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# THE EFFECTS OF VAPING ON ORAL STREPTOCOCCI AND ORAL INFLAMMATION

by

MATTHEW CALDWELL

A thesis submitted in partial fulfillment of the requirements  
for the Honors in the Major Program in Biomedical Sciences  
in the College of Medicine  
and in the Burnett Honors College  
at the University of Central Florida  
Orlando, Florida

Spring Term, 2020

Thesis Chair: Dr. Claudia Andl

## ABSTRACT

E-cigarette (e-cig) use is rising, but much is unknown about the effects of its vapor. This vapor contains chemicals such as propylene glycol, a known antimicrobial, and nicotine, whose derivatives are carcinogenic. Here, we study the effects of vaping on resident bacteria of the oral cavity and on oral cell inflammation. Oral streptococci are major residents in the oral cavity, with *S. mutans* the primary cause of dental caries. Growth and biofilm formation have been shown to be enhanced upon exposure to traditional cigarette smoke *in vitro*. In this study, we analyzed the effects of e-cig vapor on growth and biofilm formation in *S. mutans*, *S. sanguinis*, and *S. gordonii*. Organisms and oral epithelial cells were treated using nicotine-free and 3mg nicotine vapor, as well as double-shot menthol freeze flavored 3mg nicotine vapor in a vape chamber designed to phenocopy physiologically relevant exposure. Nicotine-independent inhibition of growth occurred upon exposure in all three bacterial species. Interestingly, biofilm formation was enhanced in the *S. mutans* while decreased in *S. sanguinis* and *S. gordonii*. Epithelial cells showed activation of survival pathways by Western Blot upon exposure to only e-cigarette vapor as well as co-culturing of bacterial and oral epithelial cells. The pioneer colonizers *S. gordonii* and *S. sanguinis* generally antagonize the pathogen *S. mutans*, which can become a predominant member of the community under appropriate conditions, leading to dental caries formation. The observed decrease in the biofilm formation of the commensals *S. sanguinis* and *S. gordonii* upon e-cig vapor exposure indicates the opportunistic colonization of *S. mutans*, whose biofilm-forming abilities increased. Following e-cig usage, dental caries, and cancer, in the oral epithelium may result from this dysbiosis of the microbiome.

## ACKNOWLEDGEMENTS

Thank you Dr. Andl for this opportunity and the mentorship that you provided.

Thank you, Alma, Cheyanne, Gregoire, Cedric, Alex, and Josh for being fantastic mentors and peers, helping me establish myself in the lab.

Thank you Dr. Teter for mentorship and for introducing me to biomedical research through the Program for Undergraduate Research Experience.

Thank you Dr. Altomare for being a member of this committee and helping make this thesis possible.

Thank you Dr. Moore and his lab for providing the means of completing experiments and guidance while doing so.

And finally, thank you to my friends and family who have supported me.

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## INTRODUCTION

In the span of a few years, the use of traditional cigarettes has fallen while the use of e-cigarettes continues to rise.<sup>1</sup> The e-cigarette market has an advantage in the fact that it is variable and evolving, with an increase in teen use being evident.<sup>1, 2</sup> Smoking has been shown to cause cancer, as well as enhance bacterial growth in streptococci species *in vitro*, specifically *Streptococcus mutans* and *Streptococcus sanguinis*.<sup>3</sup> Tar is not found in e-cigarette vapor, but nicotine is, with the amount varying (typically 3mg/mL to 12 mg/mL) between different vape juices. While nicotine itself is not a carcinogen, its nitrosamine products, N-nitrosornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are. In addition to this, the propylene glycol present in vape juice, meant to act as a base for carrying flavor and nicotine, has antimicrobial properties. This leads one to question whether the oral microbiome is affected by the use of e-cigarettes. The most common cariogenic bacteria in the oral cavity streptococci and lactobacilli species.<sup>9</sup> Changes in the composition and diversity of the oral microbiota contribute to disease etiology and progression through the allowance of organisms to spread outside their niche. Because cigarette smoke causes cancer and increases bacterial growth, one must ask if e-cigarette use has a similar effect, and what implication it will have on the partaking youth.<sup>3</sup>

## BACKGROUND AND SIGNIFICANCE

In 2018 alone, it is estimated that 51,140 people will be diagnosed with oral cancer, and 10,030 will die from it.<sup>12</sup> Cigarette use has been shown to be a risk factor for oral cancers, and while the amount of cigarette users is declining, a new trend is rising: e-cigarettes.<sup>2, 11</sup> Originally introduced as a cessation tool to stop smoking, e-cigarettes have become popular with youth, yet little is still known about the effects of e-cigarette usage, and what is known is disputed.<sup>45</sup> Some studies suggest that e-cigarette use is safe for oral cells, while other studies raise the possibility that consumption of the vapor induces a toxicological effect.<sup>13, 14</sup> This same study that suggests the toxicological response also breaks down what is in vape juice commonly consumed by users. Besides the well-known substances that are present, such as propylene glycol (PG), vegetable glycerin (VG), and nicotine, many compounds were found in the flavoring.<sup>14</sup> Another study that analyzed the flavoring chemicals in multiple vape juices found different chemicals from the previously mentioned study.<sup>15</sup> (**Table 1**)

**Table 1:** Compounds Found Within E-cigarette Vapor

Universal Compounds	Flavoring Compounds (Leigh, N.J., 2016)	Flavoring Compounds (Canistro, D., 2017)
Propylene Glycol	3-Methylcyclopentane-1,2-dione	Acetol
Vegetable Glycerin	(E)-beta-Damascone	3-Hexen-1-ol
Nicotine	Allyl-cyclohexylpropanoate	Diacetyl
	Methyl 3-hydroxyhexanoate	Methyl propionate
	Menthol	
	Limonene	
	Carvone	
	Coccol	
	Benzyl alcohol	
	gamma-Decalactone	
	Methyl cinnamate	
	1,2-Propanediamine	

*Adapted from Leigh, N.J. Flavorings significantly affect inhalation toxicity of aerosol generated from electronic nicotine delivery systems (ENDS) and Canistro, D. E-cigarettes induce toxicological effects that can raise the cancer risk*

Tobacco users seeking help in quitting their usage of cigarettes have been turning to e-cigarettes as an alternative, without knowing exactly how these new products affect them. Studies have shown that PG and VG mixtures are relatively benign and have limited effects, but once combusted through an e-cigarette and nicotine is added, this changes.<sup>16, 17</sup> As seen in **Table 1**, the chemicals found in flavoring can vary greatly, but PG, VG, and nicotine were constants throughout. These are the compounds that should be explored more thoroughly, as every e-cigarette user consumes PG and VG, and those who opt for nicotine in their mixture will consume it as well. Not knowing much about the short and long-term effects of e-cigarette use means that the future is uncertain for all the young users who have taken up the habit. The research that is being proposed will shed light on this and give a clearer idea of exactly how this generation of users will be impacted.

It is well known that an imbalance in the oral microbiome can cause disease both in the oral cavity, as well as elsewhere in the body. For instance, *Streptococcus pyogenes* causes strep throat but can also cause rheumatic fever, which can damage the heart, as well as other tissues. Cariogenic bacteria are bacteria that cause dental caries, or cavities, in the mouth. Dental caries created by these bacteria can lead to gingivitis, which is gum inflammation, and periodontitis, which is chronic gum inflammation and loss of gum tissue, bone, and teeth. Periodontitis is associated with an increased risk for oral cancer, meaning the presence of the disease is a risk factor.<sup>24</sup> Often, cariogenic bacteria have virulence factors: the ability to form biofilms, acid tolerance, synthesize water-insoluble glucan from sucrose, which aids in adherence, and acidogenicity, which is the ability to form acid.<sup>18, 19</sup> Coincidentally, the most common sugar found within e-cigarette liquid is sucrose.<sup>33</sup> The most common cariogenic bacteria are

streptococci and lactobacilli, with *Streptococcus mutans* being the most prevalent of all.

*Streptococcus mutans* is a Gram-positive cocci that is present in the oral cavity.<sup>9</sup>

While not currently being directly linked to oral cancers, the presence of dental caries and the bacteria that cause them are indicative of poor hygiene and lead to diseases that are associated with oral cancer.<sup>22, 24</sup> Despite this, some studies suggest that these cariogenic bacteria are beneficial to the host, while other studies indicate that there is an association between the increased presence of these bacteria and oral cancers.<sup>4, 22</sup> If bacterial growth is impacted in the presence of vapor, an associated change in the microbiome and subsequent disease may occur. This directly impacts the demographic of individuals who vape in another way besides just the oral epithelium. Our study aims to provide evidence regarding interactions between *Streptococcus mutans*, *Streptococcus sanguinis*, and *Streptococcus gordonii* and e-cig vapor individually.

While *S. mutans* is the primary culprit of dental caries, *S. sanguinis* is associated with positive oral health<sup>25</sup>. *S. mutans* and *S. sanguinis* engage in constant warfare within dental plaques, with each secreting substances to inhibit the growth of the other. *S. sanguinis* secretes hydrogen peroxide to prevent the colonization of *S. mutans*, and *S. mutans* secretes mutacins I and IV to antagonize *S. sanguinis*.<sup>26</sup> Additionally, *S. gordonii* antagonizes *S. mutans* using H<sub>2</sub>O<sub>2</sub> and antagonizes *S. sanguinis* through preventing early colonization.<sup>27</sup> Although H<sub>2</sub>O<sub>2</sub> is a reactive oxygen species and harmful to most bacteria, including its creator *S. sanguinis*, *S. sanguinis* has Dps and TrxB to protect itself, while *S. gordonii* has SodA<sup>28</sup>. *S. mutans* form of defense against H<sub>2</sub>O<sub>2</sub> is Dpr, which production is increased when in the presence of *S. sanguinis* and *S. gordonii*, and allows it to survive colonization alongside them.<sup>29</sup>

An imbalance between these organisms resulting from e-cigarette vapor could skew the makeup of dental plaques, allowing one organism to reign supreme. Previous studies have shown that whichever organism has the advantage of colonizing first will be able to outcompete the others *in vivo*.<sup>26</sup> *In vivo*, this could mean an increased risk of caries and dysbiosis if *S. mutans* outcompetes the others in biofilm formation and colonization. The oral microbiome is extraordinarily heterogeneous and composed of many species of bacteria, so any disruption to the balance may lead to oral and systemic disease within the body.<sup>46</sup>

This study analyzes how oral streptococci growth and biofilm formation are altered upon exposure to e-cigarette vapor with and without nicotine, and with or without flavoring. Additionally, we examine how this same exposure affects oral epithelial cell stress and inflammatory pathways. Finally, oral epithelial cells and *S. mutans* were co-cultured together at a and exposed to the same treatment to determine the induction of stress and apoptotic pathways.

## MATERIALS AND METHODS

### Cell Culture

#### *OKF6 Oral Epithelial Cells*

Keratinocyte Serum-Free Medium (KSFM) containing 1 ng/ml Epidermal Growth Factor (EGF), 0.05 mg/ml Bovine Pituitary Extract (BPE), and 1% penicillin streptomycin antibiotics (Gibco™, for Life Technologies, Inc., Carlsbad, CA) were used to culture OKF6 human oral epithelial cells. Cells were incubated at 5.0% CO<sub>2</sub> at 37 degrees Celsius.

#### *Streptococcus mutans and Streptococcus gordonii*

*Streptococcus mutans* (ATCC® 25175™) and *Streptococcus gordonii* (ATCC® 51656™) were grown in the same culture conditions. Bacteria were grown overnight in 10 ml tryptic soy broth TSB at 37 degrees Celsius. It was then diluted and plated on Trypticase Soy Agar with 5% sheep blood (TSAII), BD 221261 and grown overnight. One optimal colony was then selected and grown overnight in 10 ml TSB at 37 degrees Celsius. The following day, this growth was resuspended and allocated into 1.5 ml tubes in amounts of 600 microliters, referred to as snaps. The tubes were then placed in liquid nitrogen for 24 hours, removed, and then stored at -80 degrees Celsius.

#### *Streptococcus sanguinis*

*Streptococcus sanguinis* (ATCC® 10556™) was grown overnight in 10 ml TSB in 5.0% CO<sub>2</sub> at 37 degrees Celsius. It was then diluted and plated on Trypticase Soy Agar with 5% sheep blood (TSAII), BD 221261 and grown overnight. One optimal colony was then selected and grown

overnight in 10 ml TSB in 5.0% CO<sub>2</sub> at 37 degrees Celsius. The following day, this growth was resuspended and allocated into 600 microliters snaps. The tubes were then placed in (insert liquid nitrogen specs) for 24 hours, removed, and then stored at -80 degrees Celsius in (insert freezer specs).

#### *Determining Bacterial Concentration in Snap*

Bacteria were plated on Trypticase Soy Agar with 5% sheep blood (TSAII), BD 221261 plates after being diluted from 600 microliter stocks to 10<sup>-4</sup>, 10<sup>-5</sup>, and 10<sup>-6</sup>. Plates were then incubated for 24 hours in proper conditions for the organism and colonies on each plate were counted, multiplied by their dilution factor, and then averaged to determine bacterial concentration of the snap.

#### *Co-Culturing OKF6 Epithelial Cells and Streptococcus mutans*

700,000 OKF6 epithelial cells were plated in 6 well plates containing 2 mL of KSFM with EGF, BPE, and antibiotics. After growing overnight, they were washed twice with 1 ml PBS and placed in 1 ml KSFM without penicillin streptomycin antibiotics. Following this, *Streptococcus mutans* was then introduced at a multiplicity of infection (MOI) of 1, making a ratio of one OKF6 cell per bacterial cell. Co-cultures were then grown for five hours and proteins were harvested using IP Lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton x-100, 1% NP-40) supplemented with cOmplete™ EDTA-free protease Inhibitor Cocktail tablet.

## Exposure to E-Cigarette Vapor

### *Puff Delivery and Duration*

A vape chamber was created using a Intex Quick-Fill AC Electric Air Pump, tubing, Komax Biokips Extra Large Food Storage Container, and a SMOK® G-PRIV Baby e-cigarette. The e-cigarette juice selected is a 70:30 mix of vegetable glycerin to propylene glycol as the base purchased from VAPORFI®. The nicotine free condition contains only the base. The 3mg nicotine condition contains 3mg/ml added to the base. The flavoring condition contains triple-shot Menthol Freeze with 3mg/ml nicotine or Frutti Tutti with 6mg/ml nicotine as specified in the figure.

Puffs lasted for 10 seconds, as measured by the vape's feature to monitor puff length, at a wattage of 60.3 watts, a resistance of 0.42 ohms, a voltage of 5.01 volts, and a current of 11.8 amps. For the air control conditions, only the pump was run for 10 seconds with no vapor. Following the 10 second puff, cells were incubated within the vape chamber for 5 minutes, and then returned to proper incubation conditions within an incubator.

### *Bacterial Exposure to E-cigarette Vapor*

Bacteria were grown overnight in 10 mL of TSB in proper conditions for the organism. The following day, bacteria were diluted 1:10 in new TSB and 2ml of the dilution was placed into 100 mm plates for each condition. Plates were then exposed as specified in *Puff Delivery and Duration*. Following exposure, a 96 well plate was inoculated with 100 microliters of treated bacteria per well and incubated in proper conditions.



#### *Oral Epithelial Cell Exposure to E-cigarette Vapor*

Cells were counted and plated at a number of 500,000 cells per well in 100 mm plates and allowed to reach confluency overnight. Media was then removed, and cells were placed in 1 mL of KSFM and treated as specified in *Puff Delivery and Duration*. Cells were then washed with PBS and incubated for either 30 minutes or 24 hours.

#### *Co-Culture Exposure to E-cigarette Vapor*

Oral epithelial cells were plated at a number of 700,000 cells per well in a 6 well plate and allowed to reach confluency overnight. Media was removed and cells were washed with PBS and 2 mL of antibiotic free KSFM was added. *S. mutans* was then added at a MOI of 1 to wells requiring organism. Wells were then treated as specified in *Puff Delivery and Duration*, with wells not requiring treatment being wrapped in parafilm. Co-cultures were then incubated for 5 hours before protein was harvested.

## Analysis

### *Biofilm Analysis*

Following a 24 hour incubation after treatment, plates were taken out of the incubator, washed 3 times, and placed back in the incubator to dry for 20 minutes. After the plate was dried, 100 microliters of safranin were added to each well containing organism and allowed to stain the biofilm for 20 minutes. Plates were then washed 3 times and stored in the incubator for 20 minutes to dry. Finally, biofilms were resuspended in 100 microliters of an 80:20 ethanol:acetone mix and their absorbance measured at an OD of 490nm. Results were graphed and analyzed via Graphpad Prism using t-tests.

### *S. mutans and S. gordonii Growth Analysis*

Immediately after treatment, 100 microliters of bacteria per well were plated in a 96 well plate and placed in a Biotek® Synergy Plate Reader. Readings were done every 5 minutes at 600 OD for 24 hours while incubated at 37° C. Data was graphed and analyzed via Graphpad Prism using multiple t-tests.

### *S. sanguinis Growth Analysis*

Immediately after treatment, 100 microliters of bacteria per well were plated in a 96 well plate, covered in 50 microliters of mineral oil, and placed in a Biotek® Synergy Plate Reader. Readings were done every 5 minutes at 600 OD for 24 hours while incubated at 37° C. Data was graphed and analyzed via Graphpad Prism using multiple t-tests.

### *Trypan Blue Assay*

200,000 OKF6 oral epithelial cells per well were plated in a 6 well plate and grown overnight. Cells were then washed with PBS and 1 mL of antibiotic free KSFM was added. An MOI of 1 or 5 of *S. mutans* was added to the wells, while a control well was kept. Cells were then incubated, with wells being harvested at the 3 hour, 5 hour, and 7 hour time points. To be harvested, cells were washed with PBS and 500 microliters of trypsin was added for 5 minutes. 1 mL of soybean trypsin inhibitor (STI) was then added to neutralize the trypsin, and cells were centrifuged for 5 minutes. STI was then removed and 100 microliters of PBS was added. Cells suspended in PBS were then mixed with trypan blue in a 1:1 ratio and counted, using 4 different fields that were then averaged together.

### *Western Blot*

Proteins were analyzed via SDS PAGE followed by Western Blotting. Protein concentration was quantified utilizing a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA), then separated using an acrylamide gel via SDS PAGE. Gels were then transferred to a PVDF membrane, and primary and secondary antibodies were used to visualize the membranes. The proteins analyzed are presented in **table 2**.

Table 2: Proteins Probed For During Western Blotting

Proteins	Function	Time Analyzed
p-Erk 1/2	Stress Response	30 minutes/5 Hours
t-Erk 1/2	Stress Response	30 minutes/5 Hours
COX-2	Inflammation	24 hours
$\alpha$ -tubulin	Loading Control	Always

## RESULTS

### Development of an Optimal Vape Chamber

Through vaping, e-cigarette liquid is heated to approximately 300 °C before inhalation, which may induce chemical transformations that could alter its toxicity. To deliver the e-cigarette vapor with and without nicotine and flavoring, we modified a vape chamber for the specific delivery of vapor to epithelial cells and bacteria, mimicking human inhalation of vaporized e-cig juice.

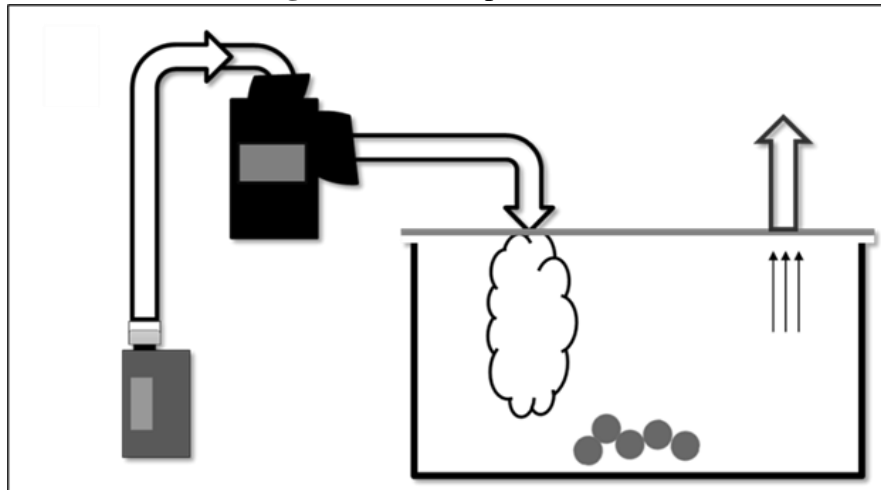
The initial vape chamber designed and generously donated by the Moore lab utilized Arduino software to trigger a coil to ignite and utilize capillary action to suck vape juice to be ignited. Two shortcomings of this design were the lack of airflow necessary to mimic inhalation and the possibility of burned metals from the coil being introduced into the vapor.

To improve on the existing design, an air pump was used to directly suck air through the vape and expel it into a chamber covered by an acrylic lid. However, in this iteration, clean air did not have a direct means of escape, resulting in air and vapor being blown out from the sides of the lid. This led an unspecified amount of air escaping. While functional, the design could still be improved.

The final version is the one that utilizes an Intex Quick-Fill AC Electric Air Pump, 110-120 Volt, Max. Air Flow 21.2CFM and a SMOK® G-PRIV Baby e-cigarette. Liquid is combusted at 60.3 watts, with a resistance of 0.42 ohms, a voltage of 5.01 volts, and a current of 11.8 amps. The chamber design allows air not containing vapor to leave through a pipe that may be closed once the duration of the puff has concluded. This prevents air escaping unnecessarily. Additionally, air no longer escapes from the sides of the lid, allowing the denser vapor to sit within the chamber.

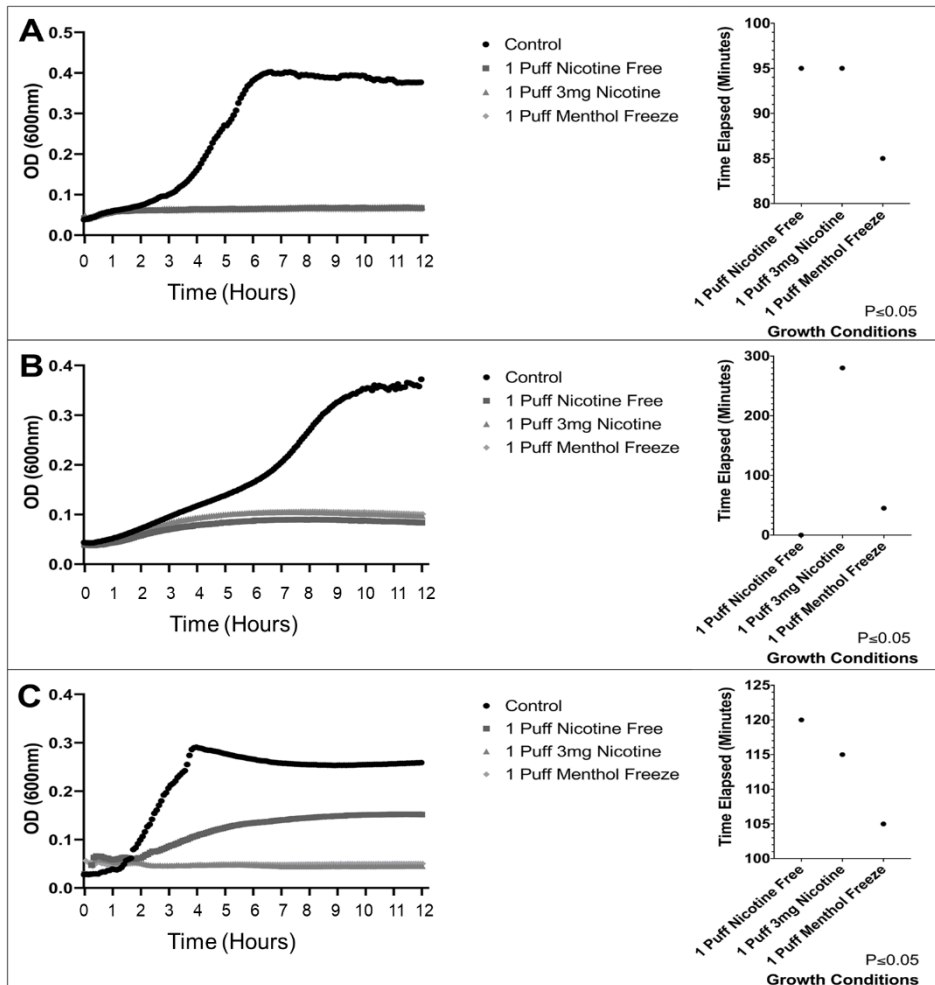
This iteration allows the air in the chamber to be recycled once per second that the air pump is running. In conclusion, this design allows the chamber to closely mimic physiological conditions. **(Figure 1)**

**Figure 1: The Vape Chamber**



## Oral Streptococci Growth is Significantly Decreased Upon Exposure to E-cigarette Vapor

To determine the effects of e-cigarette vapor on the growth of select bacterial residents of the oral cavity, *S. mutans*, *S. gordonii*, and *S. sanguinis* were all treated individually with nicotine free, 3mg/ml nicotine, and flavored e-cigarette vapor, as specified in *Puff Delivery and Duration*. Bacteria were grown for 24 hours *in vitro* following exposure and had OD measured at 600nm for the duration of the growth, with only the first 12 hours shown in **figure 2**. Readings were performed every 5 minutes, with each condition for each organism containing four replicates



**Figure 2:** Growth of oral streptococci following exposure to e-cigarette vapor. *S. mutans* (A), *S. gordonii* (B), and *S. sanguinis* (C) all experience decreased growth following exposure to nicotine free, 3mg/mL nicotine, and flavored e-cigarette vapor.

averaged together to provide the curves in **figure 2**. Only the first 12 hours are shown in **figure 2** due to the organism entering stationary phase for the rest of the growth study.

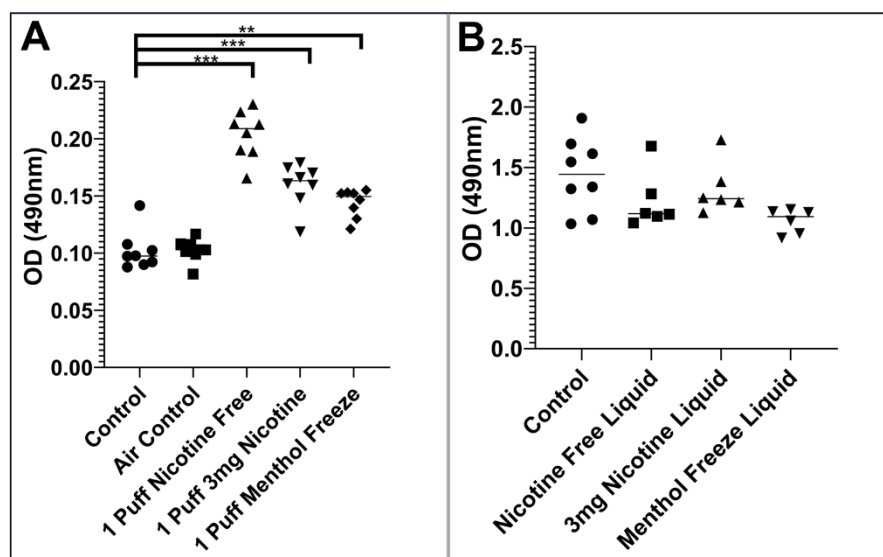
Following a multiple t-test comparing each condition including vapor to the control, the timepoint that a growth began to significantly differ from the control for the rest of the study was identified, indicating a change due to exposure to e-cigarette vapor. P-values steadily decreased for the duration of the growth, with the time elapsed shown in **figure 2** being the first 5 minute interval at which a p-value of less than 0.05 occurred.

All conditions in which *S. mutans*, *S. sanguinis*, and *S. gordonii* were exposed to any form of e-cigarette vapor resulted in significantly decreased growth. E-cigarette vapor therefore has an inhibitory role in the growth of oral streptococci *in vitro*, but it cannot be concluded whether it is bacteriostatic or bacteriocidal here. *S. sanguinis*, unlike *S. mutans* and *S. gordonii*, requires 5% CO<sub>2</sub> to grow in healthy conditions as specified by the ATCC. This posed a unique challenge as the plate reader used for these growth studies did not support CO<sub>2</sub> injection. The solution was to cover *S. sanguinis* wells in mineral oil to simulate the necessary conditions and allow it to grow outside of the incubator.

### Oral Pathogen *S. mutans* Biofilm Formation is Significantly Enhanced Following Exposure to E-cigarette Vapor, Not E-cigarette Liquid

Human oral bacteria interact with their environment by attaching to surfaces such as tooth enamel. As each bacterial cell attaches, it forms a new surface to which other cells can adhere ultimately forming a biofilm that retains the bacteria in the oral cavity through binding to the tooth allowing colonization. This is the preferred method of colonization for species such as *S. mutans*, who may undergo both sucrose-dependent and independent forms of attachment to initiate biofilm formation.

To determine the effects of e-cigarette vapor on the ability to form biofilms, *S. mutans* grown statically overnight then exposed to nicotine free, 3mg/ml nicotine, and menthol freeze e-cigarette vapors as specified in *Puff Delivery and Duration*. Additionally, *S. mutans* was exposed to nicotine free, 3mg/ml nicotine, and flavored e-cigarette liquid. Bacteria was then allowed to grow overnight. The following day, biofilms were stained with safranin and the OD was measured at 490 nm (**figure 3**).



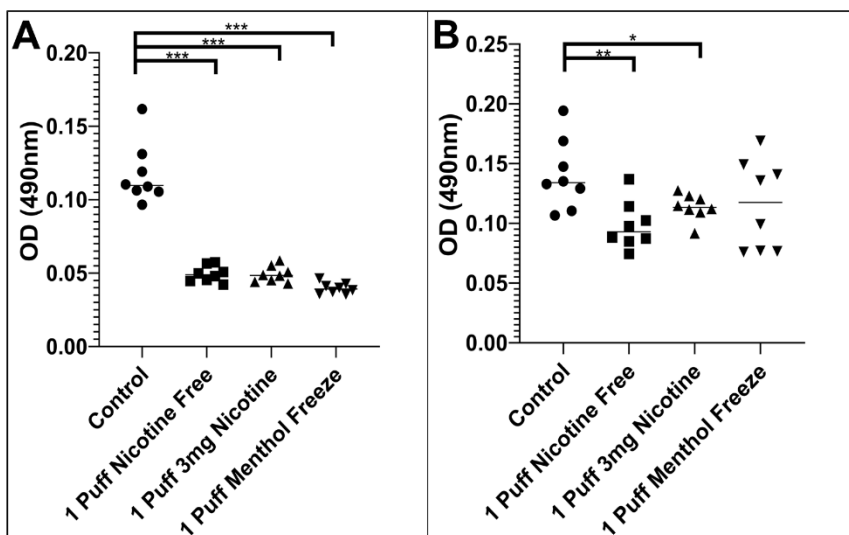
**Figure 3:** *S. mutans* biofilm formation following exposure to e-cigarette vapor and liquid. **A:** *S. mutans* biofilm formation is significantly enhanced in all conditions including e-cigarette vapor when compared to the control. **B:** There is no significant difference between the control and conditions including e-cigarette liquid when compared to the control.



Exposure to e-cigarette vapor, regardless of the presence of nicotine or flavor, resulted in a statistically significant increase in biofilm formation (**figure 3A**). The effect was dependent on a chemical transformation induced by a high temperature combustion of the e-cigarette liquid components into vapor. This is determined by the observation that enhanced biofilm formation was only shown in the vaping conditions, not upon the addition of e-cigarette liquid to the media (**figure 3B**). Each condition contained 8 replicates, with outliers identified and removed via the ROUT method. Each test has a duplicate. Statistical significance was determined utilizing a non-parametric t-test.

### Oral Commensals *S. sanguinis* and *S. gordonii* Experience Significantly Decreased Biofilm Formation Following Exposure to E-cigarette Vapor

To identify if e-cigarette vapor has the same effect on commensal biofilm formation as it does on *S. mutans*, an oral pathogen, commensals *S. sanguinis* and *S. gordonii* were grown overnight in ATCC specified conditions, then exposed individually to nicotine free, 3mg/ml nicotine, and menthol freeze e-cigarette vapors as specified in *Puff Delivery and Duration*. Bacteria were then allowed to grow overnight, and biofilms were stained with safranin and the OD was measured at 490nm (**figure 4**).



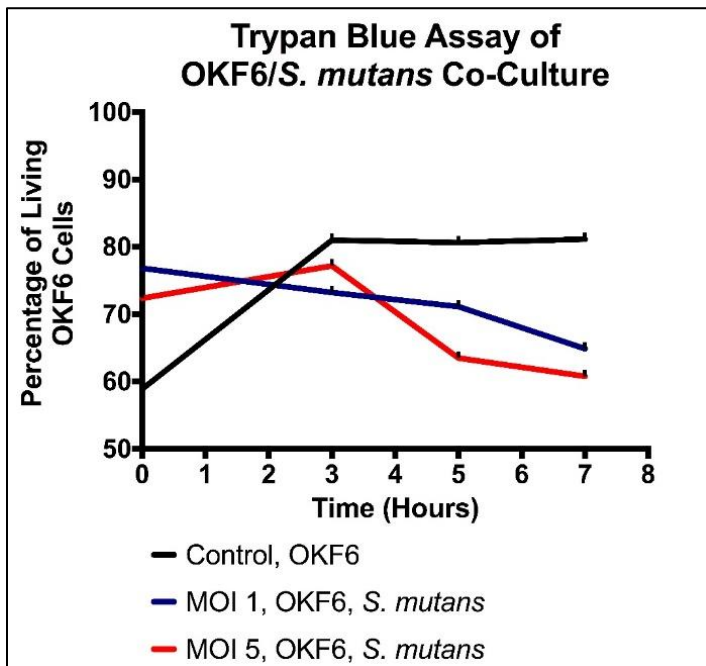
**Figure 4:** *S. sanguinis* and *S. gordonii* biofilm formation following exposure to e-cigarette vapor. **A:** *S. sanguinis* biofilm formation is significantly decreased following exposure to nicotine free, 3mg/ml nicotine, and flavored e-cigarette vapor. **B:** *S. gordonii* biofilm formation is significantly decreased following exposure to nicotine free and 3mg/ml nicotine e-cigarette vapor.

Both commensals *S. sanguinis* and *S. gordonii* showed a distinct difference in biofilm formation compared to the pathogen *S. mutans* in the presence of e-cig vapor. For both bacterial strains, we observed decreased biofilm formation, indicating that *S. mutans* could have an advantage in colonizing the oral cavity of an e-cigarette user. Factors that may contribute to this include the decrease in the innate ability to attach in *S. sanguinis* and *S. gordonii* compared to *S. mutans*, due to the preference for sucrose-independent attachment within each. Each condition contained 8

replicates, with outliers identified and removed via the ROUT method. *S. sanguinis* was done in triplicate, while *S. gordonii* was done in duplicate. Statistical significance was determined utilizing a non-parametric t-test.

### A Multiplicity of Infection of 1 is Optimal for Co-Cultures Between *S. mutans* and OKF6 Oral Epithelial Cells

The purpose of infecting oral epithelial cells (OKF6) at different multiplicity of infection was to determine a viable ratio of bacterial to epithelial cells for pro-longed co-culturing. OKF6 oral epithelial cells were exposed to an MOI of 1 and MOI of 5 of *S. mutans* over the course of 7 hours. At the 0, 3, 5 and 7 hour timepoints, cells were harvested and exposed to trypan blue to determine cell death (**figure 5**). 5 hours was determined as the point at which an MOI of 1 and an MOI of 5 resulted in differences in terms of OKF6 viability. While non-infected control OKF6 cells continued upon exponential growth to enter the stationary/stagnant phase, MOI 1 and 5 reduced epithelial cell viability over time, especially after the 5 hour time point. This experiment was performed in duplicate.

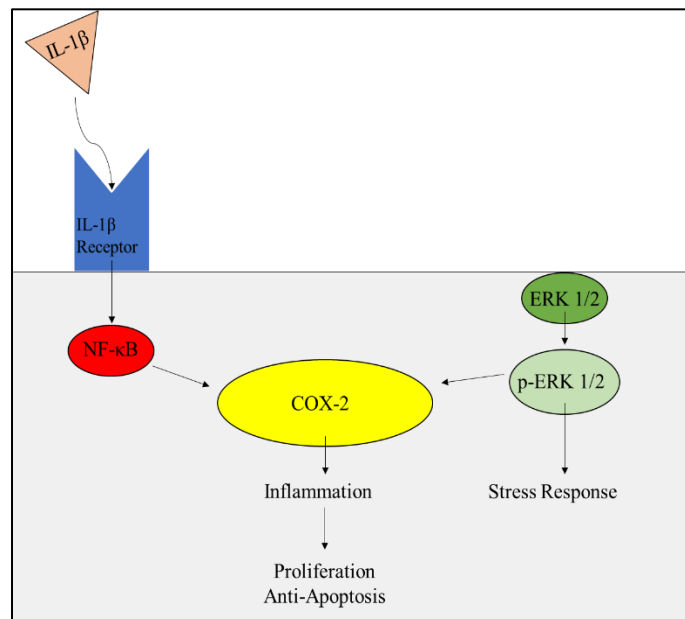


**Figure 5:** Oral epithelial cell trypan blue assay. At 5 hours, there is greater cell death when epithelial cells are cultured with an MOI of 5 of *S. mutans* than an MOI of 1, allowing this time point and multiplicity of infection to be chosen. The starting percentages differ because of the inherent drawbacks of cell culture, which is placing the same number of cells in exactly the same conditions.

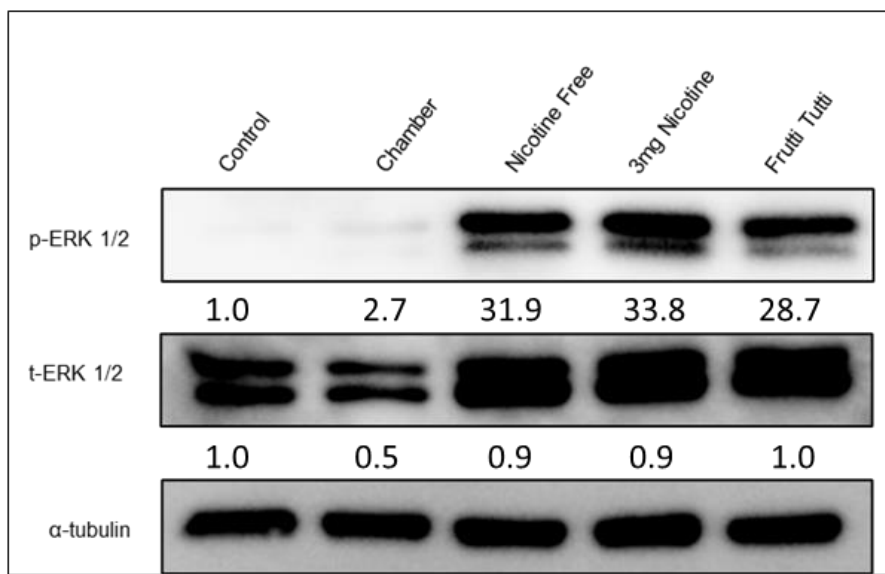
## Oral Epithelial Cells Experience a Stress Response 30 Minutes Following Exposure to E-cigarette Vapor

The ERK (extracellular-signal-regulated kinase) signaling pathway controls several cellular processes such as cell growth, proliferation, differentiation and apoptosis. The starting point of the ERK cascade is binding of a ligand (a growth factor or cytokine), known as a mitogen, to a tyrosine kinase receptor mediating downstream phosphorylation events that lead to activation of the pathway. Multiple kinases including mitogen-activated protein kinase kinase (MAPKK) and others will phosphorylate subsequent kinases in the cascade ending with the final enzyme, a mitogen-activated protein kinase called ERK in this event. Activation of ERK is measured by its phosphorylation upon which it is translocated to the nucleus where it activates transcription. A simplified version of this pathway is depicted in **figure 6**. Also depicted is the production of downstream product COX-2, which promotes inflammation, proliferation, and prevents apoptosis.

**Figure 6: COX-2 Signaling Pathway**



The observation of p-ERK 1/2 indicates a stress response within epithelial cells following a stimulus, in this case e-cigarette vapor. At 30 minutes following exposure to nicotine free, 3mg/ml nicotine, and flavored e-cigarette vapor, OKF6 oral cells protein was harvested. Western Blotting was used to compare expression and activation of p-ERK 1/2 and t-ERK 1/2 , using  $\alpha$ -tubulin as a loading control to ensure equal loading of wells. (**Figure 7**)



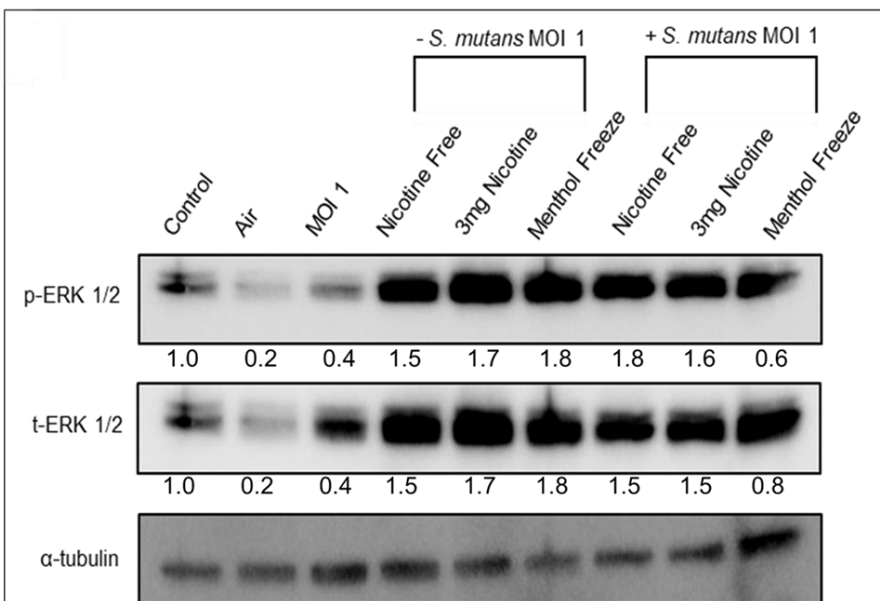
**Figure 7:** Oral epithelial cell stress response 30 minutes after exposure to e-cigarette vapor. OKF6 oral epithelial cells experience an average 31.46 fold increase in p-ERK 1/2 30 minutes after exposure to nicotine free, 3mg/ml nicotine, and flavored e-cigarette vapor.

OKF6 oral epithelial cells experience an average 31.46 fold increase in p-ERK 1/2 30 minutes after exposure to nicotine free, 3mg/ml nicotine, and flavored e-cigarette vapor. This indicates that e-cigarette vapor, regardless of nicotine or flavoring, promotes an immediate stress response within oral epithelial cells. Samples were harvested and stored at -80° C prior to protein concentrations being determined and SDS PAGE. ImageJ was used to standardize band intensity to the loading control for comparison. Western Blots were performed in triplicate.

### Oral Epithelial Cells Experience a Stress Response at 5 Hours Following Exposure to E-cigarette, However Co-Culturing Does Not Alter Stress Response

At 5 hours, ERK 1/2 phosphorylation in OKF6 cells was measured following exposure to nicotine free, 3mg/ml nicotine, and flavored e-cigarette vapor to determine whether a prolonged stress response was present. Conditions include a control, cells exposed to a 10 second puff of air with no vapor, an MOI of 1 of *S. mutans*, cells exposed to the various e-cigarette vapors, and cells exposed to the various e-cigarette vapors along with an MOI of 1 of *S. mutans*. Western Blotting was used to compare expression and activation of p-ERK 1/2 and t-ERK 1/2, using  $\alpha$ -tubulin as a loading control to ensure equal loading of wells.

**(Figure 8)**



**Figure 8:** Oral epithelial cell stress response 5 hours after exposure to e-cigarette vapor and co-culturing. OKF6 oral epithelial cells experience an increase in p-ERK 1 and 2 5 hours after exposure to nicotine free, 3mg/ml nicotine, and flavored e-cigarette vapor. Co-culturing with *S. mutans* has no compounding effect on whether a stress response occurs in response to e-cigarette vapor.

Cells experienced a stress response in the presence of e-cigarette vapor regardless of the presence of *S. mutans*. The addition of *S. mutans* provided no compounded effect to the stress response in epithelial cells exposed to e-cigarette vapor, and the presence of *S. mutans* alone did not promote

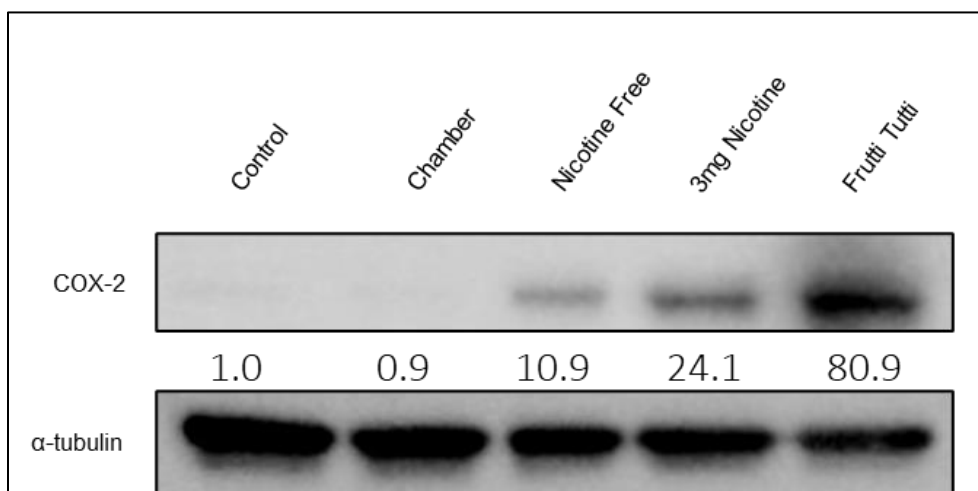
a stress response. Heat killed *S. mutans* did not trigger a stress response as well. Samples were harvested and stored at -80° C prior to protein concentrations being determined and SDS PAGE. ImageJ was used to standardize band intensity to the loading control for comparison. Western Blots were performed in triplicate.



### Oral Epithelial Cells Experience an Inflammatory Response 24 Hours Following Exposure to E-cigarette Vapor

MAPK signaling has been shown to regulate the expression of a prominent mediator of inflammation, COX-2. It has been shown that aerosols in vaping products alter the host response, prompting gum inflammation and making epithelial cells in the mouth susceptible to infection.<sup>47</sup> This provided a rational to assess if COX-2 is activated as a potential downstream target of ERK in the context of e-cig vapor-mediated inflammation.

At 24 hours following exposure to nicotine free, 3mg/ml nicotine, and flavored e-cigarette vapor, OKF6 oral cells protein was harvested. Western Blotting was used to compare expression of COX-2, using  $\alpha$ -tubulin as a loading control to ensure equal loading of wells. **(Figure 9)**



**Figure 9:** Oral epithelial cell inflammatory response 24 hours after exposure to e-cigarette vapor. OKF6 oral epithelial cells experience increased expression of the inflammatory protein COX-2 24 hours after exposure to nicotine free, 3mg/ml nicotine, and flavored e-cigarette vapor.

The expression of the inflammatory protein COX-2 was increased in OKF6 oral epithelial cells upon exposure to nicotine free, 3mg/ml nicotine, and flavored e-cigarette vapor indicating that even in the absence of nicotine, an inflammatory signaling is induced. The increased signal of

COX-2 in flavored vape suggest the strongest host-response to the insult of the additional chemicals present due to the flavoring. Samples were harvested and stored at -80° C prior to protein concentrations being determined and SDS PAGE. ImageJ was used to standardize band intensity to the loading control for comparison. Western Blots were performed in triplicate.

## DISCUSSION

Biofilms in the oral cavity are made of excreted polymeric compounds (EPS), microbes, DNA, RNA, and proteins, with only 5% of the biofilm being composed of microorganisms.<sup>30</sup> Oral pathogen *S. mutans* has the ability for sucrose-dependent attachment, using glucan-binding proteins and glucosyltransferases, as well as sucrose-independent attachment, using antigen I/II.<sup>31</sup> Conversely, sucrose-dependent adherence is difficult for *S. sanguinis* and *S. gordonii*. Opting to use sucrose-independent adherence, *S. sanguinis* does so through *SrpA*, and *S. gordonii* does so through *GspB* and *Hsa*, all of which are sialglycan-binding adhesins.<sup>32</sup>

E-cigarette liquids have been found to contain high amounts of sucrose.<sup>33</sup> Because of this, it is possible *S. mutans*, who may easily undergo both sucrose-dependent and independent attachment to smooth surfaces, such as the bottom of a 96 well plate, may have more biofilm forming prowess than *S. sanguinis* and *S. gordonii*.<sup>31</sup> In addition, previous studies have shown that *S. mutans* has more effective adherence strategies, in general, than *S. sanguinis*, even going so far as to bind more of its synthesized glucosyltransferases. The end result is that adherence of *S. mutans* is 3 times as effective as that of *S. sanguinis* to smooth surfaces.<sup>34</sup>

Regarding why the biofilm forming abilities of *S. mutans* are enhanced upon exposure to e-cigarette vapor, while *S. sanguinis* and *S. gordonii* biofilm forming abilities are decreased, the answer may lie within components of the e-cigarette vapor and the nature of biofilms.

Formaldehyde results from the combustion of propylene glycol and glycerol in e-cigarette vapors.<sup>35</sup> Formaldehyde is also a known antimicrobial agent, being used as a disinfectant clinically.<sup>36</sup> A previous study has shown that formaldehyde, in larger quantities, will inhibit biofilm formation and decrease biomass.<sup>37</sup> This may explain why *S. sanguinis* and *S. gordonii*

experience decreased biofilm following exposure to e-cigarette vapor, along with their less efficient surface adherence.

As for *S. mutans*, because of its increased ability to adhere to surfaces, it can begin establishing a biofilm sooner. The biofilm acts as protection, allowing optimal growth conditions for the organism, as well a quorum sensing and transformation rates 10-600 times higher than planktonic cells.<sup>38</sup> Therefore, within the biofilm, *S. mutans* may respond to environmental hazards quicker, and alter its biofilm to survive the environment. Additionally, if the conditions created by exposure to e-cigarette vapor are harsher than normal, biofilm formation may be encouraged further compared to regular conditions, leading to an increase in colonization.

Future studies could focus on interspecies interactions in response to e-cigarette vapor. Studies have shown *S. mutans* or *S. sanguinis* dominance *in vitro* is dependent on which organism is inoculated first.<sup>39</sup> It would be interesting to replicate this after the organisms have been exposed to e-cigarette vapors. Future experiments could also include examining the ratio of live and dead bacterial cells following exposure to e-cigarette vapor, both planktonically and within the biofilm. Finally, gene expression of sucrose-independent and dependent factors could be analyzed in each organism to better understand which is being utilized following exposure.

If *S. mutans* manages to outcompete pioneer colonizers *S. sanguinis* and *S. gordonii in vivo* following exposure to e-cigarette vapor, it could occupy a much larger niche. This would allow more acid production, destroying the enamel of the tooth, and eventually leading to an increased presence of dental caries.

Within the oral epithelium, infection by bacteria can lead to the phosphorylation of ERK. p-ERK ½ expression is often associated with a stress response that mediates inflammation through the

activation of transcription factors that produce pro-inflammatory proteins.<sup>40</sup> One of these inflammatory proteins is COX-2, which induces inflammation after being induced itself by cytokines and growth factors.<sup>41</sup> The presence and activation of these proteins at the 30 minute, 5 hour, and 24 hour timepoints seen in the results indicates an acute and prolonged inflammatory response following exposure to e-cigarette vapors.

Oral inflammation, as seen in periodontitis, has far reaching consequences within the body. Aside from increasing the risk of the development of oral cancers if prolonged, oral inflammation may contribute to other diseases such as obesity, rheumatoid arthritis, Alzheimer's disease, and inflammatory bowel syndrome due to leakage of pro-inflammatory cytokines.<sup>42,43</sup> One study identified that the average number of puffs during a vaping session would be more than 20 puffs per 10 minutes.<sup>44</sup> This type of exposure could allow acute responses to become chronic responses within the oral epithelium, further increasing the risk of oral cancer development.

Chronic inflammation, as seen in the case of infection, can induce the creation of mutagens by immune cells that induce DNA damage in epithelial.<sup>48</sup> This could mean devastating consequences *in vivo* if e-cigarette vapor weakens epithelial cells enough for bacterial invasion and a subsequent prolonged immune response. Inflammatory diseases, such as inflammatory bowel disease and gingivitis which may result from poor oral health and oral inflammation, lead to colorectal carcinoma and oral squamous cell carcinoma respectively.<sup>43,49</sup> Additionally, bacterial colonization outside of its niche may lead to cancer, as seen in the case of *H. pylori* and gastric adenocarcinoma.<sup>49</sup> In the case of oral pathogen *S. mutans*, should it invade epithelial

tissues after e-cigarette allows it to dominate biofilms, it could have a similar effect on the ability for oral cancers to develop.

Those who partake in e-cigarettes may not only be damaging oral health by inducing dysbiosis within the oral microbiome, but also increasing the chance of cancers, such as oral squamous cell carcinoma, to develop. Furthermore, the inflammatory nature of e-cigarette usage may have far-reaching consequences within the body in the form of exacerbating systemic issues linked to inflammation and oral health.

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