E-Cigarette Vaping Regulates Genes Involved in DNA Damage and Cancer: A Pilot Study in Humans

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1. Abstract

There is a paucity of data on how human vaping behaviors modifies E-Cigarette (E-cig) exposures, making it difficult to assess the associated risk. This pilot study aimed to investigate the toxicity of e-cigs to humans by investigating changes in DNA damage-response genes in buccal and blood samples. Samples from three subjects (2 Males and 1 Female) were evaluated using RT-qPCR. Each subject made three visits to the lab (nTOT=9 visits); buccal and blood samples were collected before and after scripted vaping 20 puffs (nTOT=18 samples); vaping topography data were collected in each session. Subjects used their own e-cig containing 50:50 Propylene Glycol (PG): Vegetable Glycerine (VG)+(3-6) mg/ml nicotine. After exposure, the tumor suppressor TP53 was significantly
(p<0.05) upregulated in buccal samples which was accompanied by changes in other DNA damage and repair genes. TP53 expression was puff volume and flow rate dependent in both tissues. In blood, the significant downregulation of N-methylpurine DNA glycosylase (MPG), a base excision repair gene, was consistent across all subjects. Ingenuity Pathway Analysis (IPA) showed that DNA repair and cell cycle pathways were the most enriched pathways after e-cig exposure in buccal swab samples. In blood, DNA repair and cancer were the most dominant pathways. This is the first study to demonstrate that vaping 20 puffs significantly alters expression of TP53 along with several other DNA damage genes in human tissues; vaping behavior is an important modifier of this response. A larger study is needed to confirm these relationships.

2. **Keywords:** DNA damage; Gene expression; Electronic cigarettes; Human buccal and blood; Vaping behaviors.

3 **Abbreviations:** ATSDR: Agency for Toxic Substances and Disease Registry; BER: Base Excision Repair; CO: Carbon Monoxide; CPT: Cell Preparation Tube; DEGs: Differentially Expressed Genes; E-cig: Electronic Cigarette; HPHCs: Harmful and Potentially Harmful Constituents; HR: Homologous Recombination; IPA: Ingenuity Pathway Analysis; MMR: Mismatch DNA Repair; NER: Nucleotide Excision Repair; NHEJ: Non-Homologous End Joining; PG: Propylene Glycol; SPA-D: Smoking Puff Analyzer Device; TSNA’s: Tobacco Specific Nitrosamines; qPCR: Quantitative Polymerase Chain Reaction; RT-PCR: Reverse Transcription-Polymerase Chain Reaction; VG: Vegetable glycerin

4. **Background**

The use of E-Cigarettes (E-Cigs) is rising rapidly. In two years (2017-2018), e-cig use increased 77.8% (from 11.7%-20.8%) among high school students and 48.5% (3.3%-4.9%) among middle school students [10]. Currently, all the major cigarette manufacturers are marketing e-cigs [1].

E-cigs may be less toxic compared to other combustible tobacco products (e.g., cigarettes and cigars) [6-7]. However, this assumption is based on the fact that some Harmful and Potentially Harmful Constituents (HPHCs) that are detected in mainstream smoke of combustible tobacco products, are not found in the aerosols of e-cigs [39; 14]. But, other carcinogenic HPHCs, including carbonyl compounds, were measured in e-cig aerosols at levels comparable to those in the mainstream smoke of combustible cigarettes [13].

E-cigarettes deliver chemicals through heating e-liquids, which mainly consist of Vegetable Glycerol (VG) and Propylene Glycol (PG). The thermal decomposition and oxidation of glycerol and propylene glycol were shown to form low molecular weight carbonyl compounds, including formaldehyde and acetaldehyde [21, 19]. The levels of these compounds are highly dependent on the voltage of e-cig device’s and the composition of e-liquids [19,38].
These compounds are able to induce DNA adducts [47, 43, 29]. DNA-DNA crosslinks [25], DNA-Protein Crosslink (DPCs) [31], and DNA-glutathione crosslinks [26]. The formation of these crosslinks increases cell proliferation leading to mutations which contribute to the carcinogenicity of these compounds [18]. Additionally, genes involved in apoptosis, immunity, metabolism, signal transduction, transportation, coagulation and proliferation were found to be up and down regulated after the exposure to these carcinogens [37]. In addition to aldehyde exposure, e-cigs containing nicotine were shown to deliver other carcinogens called Tobacco Specific Nitrosamines (TSNA’s). It is well known that nicotine is transformed to TSNA’s such as Nicotine-derived Nitrosamine Ketone (NNK) and Nitrosonornicotine (NNN) during curing and burning, both are potent human carcinogens [15-16]. The levels of 4-(methylnitrosamoino)-4-(3-pyidyl)-1-butanol (NNAL), an NNK derivative, in the urine and saliva of e-cig users was found to be only 5% of its levels found in comparable tobacco smokers [34]; assuming that nicotine transformation (to TSNA’s) does not occur in e-cig smoke [34]. This led to an assumption that e-cigs are 95% safer than conventional cigarette [12]. However, it is really not known whether the inhaled nicotine can be nitrosated and transformed to TSNA’s; especially with the ample cytochrome p450 enzymes in human cells that can quickly metabolize the TSNA’s into DNA damaging agents [15]. It has been demonstrated that TSNA’s induce DNA damage through DNA adducts formation [15, 22], Alkylphosphotriester formation [5], and induction of p53 and Ras mutations [5, 33].

Both aldehydes and TSNAs are classified by the Agency for Toxic Substances and Disease Registry (ATSDR) as human carcinogens [2]. These carcinogens have been identified as DNA damaging agents, contributing to tumor initiation and development in different tissues [47, 46, 44, 30]. DNA damage is usually corrected by the proofreading function of DNA polymerases that can recognize damaged sites in DNA; however, when a cell accumulates a large amount of DNA damage over a short time, its repair systems get saturated, and replication occurs in cells with unrepaired lesions, leading to the perpetuation of mutations that can lead to cancer [24]. Efforts have been made to understand the gene expression profile after e-cig exposure [27, 35 ,17]. Although informative, these studies either performed exposure assessment based on in vitro assays or did not account for the endogenous levels of the measured genes in vivo. Additionally, DNA damage resulting from toxicant exposure is dose dependent [37]. Therefore, it is important to incorporate measures of vaping behavior in studies of e-cig toxicity and exposure.

This pilot study aimed to examine acute e-cig toxicity through DNA damage and repair gene measurements in human buccal and blood samples as a result of scripted e-cig vaping by established e-cig users. In repeated measures, pilot study, we collected samples before and immediately after vaping so that each subject served as their own control. Thus, any endogenous effects on DNA in buccal and blood samples were eliminated by control. Associations between human vaping behaviors (puffing topography) and gene expression were also determined.

5. Material and Methods
An overview of the experimental study design is shown in Supplementary Figure 1. All samples were collected between May 18th and June 5th, 2017.

5.1. Study Participants

This study was performed at Battelle Memorial Institute/ Tobacco Exposure Research Laboratory (TERL) located in Columbus, Ohio. Advertisements in community and college newspapers were used to recruit participants. Interested participants were telephoned to explain the nature of the study, collect basic demographic data (age, gender, ethnicity) and determine the prospective participant’s eligibility to participate in the study (Supplementary Table 1).

Supplementary Table 2 shows the telephone survey used to screen for eligibility. Briefly, subjects had to be ≥ 18½ years old, non-smokers or ex-smokers that exclusively used e-cig at least 2 months before participation in the study and used e-cig at least 8 times a day. In addition, participants had to be healthy; and were excluded from the study if they had significant medical problems including respiratory allergies, a history of pulmonary disease, or asthma. Female participants that were trying to get pregnant, pregnant or breastfeeding, or not using a reliable birth control method were also excluded (Supplementary Table 2). Written consent was obtained for each subject at the start of each visit.

5.2. Human Exposure

Three e-cig users were recruited for in-laboratory scripted use of their own tank style devices. Participants were asked to complete three separate visits. During each visit, subjects were asked to vape 20 puffs, according to scripted vaping (three seconds puff every 60 seconds, for a total of 20 puffs over 20 minutes). To execute the scripted vaping, participants watched a computer screen that told them when to inhale and when to exhale.

Before vaping, a Vitalograph Breath CO Monitor was used to measure CO levels in exhaled breath, and blood and buccal epithelial samples were collected. Immediately after vaping 20 puffs, exhaled breath CO was measured, and blood and buccal samples were collected. Participant processing dates, e-cig devices, and exhaled CO levels are shown in Supplementary Table 3; blood and buccal samples collected are outlined in Supplementary Table 4.

5.3. Topography Data Collection

The user’s e-cig was connected to a Smoking Puff Analyzer Device (SPA-D, Sodim, FR) which recorded subject-specific topography, including puff volume, puff duration, flow rate, interpuff interval, pressure drop and average resistance during vaping.

5.4. Biologic Sample Collection
For buccal sampling, a cytology brush (Puritan® 2188 Histobrush) Cytology Brushes with Soft Tapered Tip, Nylon Bristles & Polypropylene Shaft) was used to collect buccal samples from the inner-cheek before and after vaping. After buccal sampling, the cytology brush was immediately immersed in a RNA later solution and removed, the sample was vortexed, and kept frozen (at -80°C) until analysis. Participant duplicates were processed the same way of samples (Supplementary Table 4). Method blanks were prepared by immersing the cytology brush in a RNA later solution and processed the same way of the sample. Including duplicates and blanks, from 3 subjects, and three visits, a total of 25 buccal samples were processed (Supplementary Table 4).

Sodium heparin mononuclear Cell Preparation Tubes (CPT) were used to collect blood samples before and after vaping, for gene expression analysis. After sampling, tubes were left for 5 minutes before centrifuging at ~4000 rpm for 15 min at room temperature for White Blood Cells (WBCs/buffy coat) collection. The buffy coat was then aspirated and added to RNA later solution, vortexed, and kept frozen (at -80°C) until analysis. Blanks were prepared using an RNA later tube and processed the same way as the samples. A total of 25 blood and 25 buccal samples, including duplicates and blanks (Supplementary Table 4), were shipped to the Battelle Eastern Science and Technology (BEST) for gene expression analysis.

5.5 Quantitative RT-PCR Analysis of Gene Expression

Details on gene expression analysis are available in the supporting materials and methods. Briefly, fifty biologic (25 buccal epithelial and 25 blood) samples that were stored in RNA Later, were centrifuged at 5000 rpm 4 °C for 10 minutes to separate the cells from the solution. The RNA extraction and purification were performed using the RNeasy Mini Kit. cDNA was synthesized using SABiosciences RT² First Strand Kit, which is then combined with SYBR Green qPCR Mastermix and distributed across the array (SABiosciences/Qiagen, Alameda, CA, USA Cat#: PARN11C). The genes featured in this kit are those associated with the transcriptional targets of DNA damage responses (Supplementary Table 5). Each array contains appropriate housekeeping genes, genomic DNA amplification, reverse transcriptase, and positive PCR controls (see Supporting Materials and Methods). The catalog numbers of the RT-PCR primers used in this study, are presented in Supplementary Table 5. The data is analyzed by an integrated Excel-based template that automatically performs at ΔΔCt-based calculations from the raw threshold cycle (Ct_{cutoff}=35) value to determine gene-specific expression. The Excel template normalizes the Ct values of
samples (collected after e-cig exposure) with the control (samples collected before the exposure to e-cig) values to calculate and report the fold-change of exposed samples compared to control.

### 5.6 Gene Network Analysis and Biomarker Identification

A core analysis including a downstream effects analysis was performed using the Ingenuity Pathway Analysis Propriety Tool (IPA; http://www.ingenuity.com, Redwood City, CA). Downstream analysis is used to identify biological processes along with gene network based on changes in expression of given genes. The calculation of the overlap P-value (p) and the activation Z-score is based upon Fischer’s exact test. We applied the following cut off: -1.5>fold change>1.5, and p<0.05.

### 5.7 Statistical Analysis

Although the number of samples measured for gene expression was relatively small, statistical analysis employed on buccal (n=9) and blood (n=9) samples identified genes that are differentially expressed relative to samples before exposure (controls). This controls for and essentially excludes any endogenous effects from the assessment of e-cig exposure-based gene regulation. The fold-change of the exposed samples compared to controls was used to identify Differentially Expressed Genes (DEGs). To test if a gene is differentially expressed, we performed a one sample t-test of the log2(fold-change) of all samples to determine whether the sample mean is statistically different from zero, which means no change.

For association between gene expression and vaping behaviors (e.g. puff volume, duration, etc.), a two sided Pearson correlation test (cor.test)(method=”pearson”, alternative=”two.sided”) in R) was performed. Any gene with a p-value less than 0.05 is considered to be significantly correlated with the vaping behavior tested.

GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) was used to present the data.

### 6. Results

#### 6.1 Genes Related to Dna Damage are Differentially Regulated in Buccal and Blood Samples After E-Cig Exposure
Eighty-four genes related to DNA damage were analyzed to understand the relationship between e-cig exposure and DNA damage (the names of genes that are included in this study, are presented in Supplementary Table 5). Using the criteria of fold change ≥ +1.5 or <0.66 (1.5-fold change downregulation) in mean gene expression of all subjects in all visits, 61 genes were differentially expressed in buccal samples, and 70 genes were differentially expressed in blood samples (Figure 1A). Of these genes, five were significantly (p<0.05) upregulated in buccal samples including Flap Structure-Specific Endonuclease 1 (FEN1), Apoptosis Inducing Factor Mitochondria Associated 1 (AIFM1), X-Ray Repair Cross Complementing 2 (XRCC2), Three Prime Repair Exonuclease 1(TREX1), and Tumor Suppressor TP53 gene (Figure 1B). In blood samples, only N-Methylpurine DNA Glycosylase (MPG) repair gene was significantly (p<0.05) down regulated (Figure 1B).

The DEGs were also classified using the Ingenuity Pathway Analysis (IPA) to examine the biological pathways and the associated functions that are triggered after e-cig exposure (Figure 2). In both buccal and blood samples, the DNA replication, recombination and repair pathway was the major pathway activated by e-cig exposure (Figures 2A, & B). However, this pathway includes a wide range of sub-pathways, including mismatch DNA repair (MMR), Nucleotide Excision Repair (NER), Base Excision Repair (BER), Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) [4]. The DEGs that are involved in the DNA repair pathway were further visualized by network analysis (Figures 2A, & B). This tool revealed the coordinated up and down regulation of genes associated with DNA damage and repair that occurred in both tissues. For example, the tumor suppressor, TP53 gene was significantly upregulated (Figure 2A) in buccal samples (Figure 1B). In blood samples, TP53 regulation was attenuated (Figure 2B).

Network analysis also showed changes in expression of other genes that are associated with DNA damage and repair after e-cig exposure. The general transcription factor IIH subunit 1 (GTF2H1) along with RAD51 Paralog B (RAD51B), X-Ray Repair Cross Complementing 3 (XRCC3), and Three Prime Repair Endonuclease 1 (TREX1), the dsDNA repair factors, were shown to be up regulated in blood (Figure 2B). On the other hand, N-Methylpurine Dna Glycosylase (MPG), the BER gene, was extremely down regulated (Figure 2B). The same network analysis of buccal samples revealed downregulation of Excision repair-1 Endonuclease Non-Catalytic (ERCC1) gene, mismatch- MutS
Homolog 3 (\textit{MSH3}) repair gene, and Double Strand DNA (dsDNA) break- X-Ray Repair Cross Complementing 6 (\textit{XRCC6}) repair gene (Figure 2A).

Other signaling pathways were also activated by e-cig vaping. In blood, cancer appears to be the second most enriched pathway after DNA replication, recombination and repair (Figure 2B). In buccal epithelium, while cancer pathway appeared among the top 15 triggered pathways after e-cig exposure, cell cycle was the second most enriched pathway after e-cig exposure (Figure 2A). Figure 3 shows that several DEGs contribute to both DNA repair and cancer; 15 genes in buccal and 43 genes in blood samples (Figures 3A-3B). Additionally, the p38 Mitogen-Activated Protein 2 Kinase-6 (\textit{MAP2K6} gene; also known as \textit{MKK6}) cancer gene was differentially expressed in the buccal and blood samples of all subjects after e-cig exposure (Figures 3A-3B). Further, the fold change of \textit{MAP2K6} gene in buccal samples was consistent (positively correlated) with its level in blood samples of each subject (S01:R²=0.963; S02:R²=0.806; and S03:R²=0.649), figures are not presented. Three other cancer genes including Mitogen-Activated Protein Kinase 12 (\textit{MAPK12}), Cell Death Inducing DFFA Like Effector A (\textit{CIDEA}), and Inositol Hexa-kis-Phosphate Kinase 3 (\textit{IP6K3}) were upregulated after e-cig exposure in blood, but not in buccal samples (Figures 3A-3B).

### 6.2. Significant Differential Expression of Genes is Consistent Among Participants

The significantly (p<0.05) and differentially expressed genes in buccal and blood samples of all subjects are presented (by subject) in Figure 4. In buccal tissue, while the upregulation of \textit{FEN1}, \textit{TREX1}, and \textit{XRCC2} was consistent across all subjects, \textit{AIFM1} and the tumor suppressor \textit{TP53} were different across the three subjects (Figure 4). In blood, differential expression of all five genes was consistent between visits, for subjects 1 and 2, but not subject 3 (S03) where greater variation in gene expression was observed (Figure 4). Differential expression of these genes was not significant in blood (Figures 1B and 4).

The \textit{MPG} gene was significantly downregulated in blood (Figure 1B); this was consistent across all subjects (Figure 4); but the differential expression of \textit{MPG} was inconsistent in buccal samples (Figure 4). However, the higher \textit{MPG} expression appeared to be accompanied by the lower \textit{TP53} expression in buccal samples (Figure 4). In order to test whether this correlation was significant, we performed a regression line of \textit{MPG} versus \textit{TP53} in buccal samples.
Figure 5A shows strong negative correlation by subject (mean average of all visits per subject; $R^2=0.880$). However, this correlation was significantly reversed in blood samples where a positive correlation was observed between MPG and TP53 expression (mean average of all visits per subject; $R^2=0.608$) (Figure 5B).

6.3. Gene Regulation is Vaping Behavior Dependent

In order to understand the effect of vaping behaviors on the DEGs, we presented the DEGs that are associated with vaping behaviors by visit (Figure 6). Average puff volume was associated with changes in expression of several genes, especially TP53 in both buccal and blood samples (Figures 6A-6B). It is clear that the greater puff volume and flow rate observed for subject 3 are associated with greater expression of several genes, including TP53 in blood samples (Figure 6B). In addition to puff volume and flow rate, TP53 levels in buccal samples appeared to also be sensitive to pressure-drop and resistance, where the lower pressure-drop and resistance were associated with the higher expression of tumor suppressor TP53 (Figure 6A). Overall, flow rate was associated with expression of a very few genes in buccal epithelium. In blood, changes in flow rate were associated with changes in expression of several genes, including MAP2K6. MAP2K6 showed higher expression in buccal and blood samples when puff volume was larger (Figures 6A-6B). Larger puff volume was also associated with increased differential expression of 8-oxoguanine glycosylase 1 (OGG1; a lung cancer [41]) gene in buccal and blood samples (Figures 6A-6B).

7. Discussion

E-cigarettes are thought to be safer than conventional cigarettes; however, very little has been documented with respect to understanding their toxicity to humans. DNA damage is one of the major concerns of exposure to carcinogens from tobacco products. After exposure to toxicants, cells adapt several pathways to repair DNA damage through the activation of genes involved in these pathways [4]. This pilot study is the first to demonstrate that even short-term e-cigarette exposure impacts expression of several genes associated with DNA damage, DNA repair, cell cycle and cancer. Genes related to DNA damage were differentially (1.5-fold change up or down) regulated in both buccal and blood samples, immediately after vaping 20 puffs using e-cigs with (50:50) PG/VG+(3-6) mg/ml nicotine. In buccal samples, the TP53 tumor suppressor gene was significantly upregulated (Figure 1B). This is important because in normal cells, TP53 is expressed at very low levels. However, upon exposure to
toxicants, TP53 levels increase, leading to cell cycle arrest at the checkpoint until the DNA damage is repaired [45]. It is not surprising that, in parallel with the TP53 upregulation in buccal, TP53-dependent genotoxic stress inducible genes that are involved in regulation of cell cycle, proliferation and differentiation, are upregulated as well (i.e. AIFM1, FEN1 and TREX1) [20,8]. It is well known that TP53 modulates the transcription of a vast number of genes that are involved in cell cycle control, apoptosis, differentiation and DNA repair [28, 42]; however, different genes respond differently to different levels of TP53, based on the type of DNA error occurs after exposure [32]. Our results show that the cell cycle is the second most enriched pathway triggered by e-cig vaping, after DNA damage pathway, in buccal samples (Figure 3A), which is consistent with the upregulation of TP53, a major cell cycle, and tumor suppressor gene [45].

The role of MPG in the BER pathway is to recognize and excise damaged nucleotides; but, under certain conditions, MPG contributes to the formation of sister chromatid exchange and chromosomal aberration [40,9]. Additionally, MPG was found to be upregulated in several types of cancers [11, 23]; it interacts with and inhibits the tumor suppressor TP53 protein and its target genes [36]. Our results demonstrated an inverse association between MPG and TP53 expression in buccal epithelium (Figure 5A; R²=0.880). However, the downregulation of MPG in blood cannot be explained by this model (Figure 5B). Larger (in vitro and in vivo) studies might clarify the correlation/interaction between MPG and TP53 genes and the effect on TP53 downstream targets in different tissues, after e-cig vaping.

MAP2K6, a cancer-related gene, was consistently upregulated in both buccal and blood samples following vaping. Although its expression was not significant in this small study, it might be used as a marker if confirmed by a larger study.

The observed impact of vaping behavior on gene levels is not surprising given that studies in combustible products have demonstrated that smoking behavior impacts exposure [3]. In this study, while puff volume was associated with changes in expression of a large number of genes in both buccal and blood samples, flow rate showed effects on several genes in blood but not in buccal (Figures 6A-6B). The tumor suppressor TP53 seemed to be sensitive to puff volume and flow rate in both tissues (Figures 6A-6B). Our results show that vaping behavior impacts gene expression, thus, e-cigarette toxicity. However, a larger sample size is needed to substantiate the findings observed here and to further our understanding on how different vaping behaviors could cause aberrant gene regulation and impact e-cigarette toxicity.

8. Conclusion

In conclusion, this pilot study shows preliminary evidence that vaping 20 puffs of an e-cigarette is sufficient to cause significant changes in expression of the tumor suppressor TP53, in addition to other cancer-related genes (e.g. MPG) in humans. This study also showed corradiated expression of MPG and TP53 following vaping, and that vaping behaviors impact gene expression changes in relevant target tissues. These preliminary results require confirmation; and a larger study in humans is needed to better understand the impact of e-cig use on these genes and pathways, as well as how vaping behavior modifies e-cig exposure and toxicity.
9. Acknowledgment

Authors would like to thank Quintella Bester, the manager of Individual and Population Health program at Battelle Memorial Institute at Columbus, Ohio, and her staff for their help in human vaping behaviors and biological samples collection.

10. Author Contribution

SH, MM, and CG contributed to the study design, and reviewed subjects to determine the eligibility. YHT and NM analyzed the data. KC, Research Associate and Certified Phlebotomists, collected and processed the biologic samples, as well as the CO and the topography data, when she was working at Battelle. CG, IJ and PC edited the manuscript and provided critical insight into the interpretation of data. When all authors read, revised, and approved the manuscript, SH, PC and CG made the decision to submit the study for publication.

11. Funding

Research reported in this publication was supported by grant number P50CA180523 from the National Cancer Institute and FDA Center for Tobacco Products (CTP) awarded to the University of Maryland. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the Food and Drug Administration.

12. Conflict of Interest

Author KC was employed by Gad Consulting Services. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

13. Data Availability

The original contributions presented in the study are included in the Supplementary Tables 6A, B. Further inquiries can be directed to the corresponding author/s.

14. References


