ULC, Kitchener, ON, Canada), and a modification of Health Canada Method T-502 was used. There was no cytotoxicity at a concentration of 0.1%. In addition, each e-liquid was analyzed by GC-MS, and none of the compounds reported to cause e-liquid cytotoxicity such as allyl alcohol and diacetyl were found. However, one e-liquid was found to contain eugenol and cinnamaldehyde, compounds not usually used in cigarettes sold in the US.

Introduction

Since the abstract for this poster was submitted for presentation at this meeting, two other publications have dealt with the cytotoxicity of e-liquids: Farsalinos *et al.*, 2013, and Behar *et al.*, 2014. The authors of those two papers took very different approaches to the overall process of assaying cytotoxicity and the relevance of their experimental results assessing the potential of e-cigarette use to cause adverse health effects in both users as well as those who may be exposed to indoor air pollution resulting from the use of e-cigarettes. However, the finding of cytotoxicity in e-liquids is surprising since the major components of those mixtures (e.g., propylene glycol and/or glycerol) are not cytotoxicity and nicotine, if used, is only slightly cytotoxic (Bahl *et al.*, 2012). Most flavors used in contemporary tobacco cigarettes are not cytotoxic.

Introduction (con't)

Since flavors used in most contemporary cigarettes are not expected to be cytotoxic (cytotoxicity of mainstream cigarette smoke is due to the pyrolysis/combustion products of the tobacco), the findings by Bahl *et al.* (2012) were unexpected. The e-liquids analyzed by Bahl were obtained from several suppliers, but the majority came from Johnson Creek (PG-based) or its Red Oak division (glycerol-based). The data on the samples tested are shown in Table 1 below.

Table 1. Concentration and cytotoxicity data from Bahl et al., 2012

Refill Fluid	Nicotine Concentration (mg/mL)	hESC IC_{50}	mNSC IC_{50}	hPF IC_{50}
Tennessee Cured	18	0.32	0.09	>1
Marcado	18	0.08	0.09	0.82
Valencia	18	0.22	0.31	>1
Swiss Dark	18	0.11	0.16	0.30
Propylene Glycol	0	>1	>1	>1
Nicotine in PG	100	0.23	0.31	0.35

In Table 1, hESC refers to human embryonic stem cells, mNSC refers to mouse neural stem cells, and hPF refers to human pulmonary fibroblasts. The MTT assay was used, and the IC_{50} values were determined from the dose–response curves (Bahl *et al.*, 2012).

These data have asked more questions than they have answered. For example:

- What were the cytotoxic agents in these e-liquids?
- Were the assay methods used more sensitive than the Neutral Red Uptake (NRU) method with CHO cells (i.e., Health Canada Method T-502, Health Canada 2004) that is used for many tobacco-related cytotoxicity studies?
- Were the major ingredients of the flavor cytotoxic or was the cytotoxicity due to unknown impurities?
- Do these data have any role in assessing the potential adverse health effects from using those e-liquids?

The purpose of the research presented here was to answer those questions.

Materials and Methods

Materials. The following e-liquids were obtained directly from Johnson Creek Enterprises, LLC, Johnson Creek, WI, August 2013: Red Oak Tennessee Cured™, Marcado™, Valencia™, Swiss Dark™, and Silverthorn®. All samples were in glycerol and were reported to contain 1.8% nicotine. An additional sample from another manufacturer, V2 Platinum E-Liquid Chocolate was obtained in May 2013, and was reported to contain 1.2% nicotine in propylene glycol.

Methods. The Neutral Red Uptake (NRU) assays were purchased from Labstat International ULC, Kitchener, ON, Canada. E-liquid concentrations used in the assay were 0.1%

Materials and Methods (con't)

GC-MS analyses were obtained from two laboratories. Both laboratories used bench-top GC-MS systems, and one lab used a wax-type capillary column while the other used a 5-MS-type capillary column. Instrument parameters were typical for flavors and flavor-related substances.

Results and Discussion

The five e-liquids were assayed along with concurrent controls of glycerol and 1:1 (*v*/*v*) PG/glycerol. A summary of the NRU cytotoxicity data from Labstat is shown below in Table 2.

Table 2. NRU relative absorbance data from Labstat International, 2013

Refill Fluid	Sample Grand Mean	Growth Medium Mean	SLS 110 (µg/mL)	SLS 200 (µg/mL)
Tennessee Cured	102	103	14	1
Marcado	103	107	17	1
Valencia	120	115	18	1
Swiss Dark	103	100	12	2
V2 Chocolate	108	106	12	1
PG + Glycerol (1/1)	102	104	13	1
Glycerol	104	106	16	1

The data in Table 2 do not indicate cytotoxicity. There is no explanation with currently available information as to why the results were not similar to those reported by Bahl and coworkers. Perhaps the manufacturer learned of the cytotoxicity and took corrective action. A summary of the GC-MS data is shown in Table 3 below. Visual estimates of GC-MS peak area were made and are described using the legend at the bottom of Table 3.

Table 3. Summary of GC-MS data for Red Oak E-liquids

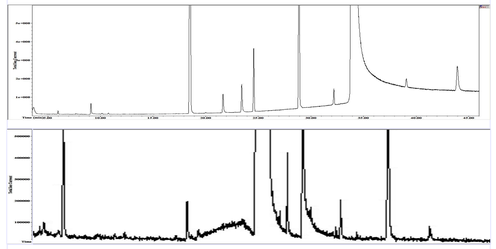
Retention Time (min)	Peak CAS RN	Peak Name	Tennessee Cured	Marcado	Valencia	Swiss Dark
3.80	513-86-0	Acetoin	T			
5.98	64-19-7	Acetic acid	VS	T		
7.94	78-70-6	Linalool			T	
8.22	111-87-5	Octyl alcohol			T	
9.11	57-55-6	Propylene glycol	T	T	T	T
10.15	87-44-5	Caryophyllene		T		
10.23	107-92-6	Butyric acid	T			
10.76	22047-25-2	Acetylpyrazine		T		
16.82	765-70-8	Methyl cyclopentenolone	T			
18.53	54-11-5	Nicotine	L	L	L	L
21.66	118-71-8	Maltol		VS		
23.44	4940-11-8	Ethyl maltol	S	VS	T	T
24.58	104-55-2	Cinnamaldehyde		S		
28.89	97-53-0	Eugenol		M		
32.19	91-10-1	2,6-Dimethoxyphenol	T	T	T	T
34.32	56-81-5	Glycerol	XL	XL	XL	XL
39.09	91-64-5	Coumarin		T		
43.92	121-33-5	Vanillin		VS		

XL = Extra large, L = Large, M = Medium, S = Small, VS = Very small, T = trace

Three of the four brand-styles of Red Oak e-liquid were rather lightly flavored in comparison with the fourth one, Marcado. The Marcado product contained cinnamaldehyde, a compound that has been reported to cause e-liquids to be cytotoxic (Behar *et al.*, 2014). It may well be that the level of cinnamaldehyde in the sample used in this research was less than the level used in the sample assayed by Bahl, *et al.*, 2012). GC-MS preferably under two conditions is the gold standard for the analyses of flavors.

Results and Discussion (con't)

Good product stewardship requires complete information about ingredients and materials and their expected performance under normal and abusive conditions before marketing a product. Use of bioassays and chemical analyses should be secondary steps to assure that ingredients and materials are within specifications. GC-MS traces obtained for Marcado are shown below.



The top trace was from a run on a wax-type column while the bottom was from a run on a 5-MS-type column after the e-liquid had been persilylated with a mixture of BSTFA/DMF. The point here is not so much the GC-MS analyses, but that different analytical conditions may yield different conclusions about a given e-liquid. Moreover, just because a e-liquids is not cytotoxic and/or does not contain ingredients that raise other toxicological concerns does mean that it will perform satisfactorily in all devices under all operating conditions.

Conclusion

The e-liquid cytotoxicities reported by Bahl and colleagues (2012) could not be duplicated using the available samples and the Neutral Red Uptake assay. These differences in results may have been caused by a reformulation of the products between after the first assays were done.

References

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