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Limited mutagenicity of electronic cigarettes in mouse or human cells *in vitro*

Running title: Examining e-cig mutagenicity

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ABSTRACT

Objectives: Electronic cigarettes (e-cig), which are promoted as safe alternatives to tobacco cigarettes or as aides to smoking cessation, are becoming increasingly popular among adult chronic smokers and adolescents experimenting with tobacco products. Despite the known presence of toxicants and carcinogens in e-cig liquid and vapor, the possible carcinogenic effects of e-cig use in humans are unknown. **Materials and Methods:** We have utilized two validated *in vitro* model systems to investigate whether e-cig vapor induces mutation in mouse or human cells. We have exposed transgenic mouse fibroblasts *in vitro* to e-cig vapor extracts prepared from three popular brands, and determined the induction of mutagenesis in a reporter gene, the *cII* transgene. Furthermore, we have treated the pSP189 plasmid with e-cig vapor extract, transfected human fibroblast cells with the e-cig-treated plasmid, and screened for the induced mutations in the *supF* gene. **Results and Conclusion:** We observed no statistically significant increases in relative mutant frequency in the *cII* transgene or *supF* gene in the e-cig treated mouse or human cells, respectively. Our data indicate that e-cig vapor extracts from the selected brands and at concentrations tested in this study have limited mutagenicity in both mouse and human cells *in vitro*.

Keywords: *cII* transgene, mouse embryonic fibroblasts, mutation, *supF* assay, vaping

1. INTRODUCTION

Electronic cigarettes (e-cig) are battery-powered devices that heat solutions usually containing nicotine and flavorings into inhalable vapor [1]. E-cig are promoted as safe alternatives to conventional tobacco cigarettes and/or as aides to smoking cessation. E-cig are highly popular among adult chronic smokers who seek to transition to putatively harm-reducing tobacco substitutes [2]. E-cig are also gaining rapid acceptance among adolescents, especially those who have never used combustible cigarettes [3]. According to the World Health Organization (WHO), while e-cig represent an evolving frontier filled with promises and challenges for tobacco control, research is needed to empirically address the safety of e-cig and their efficacy in aiding smoking cessation [4, 5]. Whilst e-cig vapor is likely to be less toxic than cigarette smoke, it remains to be determined whether e-cig are indeed a modified-risk tobacco substitute and/or an effective smoking cessation tool [5]. It is conceivable that e-cig may have potential utilities for tobacco harm-reduction and/or smoking cessation. However, it is equally plausible that e-cig use, otherwise known as ‘*vaping*’, may pose a threat to regular vapers and others, *e.g.*, fetuses of vaping pregnant mothers or bystanders exposed to secondhand vapor released into the environment [5]. There is also concern that e-cig may serve as a gateway to nicotine addiction and lead to smoking, especially in adolescents [6]. The latter is ascribed to the large variety of e-cig flavorings, many of which (*e.g.*, chocolate- and candy-flavors) being highly attractive to children and youth. It has been reported that by January 2014, there were 7,764 unique e-cig flavors in the market [7].

Chemical analyses of e-cig liquid and vapor have shown that many toxicants and carcinogens present in cigarette smoke are also found in a range of e-cig products, albeit in generally lower concentrations [8-13]. Yet, empirical data on the possible cancer-causing effects

of e-cig use are lacking [1]. This is a critical omission because the Food and Drug Administration's (FDA) recent ruling will require e-cig manufacturers to provide warning labels on their products if their use is empirically linked to negative health outcomes [14-16]. Exploring the carcinogenic potential of e-cig use in humans will clarify whether the increasing number of smokers-vapers (*i.e.*, dual users) or exclusive vapers (*e.g.*, former smokers) who perceive these products as harmless or less harmful than tobacco cigarettes are still putting themselves at risk for cancer, or conversely are benefiting from their transition to vaping. Data pointing to a carcinogenic effect of e-cig could be used to counter the prevailing perception that e-cig use is healthier than smoking [17-21]. Conversely, if the data show no or less carcinogenic effect of e-cig as compared to tobacco smoke, they could lead to evidence-based promotion of vaping as an alternative nicotine delivery method for tobacco harm reduction [22-25].

A pre-requisite for human studies on e-cig carcinogenicity is the availability of biomarkers that can inform about e-cig exposure and early effects of relevance to cancer [1]. These biomarkers can be developed and validated in *in vitro/in vivo* model systems. Towards this goal, the present study utilizes two validated *in vitro* model systems [26-37] to investigate whether exposure to e-cig vapor induces mutation in mouse or human cells. Here, we have exposed transgenic mouse fibroblasts *in vitro* to e-cig vapor extracts prepared from three different brands, and subsequently determined the induction of mutagenesis in a reporter gene, the *cII* transgene. Furthermore, we have treated the pSP189 plasmid with e-cig vapor extract, transfected human fibroblast cells with the e-cig-treated plasmid, and subsequently performed the *supF* mutagenesis assay to determine whether *in vitro* treatment with e-cig vapor elicits a mutagenic response in human cells. Our data show that e-cig vapor extracts from the selected

brands and at concentrations tested in this study have limited mutagenicity in both mouse and human cells *in vitro*.

2. MATERIALS AND METHODS

2.1. Preparation of e-cig vapor extracts

We generated e-cig vapor extracts from three popular brands using our previously published protocol [38]. Briefly, e-cig vapor from blu eCigs (16 mg/ml nicotine), NJOY (18 mg/ml nicotine), and V2 Cigs (18 mg/ml nicotine) were produced using a smoking machine that was connected with Cole Parmer MasterFlex Tygon tubing (Vernon Hills, IL) to a MasterFlex peristaltic pump (3 Amp, 115Vac, 50/60 Hz; Barnart Company, Barrington, IL; Model #7520-00) [39]. The line between the smoking machine and the pump contained a T-connector (Fisher Scientific) that held the electronic cigarette. The peristaltic pump was warmed up for a minimum of 15 minutes before collecting vapor into a round bottom flask that contained methanol and was submerged into an ice bath. The smoking machine was calibrated to draw 4.3 seconds of vapor [40] at a frequency of 10 puffs/hour. With this method, 1 puff was collected in a round bottom flask with the solvent within an ice bath over the course of 6 minutes to allow for the entire puff to fully settle and be collected. Although only 10 puffs could be collected per hour, the concentration of aerosol solutions used in this study are high enough to detect an effect as shown by our earlier studies (*e.g.*, [41]). For blu eCigs, NJOY, and V2 Cigs, an adequate air flow rate to activate each device was determined and found to be 21 ml/sec (90 ml puff volume), 15 ml/sec (65 ml puff volume), and 10 ml/sec (43 ml puff volume), respectively. Each vapor extract was

produced then filtered and aliquoted. All aliquots were evaporated to dryness in a Savant Speed Vac SVC-100H (Savant Instruments, Inc., Farmingdale, NY). The dried extracts were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich Inc., Saint Louis, MO). Extract concentrations were expressed as total puff equivalents (TPE), which represent the number of puffs of vapor dissolved per milliliter of solvent.

2.2. Cell culture and e-cig treatment

Culturing and chemical treatment of mouse cells and the *cH* mutation detection assay were performed according to our published protocols [42, 43]. We first examined the cytotoxicity of e-cig vapor extracts prepared from three different brands in mouse embryonic fibroblasts by establishing a dose-response survival curve. Briefly, early passage Big Blue® mouse embryonic fibroblasts were grown as monolayers at approximately 50-60% confluence in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Prior to chemical treatment, the media were removed, and cells were washed thoroughly with phosphate buffered saline (PBS). The culture dishes were filled with serum free DMEM plus increasing concentrations of each e-cig vapor extract, and incubation was carried out at 37°C for 24 hours in the dark. Immediately after treatment, the cells were harvested by trypsinization and evaluated for cytotoxicity using the trypan blue dye exclusion technique. Because only mitotically active and dividing cells can manifest mutation consequent to exposure to genotoxic agents [44], we set a maximum threshold limit of cytotoxicity ($\leq 20\%$) for each of the three tested e-cig vapor extracts. We have routinely used comparable cytotoxicity thresholds while investigating the mutagenicity of carcinogens [34-36, 42]. The set limit of cytotoxicity was reached at a

concentration of 25 total puff equivalents (TPE) for all the three tested e-cig brands. We then treated transgenic mouse embryonic fibroblasts with high- and low concentrations of each of the three e-cig vapor extracts, resulting in ~80% and 95%, respectively, cell viability post-treatment. For control purposes, counterpart cells were similarly treated with serum free DMEM plus solvent DMSO at the highest concentration used. At the end of the 24-hour treatment, media were removed, cells were washed 3x with PBS, and subsequently cultivated in complete growth medium for 7 days, while being passed once (1 to 3) on day three. Upon completion of the culturing period, cells were harvested by trypsinization, pelleted by centrifugation, and stored at -80°C until analysis. At the time of harvesting (*i.e.*, day 7 post-treatment), all cultures had undergone 3-4 population doublings, a requisite for fixation of mutations into the genome [44]. As a positive control, counterpart cell cultures were treated with two tobacco carcinogens, benzo[*a*]pyrene (B[*a*]P) and 4-aminobiphenyl (4-ABP), both of which require metabolic activation to exert mutagenic effects [45, 46]. B[*a*]P and 4-ABP have different mutagenic potencies, with the former being a stronger mutagen [45, 46]. To keep a consistent cytotoxicity threshold, control cultures were treated with 5 μ M of B[*a*]P and 10 μ M of 4-ABP that resulted in 80% cell viability post-treatment. Both B[*a*]P and 4-ABP were purchased from Sigma-Aldrich Inc. (Saint Louis, MO). The B[*a*]P- and 4-ABP-treated cultures were processed similarly to the e-cig treated cultures, as described above. All experiments were conducted in triplicate.

2.3. Genomic DNA isolation

Genomic DNA was isolated using a standard phenol and chloroform extraction and ethanol precipitation protocol [47]. The DNA was dissolved in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.5), and kept at -80°C until further analysis.

2.4. *cII* Mutation analysis

Genomic DNA of transgenic Big Blue® rodents contains multiple copies of the chromosomally integrated λ LIZ shuttle vector, which carries two reporter genes, namely the *cII* and *lacI* transgenes [32]. The *cII* mutation detection system is based on the recovery of the coliphage vector from the genomic DNA, followed by phenotypic expression using a temperature-sensitive bacterial assay [31]. Briefly, the recovered vector is packaged into viable bacteriophages, and the infective λ phage particles are introduced into an indicator host *Escherichia coli* (*E. coli*). The λ phages can multiply either lytically or lysogenically in the host *E. coli* depending on *cII* transcription status [48]. The *cII* protein is required for activation of the *cI* repressor and lambda integrase, both of which being essential for lysogenization [48]. Mutated *cII* protein, however, causes the infected *E. coli* to undergo lysis, thereby forming plaques on an agar lawn [31]. The λ LIZ shuttle vector also harbors a *cI857* temperature sensitive (*ts*) mutation, which makes the *cI(ts)* protein labile at temperatures higher than 32°C [31]. Thus, all vector-bearing phages, irrespective of the *cII* mutation status, multiply lytically in the host *E. coli* at incubating temperatures exceeding 32°C [32]. This temperature sensitivity is the basis for the *cII* selection assay in which phenotypic expression of the *cII* mutants is achieved under selective incubation

condition, *i.e.*, 24°C [31]. Under non-selective incubation condition, *i.e.*, 37°C, both wild type and mutant *cII* are expressed [31]. The ratio of plaques formed under the selective condition to those formed under the non-selective condition is commonly referred to as the “*cII* mutant frequency”, which denotes mutation rate in the *cII* transgene [44].

Briefly, genomic DNA from Big Blue® mouse embryonic fibroblasts treated *in vitro* with e-cig vapor extracts and control were used to recover the λ LIZ shuttle vectors containing the mutational reporter *cII* transgene. The recovered vectors were then packaged into viable phage particles using the Transpack Packaging Extract kit (Stratagene Corp., Acquired by Agilent Technologies Inc., Santa Clara, CA). After pre-adsorption of the phages to G1250 *E. coli*, the bacterial culture was grown on TB1 agar plates. To select for *cII* mutants, screening plates were incubated at 24°C for 48 hours, whereas titer plates were incubated under non-selective condition, *i.e.*, 37°C overnight, to express both the wild type and mutant *cII*.

2.5. *supF* Mutation analysis

Treatment of the pSP189 plasmid, transfection into human cells, and the *supF* mutagenesis assay were performed according to our published protocol [34]. The pSP189 shuttle vector, containing the *supF* gene as a mutational target [49], and the indicator *E. coli* strain MBM7070, were generous gifts from Dr. Michael Seidman of the National Institute on Aging (NIA), National Institutes of Health (NIH), Baltimore, MD. The mutagenesis assay is based on the ability of the *supF* gene, which encodes a suppressor tRNA, to bypass an amber mutation in the *lacZ* gene of the MBM7070 genome, thereby restoring β -galactosidase activity [30]. MBM7070

transformants with wild-type *supF* bearing-plasmids retain β -galactosidase activity and, in the presence of the inducer of β -galactosidase, isopropyl β -D-thiogalactoside (IPTG), and the chromogenic substrate, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), form blue colonies on an agar lawn. Conversely, colonies resulting from the transformation of bacteria with mutant *supF* bearing-plasmids remain *lacZ*⁻ and appear white on agar plates [27, 28, 30]. The mutation frequency is calculated by scoring the number of white colonies *versus* total number of screened colonies [34, 50].

The *supF* mutation assay was performed as previously described with some modifications [30, 34, 51]. Briefly, the pSP189 plasmid was incubated with V2 e-cig vapor extract, at a concentration of 8 and 80 TPE/ml, respectively, for 5 hours at 37°C in the dark. DMSO only-treated plasmid was used as control. Following phenol/chloroform extraction and ethanol precipitation, the treated plasmids and controls were transfected into cultures of SV-40-transformed human fibroblasts (GM4427) (American Type Culture Collection, Manassas, VA) using the Lipofectamine® 2000 reagents and the manufacturer's protocol (Invitrogen, Carlsbad, CA). After a 72-hour incubation period, plasmid DNA was extracted using the QIAprep® Spin Miniprep kit (QIAGEN, Valencia, CA), and digested with DpnI (New England Biolabs, Ipswich, MA), to remove unreplicated plasmid [27, 30]. The rescued pSP189 progeny was then used to transform CaCl₂-competent MBM7070 bacteria, which carry a mutated *lacZ* gene [27, 28, 30]. The transformed bacteria were diluted in 250 ml SOC medium and plated on LB-agar plates containing 100 μ g/ml ampicillin and spread with a ready to use X-Gal/IPTG solution (Firozeh, Growcells/Molecular Biologicals International, Inc., Irvine, CA). After an overnight incubation at 37°C, wild-type (blue) and mutant (white) colonies were counted to determine the *supF* mutant frequency. As positive control, the pSP189 plasmid was irradiated with ultraviolet C light

(UVC) for 3 seconds using a germicidal lamp according to our published protocol [52]. The UV-irradiated plasmid was processed and analyzed by the *supF* mutation assay similarly to the e-cig treated samples, as described above.

2.6. Statistical Analysis

Results are expressed as means \pm SD. Comparison of all variables between treatment and control groups was done using the Wilcoxon-Mann-Whitney test. All statistical tests were two-sided. Values of $P < 0.05$ were considered statistically significant.

3. RESULTS

3.1. Mutagenicity of e-cig vapor in mouse cells

Big Blue® mouse embryonic fibroblasts carrying the λ LIZ shuttle vector containing the mutational target *cII* transgene [31] were treated with e-cig vapor extracts *in vitro*, and subsequently screened for mutation induction in the *cII* gene. Treatment of cells with e-cig vapor extracts prepared from three popular brands at two different concentrations, yielding low or high cytotoxicity, *i.e.*, $\sim 5\%$ and $\leq 20\%$, respectively, did not elicit a significant mutagenic response (Table 1). The *cII* mutant frequency in e-cig treated cells was only slightly different from that of control. Marginal differences in the *cII* mutant frequency between e-cig-treated cells and controls varied between 22-24%, but in no case did the differences reach a statistically significant level. Conversely, treatment of counterpart cells with two tobacco carcinogens (serving as positive

controls), benzo[*a*]pyrene (B[*a*]P) and 4-aminobiphenyl (4-ABP) [45], resulted in statistically significant increases in the *cII* mutant frequency relative to background ($P < 0.05$). The background mutant frequency of the *cII* transgene was elevated 2.5- and 4-fold in the 4-ABP- and B[*a*]P-treated cells, respectively.

3.2. Mutagenicity of e-cig vapor in human cells

The pSP189 plasmid carrying the mutational target *supF* gene was treated with e-cig vapor extract *in vitro*, and subsequently transfected into human fibroblasts wherein mutagenic effects manifest as increase in relative *supF* mutant frequency [27]. Human cells transfected with the e-cig-treated plasmid did not show a significant mutagenic response (Table 2). The mutant frequency of the *supF* gene in cells transfected with e-cig-treated plasmid was only marginally but not significantly different from that of controls (*i.e.*, cells transfected with solvent-treated plasmid). In contrast, counterpart cells transfected with ultraviolet (UV)-irradiated plasmid [34] (serving as positive control) showed a statistically significant increase in relative *supF* mutant frequency, which was 10-fold over the background ($P < 0.05$).

4. DISCUSSION

Accumulating evidence shows that e-cig solutions—both in liquid form and after being heated into vapor—contain many of the same toxicants and carcinogens as those found in cigarette smoke [8-13]. The presence of carcinogenic compounds in e-cig products is alarming and

deserves further investigation as to whether it may constitute a cancer risk to humans [1]. To investigate the carcinogenic potential of e-cig, we have utilized two validated *in vitro* model systems [26-37] to determine whether e-cig vapor induces mutation in mouse or human cells. Both model systems have been extensively used for mutagenicity testing of various carcinogens, including tobacco smoke and many of its constituent compounds [29, 34, 42, 43, 53-57].

In the present study, *in vitro* exposure to e-cig vapor extracts prepared from three different brands did not cause significant mutagenic effects in transgenic Big Blue® mouse embryonic fibroblasts. To demonstrate the competency of mouse embryonic fibroblasts to metabolize inert carcinogens and convert them to DNA reactive agents capable of inducing mutation, we similarly exposed these cells to two prominent tobacco carcinogens, including B[a]P and 4-ABP, which require metabolic activation to yield promutagenic DNA adducts with distinct mutagenic potencies [45, 46]. In both cases, we detected significant mutagenic effects as reflected by the 2.5- and 4-fold increases, respectively, in relative *cII* mutant frequency in the 4-ABP- and B[a]P-treated cells, respectively. Likewise, e-cig-treated pSP189 plasmid transfected into human fibroblasts did not elicit a significant mutagenic response in the *supF* mutational target gene. The *supF* assay is commonly used to screen for *direct* mutagens, including those present in tobacco smoke, complex mixture of chemicals (*e.g.*, polluted air), or physical agents (*e.g.*, sunlight UV) [29, 30, 34, 43, 56-62]. Unlike e-cig vapor, UV radiation was significantly mutagenic as reflected by the 10-fold increase in relative *supF* mutant frequency in human cells transfected with UV-irradiated plasmid. Altogether, our data indicate that e-cig vapor extracts from the selected brands and at concentrations tested in this study do not significantly induce mutations directly and/or indirectly in mouse chromosomal DNA or directly in cell-free DNA replicating in human cells.

Thorne *et al.* [63] and Misra *et al.* [64] have recently reported that e-cig aerosol is not mutagenic in the Ames assay in bacterial strains TA98 and TA100. The limited mutagenicity of e-cig vapor found in this *in vitro* study as well as in those reports [65, 66] may imply that e-cig is worthy of further consideration as a putatively modified-risk tobacco substitute. The view that e-cig may pose a reduced risk as compared to combustible cigarettes is consistent with the lower concentrations of most toxicants and carcinogens in e-cig vapor than cigarette smoke [8-13]. For example, the average ratios of carcinogenic compounds in e-cig vapor to those in cigarette smoke are 1:9 for formaldehyde, 1:15 for acrolein, 1:120 for toluene, 1:40-380 for tobacco-specific nitrosamines, and 1:450 for acetaldehyde [67-71]. The detected levels of lead and chromium in e-cig vapor are within the ranges known for cigarette smoke; however, nickel concentrations are 2-100 times higher than those in cigarette smoke [38]. Proponents of e-cig interpret the lower content of toxicants and carcinogens in e-cig vapor as compared to cigarette smoke as an indication of harm-reducing potentials. Opponents of e-cig, however, have a different perspective on the comparative levels of carcinogens in e-cig vapor and cigarette smoke. The latter argue that because it is generally accepted that there is no minimum threshold of toxicity for carcinogens, lower levels of carcinogens in e-cig vapor do not equate to no carcinogenic potential. Altogether, the herein findings are more in agreement with the former view that e-cig may putatively have harm-reducing potentials. Nonetheless, we caution against over interpretation of the results, considering the limitations of our study (*see*, below).

In humans, regular vaping results in cumulative exposure to a wide range of e-cig derived toxicants and carcinogens at concentrations that are much higher than those amenable to testing *in vitro*. Thus, short term *in vitro* studies may not fully recapitulate human exposure to toxicants and carcinogens incurred in the course of chronic vaping. This limitation may, at least partially,

explain the non-significant mutagenicity of e-cig vapor observed in the present study. Indeed, the concentrations of vapor extracts tested in our study correspond to much lower doses of e-cig vapor to which human users are exposed in real life. Such inherent shortcoming of the *in vitro* model systems could be addressed *in vivo* by inhalation experiments in appropriate animal models [32, 44]. Furthermore, carcinogenic compounds inhaled during vaping undergo biotransformation in human lungs and other tissues and organs to produce DNA reactive species that may lead to mutagenesis [25, 72, 73]. The metabolic capacity of human cells to activate e-cig derived carcinogens *in vivo* may not be similar to that *in vitro* by various cell types. While we demonstrated the proficiency of mouse embryonic fibroblasts to activate two prototype tobacco smoke carcinogens *in vitro*, we cannot rule out the possibility that this cell type is not as efficient as human cells *in vivo* to metabolize e-cig derived carcinogens.

Currently, there are hundreds of e-cig devices and thousands of e-cig juices whose product features and characteristics are unknown [7]. This makes it difficult, if not impossible, for researchers to know which e-cig product(s) is the most appropriate to use in their studies. In the present study, we have tested 3 brands of cig-a-like products, which were popular at the time of launching this study. Since then, the e-cig technology has been evolving constantly. Advancements in manufacturing technology have led to numerous e-cig devices/models and countless number of e-juices [7]. Variation in device battery output voltages and differences in e-juice content, between brands or even within the same brand, have resulted in e-cig vapor with nonuniform chemical compositions [12, 74]. For instance, newer e-cig models with powerful batteries (*e.g.*, third-generation, mod or Advanced Personal Vaporizer) can heat e-juices to the point of causing chemical reactions, resulting in the release of toxicants and carcinogens that are substantially different, both qualitatively and quantitatively, from those produced by first- or

second-generation devices [12, 69, 70, 74]. Thus, while we appreciate the importance of our findings in 3 select e-cig brands, we cannot generalize our results to all e-cig products. Of note, the recently announced National Institute on Drug Abuse (NIDA) Standard Research E-cigarette (SREC) may provide a unique opportunity for investigators to study a reference e-cig whose product characteristics are well described [75]. The SREC has an accompanying data package, which describes the chemical composition of the e-juice and vapor, the reproducibility of its puff-to-puff output, and the human pharmacokinetics of its nicotine delivery [75]. These data should empower researchers who will use the SREC as a model e-cig in studies to evaluate the value and limitations of e-cig as a tobacco risk reduction tool.

Lastly, although tobacco smoke and many of its constituents are mutagenic in both test systems used in this study [30, 34, 42, 43, 76], the limited mutagenicity of e-cig vapor *in vitro* found in this study should not be considered as an absolute proof of no carcinogenicity. Whereas genotoxicity is a key determinant of carcinogenicity [77], nongenotoxic modes of action, including epigenetic effects, such as aberrant DNA methylation, histone modifications, chromatin remodeling, and micro-RNA derived modulation of gene expression, are alternative or complementary mechanisms of carcinogenesis [78-86]. Future investigations are needed to determine whether e-cig vapor may exert epigenetic effects of relevance to carcinogenesis. It is also important to recognize that e-cig use, in addition to possible carcinogenic effects, may have other disease-causing effects, such as pulmonary or cardiopulmonary effects [87].

5. CONCLUSIONS

We have demonstrated that e-cig vapor extracts from select brands and at concentrations tested in this study have limited mutagenicity in both mouse and human cells *in vitro*. While our results may imply a potential utility for e-cig as a putatively modified-risk tobacco substitute, the limitations of our study underscore the need for future research on the comparative health benefits or risks of e-cig use and cigarette smoking. Follow up studies, preferably in relevant *in vivo* model systems, should leverage standardized e-cig devices with fully described product characteristics, such as the newly developed NIDA reference e-cig [75].

6. ACKNOWLEDGMENTS

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7. CONFLICT OF INTEREST STATEMENT

All the authors declare no conflict of interest.

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TABLE 1. Mutant frequency of the *cII* transgene in Big Blue mouse embryonic fibroblasts treated with e-cig vapor extract and controls

Treatment	Total number of plaques (<i>pfu</i>) [‡]	Mutant plaques	Mutant frequency ($\times 10^3$) [‡]
Control (solvent)	919,259	90	9.71 \pm 2.09
Blue Cigs (x 5 TPE)	661,329	51	7.71 \pm 0.38
Blue Cigs (x 25 TPE)	477,813	55	12.07 \pm 2.08
V2 Cigs (x 5 TPE)	58,188	4	8.74 \pm 5.70
V2 Cigs (x 25 TPE)	520,335	62	12.02 \pm 0.82
NJOY Pro (x 5 TPE)	609,855	54	9.03 \pm 1.21
NJOY Pro (x 25 TPE)	479,492	36	7.59 \pm 1.43
4-aminobiphenyl (4-ABP)	24,618	4	24.45 \pm 1.61 [‡]
Benzo[<i>a</i>]pyrene (B[<i>a</i>]P)	12,869	5	38.36 \pm 2.82 [‡]

Mouse embryonic fibroblasts were treated with 5 and 25 total puff equivalents (TPE) of each of the three e-cig vapor extracts, resulting in ~95% and 80% cell viability, respectively. Counterpart cells were treated in serum free medium in the absence of e-cig extract, and used as negative control. As a positive control, cells were treated with two tobacco carcinogens, benzo[*a*]pyrene (B[*a*]P) and 4-aminobiphenyl (4-ABP) (*see*, text).

[‡]Plaque forming unit; [‡]Results are expressed as Mean \pm SD of independent assays; [‡]Statistically significant as compared to control; $P < 0.05$ by the Wilcoxon-Mann-Whitney test.

TABLE 2. Mutant frequency of the *supF* gene in human fibroblasts transfected with pSP189 plasmid pre-treated with e-cig vapor extract and controls

Treatment	Total number of colonies (<i>cfu</i>)	Mutant colonies	Mutant frequency ($\times 10^{-2}$) [†]
Control (solvent)	4,027	36	0.89 \pm 0.26
V2 Cigs (x 8 TPE/ml)	9,624	63	0.65 \pm 0.27
V2 Cigs (x 80 TPE/ml)	12,015	73	0.61 \pm 0.44
UVC ($\lambda < 280$ nm)	405	36	8.89 \pm 0.16 [‡]

pSP189 plasmid was treated with e-cig vapor extract dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich Inc.), at concentrations of 8 and 80 TPE/ml. DMSO only-treated plasmid was used as negative control. UVC-irradiated plasmid served as positive control.

[†]Colony forming unit; [‡]Results are expressed as Mean \pm SD of independent assays; [§]Statistically significant as compared to control; $P < 0.05$ by the Wilcoxon-Mann-Whitney test.