THE EFFECTS OF VAPING ON ESOPHAGEAL KERATINOCYTES

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Glossary of Abbreviations

GERD: Gastroesophageal reflux disease

PET Scan: Positron emission tomography scan

CT Scan: Computed tomography scan

EMR: Endoscopic mucosal resection

PDT: Photodynamic therapy

E-Cigarettes: Electronic cigarettes

ENDS: Electronic nicotine delivery system

TCs: Traditional cigarettes

CDC: Centers for Disease Control and Prevention

FDA: Food and Drug Administration

EPC1: EPC1-hTERT human esophageal keratinocytes

EGF: Epidermal growth factor

STI: Soybean trypsin inhibitor

USP: United States Pharmacopeial

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

DMSO: Dimethyl sulfoxide

ANOVA: Analysis of variance

UT: Untreated experimental group

T1: Treatment 1 experimental group

T2: Treatment 2 experimental group

BEAS-2B cells: Normal human bronchial epithelial cells

A549 cells: Adenocarcinomic human lung epithelial cells

LDH: Lactate dehydrogenase

OSCC: Oral squamous cell carcinoma

DNA: Deoxyribonucleic acid

SEM: Standard error of the mean

Abstract

Electronic cigarettes, also known as electronic nicotine delivery systems (ENDS), are battery powered devices that are designed to deliver nicotine by heating a liquid solution (vape juice), creating an aerosol that is inhaled by users. Electronic cigarettes are advertised as a safer alternative to smoking traditional cigarettes (TCs) as well as an effective cessation tool. This is because the vape juice solution usually contains less chemicals than TCs. However, vape juice may contain carcinogenic chemicals such as formaldehyde, acrolein, and pulegone. Smoking has long been established as a major risk factor for developing esophageal cancer, but research on the impacts of vaping is limited. The objective of this study was to determine if human esophageal epithelium demonstrate carcinogenic properties after exposure to vaping. Esophageal keratinocytes exposed to vape-treated media had a decreased rate of proliferation and viability in comparison to untreated cells. This contradicts prior research on oral cancerous epithelial cells which found increased proliferation rates and viability after exposure to vaping. These results suggest that acute exposure to vaping does not cause esophageal keratinocytes to become carcinogenic. Future research should expand on this study to determine if the trends apparent after acute exposure to vaping are also apparent after long-term exposure to vaping.

Introduction and Background

Anatomy and Physiology of the Esophagus

The esophagus is an important organ, which often gets overlooked due to the simplicity of its purpose. The esophagus is responsible for passing food from the pharynx to the stomach (Figure 1). There are four layers of the esophagus: the mucosa, submucosa, muscularis, and the tunica adventitia.[1] The mucosa is the innermost layer and is made up of a stratified squamous epithelium, lining the lumen of the esophagus. Similarly to our skin, most of these epithelial cells are keratinocytes which serve to protect our esophagus from microbial infections, provide a barrier preventing the diffusion of water, and protect the outer layers from other mechanical or environmental stressors.[2] The mucosa also has a loose connective tissue called the lamina propria, and a layer of smooth muscle called the muscularis mucosa. Superficial to the mucosa is the submucosa. The submucosa is a thick layer of connective tissue, containing blood vessels and small glands, which secrete mucus to lubricate the esophagus and protect it from acidic environments due to food or gastric acid. Superficial to the submucosa is the muscularis propria, which is made up of two layers of muscle. The inner muscle layer is circular and the outer muscle layer is longitudinal. The muscularis propria is responsible for the peristalsis of the bolus from the pharynx to the stomach. The last, most superficial layer of the esophagus is another layer of connective tissue, the tunica adventitia, which functions to connect the esophagus to nearby structures.[1]

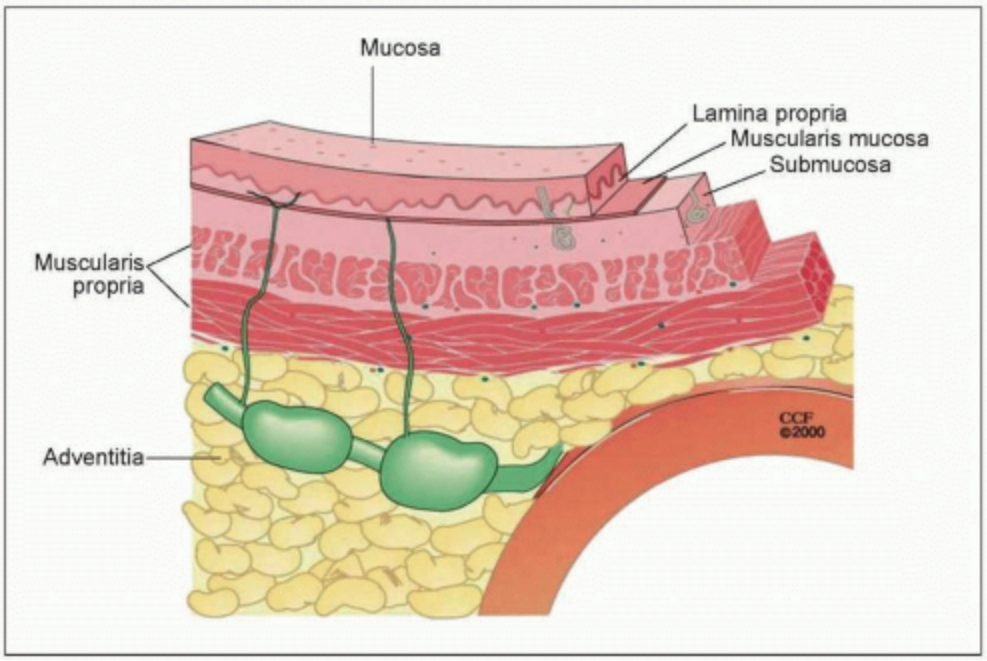

Figure 1. The four layers of the esophagus. The mucosa is the deepest layer, lining the lumen. Superficial to the mucosa is the submucosa, followed by the muscularis propria and adventitia. Image obtained from: https://abdominalkey.com/normal-esophageal-anatomy-and-physiology/

Esophageal Cancer

The American Cancer Society estimates that there will be approximately 21,000 new esophageal cancer diagnoses in 2023.[3] Like most other carcinomas, esophageal cancer is deadly, with an estimated 16,000 lives lost to it in 2023 in the United States alone. Additionally, only 20% of patients are expected to survive more than five years after being diagnosed with esophageal cancer. There are two main types of esophageal cancer. Adenocarcinomas develop inside the glands of organs, as opposed to squamous cell carcinomas which develop in the epithelium lining the organs. Esophageal squamous cell carcinoma develops in the mucosa layer of the esophagus. It is caused by abnormal growth and replication of keratinocytes, creating tumors that make it painful to swallow by blocking part of the lumen of the esophagus.[4] Adenocarcinoma also develops in the mucosa, but is caused by abnormal growth and presence of glandular cells that replace the function of keratinocytes in healthy individuals.

Risk Factors

Men are more likely to develop esophageal cancer than women, with the lifetime risk of developing the disease at 1 in 125 for men and 1 in 417 for women.[3] The most common type of esophageal cancer in the United States used to be squamous cell carcinoma, but now adenocarcinoma makes up over 70% of esophageal cancer cases nationwide.[5] Also, adenocarcinoma is more prevalent among white people than people of other races.[3] Squamous cell carcinoma on the other hand, is more prevalent in African Americans.

One risk factor for developing adenocarcinoma is having Barrett's esophagus.[6] Barrett's esophagus is characterized by some of the squamous cell epithelium lining the lumen of the esophagus being irregularly replaced by glandular cells. This commonly occurs in individuals who have gastroesophageal reflux disease (GERD), which is chronic acid reflux or heartburn.[4] GERD occurs in individuals where the lower esophageal sphincter does not function properly, allowing the contents of the stomach to reflux into the esophagus. The acidity of stomach chyme damages the squamous cell epithelium of the esophagus. Over time, these squamous cells get replaced by glandular cells, developing into Barrett's esophagus as described above. These glandular cells secrete mucus, making them more resistant to damage from chronic gastric acid reflux than squamous epithelial cells. However, people with Barrett's esophagus are more likely to develop adenocarcinoma. In fact, GERD is responsible for one-third of all esophageal cancer diagnoses. Another risk factor for developing adenocarcinoma specifically is obesity, due to increased obesity being linked to a higher incidence of GERD.[7]

Heavy alcohol consumption and tobacco use are major risk factors for all types of esophageal cancer, but are most strongly linked to squamous cell carcinoma. Furthermore,

combined usage of alcohol and tobacco products increases the risk of developing esophageal cancer more than using either exclusively.[3]

Diagnosis and Symptoms

Unlike breast, colon, and lung cancer, people do not receive routine screenings or scans to monitor the health of their esophagus. Therefore, the diagnosis process begins with patients developing symptoms. However, esophageal cancer can be asymptomatic in its early stages.[4] In fact, the most common symptom of esophageal cancer is dysphagia, which is difficulty swallowing. The major issue with this is that the cancer is typically not diagnosed in the early stages. Often, patients will only notice dysphagia after half of their esophagus is obstructed by the tumor. Other symptoms include weight loss, pain in the chest or throat, hoarseness, heartburn, and vomiting blood. Once patients have developed symptoms and inform their physician, they get screened by performing a barium swallow, PET scan, CT scan, or biopsy.[4]

Prognosis and Treatment

The first stage (stage 0) of esophageal cancer is characterized by only having the cancer present in the epithelial layers. It is commonly referred to as high-grade dysplasia.[8] Patients with stage 0 esophageal cancer are asymptomatic, so it is more commonly diagnosed in patients with Barrett's esophagus undergoing a routine biopsy. Whereas people without Barrett's esophagus will not be screened until displaying symptoms, as they are not present until later stages of the disease. Treatment options for patients with stage 0 esophageal cancer include endoscopic treatments, such as endoscopic mucosal resection (EMR) or photodynamic therapy (PDT), as well as an esophagectomy. Stage I is characterized by the cancer growing further into the mucosa and submucosa layers of the esophagus, potentially even spreading into the muscularis and tunica adventitia. Early stage I cancer can be treated with endoscopic treatments too, but most

patients will undergo chemotherapy and surgery if they are able. Stage II and stage III are characterized by the cancer growing into the muscularis and tunica adventitia of the esophagus, as well as spreading to nearby lymph nodes. Stage III cancer may have grown into other nearby organs too. For these stages, treatment usually involves chemotherapy, radiation therapy, and surgery. Stage IV esophageal cancer occurs when it has spread to distant lymph nodes and organs. It is very difficult to destroy the cancer at this point, so patients receive chemotherapy, radiation therapy, or immunotherapy to reduce their symptoms and delay its progression.[8]

As mentioned earlier, people do not receive routine screenings to diagnose esophageal cancer early, resulting in most new cases occurring in patients who already have a tumor obstructing half of the lumen of their esophagus.[4] In fact, more esophageal cancer cases are diagnosed in stage IV of the disease than any other stage.[9] As of 2020, 36.99% of newly diagnosed esophageal adenocarcinomas and 26.81% of esophageal squamous cell carcinomas were classified as stage IV. Due to this, the 5-year survival rate of esophageal cancer is only 21.7%.[10] This means that esophageal cancer has the third lowest 5-year survival rate of any cancer in the United States. However, the 5-year survival rate increases the earlier that it is diagnosed. In fact, if it is diagnosed when the cancer is still localized, patients have a 5-year survival rate of 47%.[11]

E-Cigarettes Background

In the 1960s, Herbert Gilbert announced a patent for a smokeless cigarette that used heated and flavored air instead of tobacco.[12] However, it took 40 years for the first modern electronic cigarette (e-cigarette) to be designed by Hon Lik. At first, it was advertised as a smoking cessation tool in China, but it got its international patent in 2007. Since then, e-cigarettes have boomed in popularity, with 4.5% of adults in the United States using them as of

2021.[13] The United States had an estimated 258 million adults in 2021, meaning there were approximately 11.6 million adults who used e-cigarettes.[14] E-cigarettes are also popular among adolescents. In fact, a report in 2022 found that 3.3% of middle schoolers and 14.1% of high schoolers have used e-cigarettes in the last 30 days.[15]

E-cigarettes, also known as electronic nicotine delivery systems (ENDS), are battery powered devices that are designed to deliver nicotine to the consumer's bloodstream. E-cigarettes have a heating element which turns the liquid solution (vape juice) containing nicotine, flavorings, and other chemicals into a vapor. This vapor gets inhaled by the consumer and the nicotine from it enters the bloodstream through their lungs. Although vape juice in e-cigarettes usually contains less chemicals than traditional cigarettes (TCs), they may contain chemicals such as formaldehyde, acrolein, pulegone, and trace metals like lead, nickel, and cadmium.[16] All of these chemicals are capable of damaging DNA, causing mutagenesis, and are classified as potentially carcinogenic.

History of E-Cigarette Advertisement

Since the creation of the e-cigarette, it has been heavily advertised in a couple ways. Originally, it was advertised as a tool to help people quit smoking TCs. According to the CDC, research on this has yielded mixed results and e-cigarettes have not been approved by the FDA as a smoking cessation aid.[13] However, there have been reviews that suggest that e-cigarettes can be a successful smoking cessation tool. Hartmann‐Boyce et al. suggest that TC smokers are more likely to quit when using e-cigarettes (with nicotine) in comparison to using nicotine patches, gum, or without any support.[17] One significant problem with this conclusion is the lack of randomized control trials tracking cessation for 6 months or longer. Although their database found 1,704 unique studies, only 2 of those were randomized control trials that followed-up

long-term (6 months or longer) with the participants. While e-cigarettes certainly have aided people in smoking cessation, it cannot be concluded to be a more effective and less harmful tool to quit smoking until more research has been done. Additionally, the majority of adults using e-cigarettes continue to smoke TCs, choosing to use both products instead of quitting smoking TCs.[18]

Recently, the industry has advertised it as a healthier alternative to smoking TCs. Since e-cigarettes do not burn tobacco like TCs, vaping does not produce carbon monoxide or tar which are harmful to the consumer.[19] Also, smoke from TCs usually contains a toxic mix of over 7,000 chemicals, but the vapor produced from e-cigarettes has fewer toxic chemicals.[13] This is enough evidence for the CDC to describe e-cigarettes as less harmful than TCs. Early research supports this conclusion, but the health effects of long-term use of e-cigarettes is still uncertain and needs to be studied further.

Two problems have arisen due to how e-cigarettes have been advertised. Firstly, the claims made about them being healthier than TCs and as an effective tool to quit smoking have not been studied enough to be regarded as fact. Secondly, describing vaping as the healthy way to smoke is misleading to consumers. Mostly because the long-term effects of vaping are still unknown and there are major health concerns associated with it due to the nicotine, carcinogenic chemicals, and trace metals it contains.[16] Additionally, after a study determined e-cigarettes to be 95% less harmful than TCs, the United Kingdom launched a publicity campaign shifting the tone of the message describing vaping as "95% safer than smoking cigarettes."[20] This is problematic because the positive tone of the message may encourage people who would not smoke to vape. This trend is most apparent in young adults (ages 18-24). In fact, 56% of young adults who vape have never smoked a traditional cigarette.[13]

Decades of research and education on the risk of smoking TCs has resulted in the number of smokers declining. Therefore, many people opt to start vaping since it is perceived as healthy, when it is still potentially damaging to their health long-term. In particular, vaping among adolescents has surged with approximately 2.5 million high school and middle school students vaping as of 2014.[21] This is more than triple of the number of students vaping in 2010. Furthermore, this trend has continued to increase, with 3.6 million students vaping in 2018.[22]

The Physiological Effects of Vaping and Smoking

The number of adolescents who vape has been increasing since 2010, raising concerns about the long-term health effects of vaping. The nicotine alone in the aerosol of e-cigarettes is concerning for adolescents. Extensive research has been done on the effects of nicotine on neural development. The research suggests that chronic nicotine use impacts memory and attention.[23] Additionally, the human brain becomes fully developed in an individual's mid-twenties, but Americans can purchase TCs and e-cigarettes legally at 21 years old. Nicotine addiction is a larger issue for adolescents since they are less cognitively developed and are more susceptible to peer pressure than adults. Although there are aerosol options to vape without nicotine, the majority of adolescents do not vape exclusively nicotine-free.[24] Furthermore, there are concerns about e-cigarettes being a "gateway" drug. A gateway drug is a less harmful and addictive substance that leads people to use and become addicted to other drugs. People who have vaped in the last 30 days are more likely to have used marijuana, cocaine, alcohol, or other forms of tobacco. Oftentimes, people will vape and use one of these drugs at the same time.[25]

The vape juice used for e-cigarettes is not pure nicotine and solution though. As mentioned earlier, vape juice may contain potentially carcinogenic chemicals like acetaldehyde, formaldehyde, acrolein, and trace metals like lead, nickel, and cadmium. While these chemicals

are not purposely added to vape juice, they are produced as the result of vaping. Acetaldehyde, formaldehyde, and acrolein are created by the thermal degradation of propylene glycol and glycerin, which are common ingredients in vape juice.[26] Another chemical found in vape juice, pulegone, is potentially carcinogenic and has been banned by the FDA as a food additive. However, it is still found in mint or menthol flavored vape juice for e-cigarettes and in smokeless tobacco products.[23]

It has been well established that tobacco is a leading cause of cancer development and death.[27] Tobacco usage is an independent risk factor for many cancers, including cancers of the lungs, mouth, esophagus, and stomach to name a few. The objective of this study is to determine if vaping from e-cigarettes has an effect on the phenotype, proliferation, viability, and migratory capabilities of human esophageal epithelial cells *in vitro*. We expect to observe changes to the phenotype of the cells, and measure an increased rate of cellular proliferation and cellular migration. Subjecting the esophageal keratinocytes to vaping acutely may cause abnormalities to their phenotype, proliferation, migration, and viability. Any differences raises questions about the safety and effects of exposing our esophagus to vaping chronically.

Methodology

Cell Culture

EPC1-hTERT human esophageal keratinocytes (EPC1) were gifted to Dr. Heather Lehman from Dr. Douglas Stairs (Penn State College of Medicine, Hershey, PA). EPC1-hTERT cells were grown in keratinocyte serum-free medium containing 40 μg/mL bovine pituitary extract, 1.0 ng/mL EGF, 100 U/mL penicillin, and 100 μg/mL streptomycin. The media was replaced twice weekly and cells were incubated at 37°C and 5% CO_2. Once cell cultures reached approximately 75% confluency, they were split and expanded to new plates. This occurred

weekly. To do this, the media was drawn off each plate, before adding 1mL of 0.25% Trypsin-EDTA. The cells were incubated while the trypsinization reaction occurred, approximately 10 minutes. Once all cells were detached from the plate, 3mL of soybean trypsin inhibitor (STI) was added to each plate to inhibit further action of trypsin. The cell suspension was then transferred into a 15mL tube and centrifuged at 1,300rpm for 5 minutes. After 5 minutes, the supernatant was removed and the pellet was resuspended in 2mL of media. Finally, 1mL of the suspension was added to two new petri dishes and spread across the surface with 7mL of added media.

E-Cigarette and Vape Juice

This study used an e-cigarette styled as a traditional vape pen (Figure 2). The e-cigarette is an eGo-T vape pen with a CE4 Clearomizer tank containing 1.6mL of vape juice. The vape juice used was Salebae50 Sweet Caramel Tobacco flavor, containing a nicotine concentration of 50mg/mL (Figure 3). The vape juice is made up of USP propylene glycol, USP vegetable glycerin, USP salt nicotine, natural and artificial flavors. This product had a warning label too, where it informed consumers that they may be exposed to acetaldehyde by using the product, which is known to cause cancer.

Vaping Treatment

The effect of vaping on EPC1 cells was conducted by imitating vaping using a laboratory vacuum (Figure 4). The e-cigarette was connected to a 30mL impinger containing EPC1 media. That impinger was connected to a second 30mL impinger which was empty. This second impinger was utilized to trap any media overflow. This was then connected to a flow meter which was connected to the laboratory vacuum. The vacuum valve was turned on and the flow meter was adjusted so that the vapor would be drawn out from the e-cigarette and flow through

the media in the impinger, but not too much that the media would then get pulled through to the laboratory vacuum. The button on the vape would be held for the duration of the interval, causing the vapor to be released and move through the EPC1 media. Treatment 1 of each experiment had a puff interval of 5 seconds and an interpuff interval of 5 seconds. Treatment 1 had 20 rounds of the puff and interpuff intervals. Treatment 2 of each experiment had the same puff and interpuff intervals, but it had 30 rounds instead of 20 (Table 1).

Cell Phenotype

Cell phenotype was examined using a Zeiss Axiovert 10 Inverted Microscope and photographed at each time interval for all experiments (0hr, 24hr, 48hr, and 72hr). Pictures were taken of both untreated and treated cells to determine if the morphology of the cells had been affected by the treated media in comparison to the control. These pictures were taken at 100X magnification for all experiments. For the wound healing assay, additional pictures were taken at 50X.

Cell Counting

Cells were counted using the Trypan Blue dye exclusion method to determine the effect of the treated media on the viability of the cells. The cells were trypsinized and centrifuged using the same procedure described in *Cell Culture*. Once the cells were resuspended in 1mL of EPC media, 100µL of the cell suspension was mixed in a microcentrifuge tube with 400µL of Trypan Blue dye (0.4%). 100µL of this suspension was pipetted into a hemocytometer. The microscope was focused on the gridlines of the hemocytometer at 100X magnification. The number of live, unstained cells were counted at each of the 4 sets of 16 corner squares on the hemocytometer (Figure 5). A hand tally counter was used to record the number of live cells, which was then divided by 4 to get the average. To calculate the number of viable cells per mL, the average was

multiplied by 10,000 because each large square on the hemocytometer is 1/10,000th of a milliliter. This was further multiplied by 5 to account for the Trypan Blue dye dilution to get the number of viable cells in the original suspension.

Cell Plating

To plate cells to start each experiment, the same procedure was followed as described in *Cell Counting*. The number of cells needed was divided by the number of viable cells in the original suspension, giving us the amount of cell suspension (mL) to plate. EPC1 media was added to each well before plating the cells, so the cells would distribute throughout the well evenly.

Cell Viability

A 24-well plate was plated with 30,000 cells in 500µL of EPC media per well. All cells were plated in untreated media for 24 hours to allow the cells to adhere to the plate. After 24 hours, the media was replaced in each well with 500µL of corresponding treatment and the cells were imaged. This time point was considered 0 hours in the experiment (t = 0 hours). The number of viable cells and the number of dead cells were reported at 24, 48, and 72 hours as described in *Cell Counting*. The wells were also photographed at 24, 48, and 72 hours to determine if any changes occurred in the phenotype of the cells.

Proliferation (MTT) Assay

A 96-well plate was plated with 2,000 cells per well. The cells were allowed to grow, reaching approximately 70% confluency in each well. From this point, the media in each well was replaced with treated media or new control media. At the end of each time interval (24hr, 48hr, 72 hr), 20µL MTT solution was added to the media and the cells were resuspended. The cells were then incubated for 3 hours at 37℃ and 5% CO_2. After 3 hours, the media was

removed and 200µL DMSO was added to the wells. The plate was shaken for three minutes to mix and the absorbance was read at 562nm.

Cell Migration

50,000 cells per well were plated in a 12-well plate and were checked daily until they had grown to 100% confluency. After the cells reached 100% confluency, the growth media was removed and the cell monolayer was scratched using a 200µL pipet tip. Untreated or treated media was added to each well and scratches were photographed at 50X total magnification at each time point (0hr, 24hr, 48hr, 72hr). After 72 hours, the cells were counted using the procedure described in *Cell Counting*.

Data Analysis

Each experiment conducted included three biological replicates and three technical replicates. The cell viability experiment contained two technical replicates for the control group. Cell migration was measured by calculating the scratch width using the ImageJ program. A one-way ANOVA was used to determine the significance of differences between experimental groups. $P \leq 0.05$ was considered statistically significant. If the differences were calculated as statistically significant, the Tukey-Kramer test was used to determine which differences between groups were significant (T1-T2, T1-UT, or T2-UT).

Results

Changes in keratinocyte phenotype after exposure to vaping

Esophageal keratinocytes cultured in keratinocyte serum-free medium are irregular and cuboidal in shape (Figure 6A-D). However, when the keratinocytes were cultured in vape-treated media, their phenotype changed. Keratinocytes in both treatment groups were rounded in shape after 72 hours of incubation (Figure 6H, L). Additionally, the size of the cells decreased in

comparison to the untreated group. Expected growth was observed in the untreated groups, reaching almost 100% confluency after 72 hours (Figure 6D). This was not observed in the treated groups, with no difference in growth of the cells visible after 72 hours. In fact, the cells exposed to vape juice aerosol appeared to be similar in both size and confluency when comparing them after 72 hours to when they were plated (Figure 6E, H, I, L). Additionally, there were no visible changes in size or confluency for the treated groups at 24 or 48 hours (Figure 6F-G, J-K). However, the untreated cells demonstrated continual growth in size and confluency at each time interval (Figure 6A-D).

Decreased keratinocyte viability measured after exposure to vaping

As mentioned above, the cells grown in treated media were less confluent than the cells cultured in untreated media. The number of live cells were counted at 24, 48, and 72 hours in untreated, treatment 1, and treatment 2 media to determine if this difference in confluency was due to a decreased number of live cells in the treated groups after 72 hours. Furthermore, the number of dead cells were counted to determine if vaping caused the viability of the keratinocytes to decrease. The average number of live cells counted decreased after 24 hours in treatment group 1 (6,943 cells ± 1,387) and treatment group 2 (8,333 cells ± 4,167) in comparison to the untreated group (16,667 cells ± 4,167). Furthermore, the average number of live cells counted was greater in the untreated group after 48 hours (58,333 cells ± 30,262) than in both treatment groups (T1=11,113 cells ± 3,674; T2=12,500 cells ± 2,408). This trend continued at 72 hours (Figure 7). The live cell count of both treatment groups (T1=12,500 cells ± 2,407; T2=12,503 cells ± 4,167) had decreased in comparison to the untreated group at 72 hours (66,667 cells ± 9,081). The differences in live cell count between experimental groups were not statistically significant at 24 or 48 hours (Table 2; $p=0.19$, $p=0.18$). However, the decreased live

cell count in both treatment groups after 72 hours was significant in comparison to the untreated group ($p<0.01$).

The dead cell counts at 24 hours of all the experimental groups were very low. In fact, the treatment 1 group and the untreated group did not have any dead cells counted (Table 3). However, after 48 hours, treatment 1 caused a higher number of dead cells (2,780 cells ± 1,390) than the untreated group (0 cells ± 0). At 72 hours though, the number of dead cells were highest in the second treatment group (12,500 cells ± 2,408), followed by the untreated group (8,333 cells ± 8,333), and were the lowest in the first treatment group (4,167 cells ± 2,405). None of the differences in dead cell counts between groups were statistically significant ($p=0.55$).

These dead cell counts do not account for the variation in the number of total cells in each experimental group. The percentage of cells that were live cells was calculated to account for the increased number of total cells present in the untreated group in comparison to the treatment groups. After 24 hours, both the untreated and treatment 1 groups had a live cell percentage of 100%, but the percentage of live cells was only 86% in treatment group 2 (Figure 8). At 48 hours, the untreated group still had the highest percentage of live cells (100%), followed by treatment 2 (90%), and finally treatment 1 with the lowest percentage of live cells (80%). As mentioned above, treatment 1 had a decreased total number of dead cells compared to the untreated group after 72 hours (Table 3). However, 89% of the cells counted in the untreated group were live cells, whereas only 75% of the cells counted were live cells in the treatment 1 group (Figure 8). Treatment group 2 had the lowest percentage of cell viability with only 50% of the cells counted being live cells after 72 hours.

Exposure of keratinocytes to vaping causes decreased cell proliferation

When comparing the viability of the keratinocytes after exposure to vaping, it was noted that the total number of live cells decreased in the treated groups. Therefore, the MTT assay was performed to determine if vaping caused a decreased rate of proliferation of the keratinocytes. After 24 hours, the untreated group had the greatest absorbance (0.761 ± 0.010), followed by treatment 1 (0.511 ± 0.013), and treatment 2 had the lowest absorbance (0.338 ± 0.011). These results demonstrate that keratinocytes exposed to vaping have decreased metabolic activity, reflecting a decreased rate of proliferation in comparison to the untreated group. Additionally, cells exposed to vaping longer (treatment 2) had a significantly lower rate of proliferation than the cells with less exposure to vaping (treatment 1; $p<0.01$). This trend continued at 48 hours too (Table 4). The absorbance increased in the untreated group (0.856 ± 0.017) and decreased in both treatment groups (T1=0.403 ± 0.006; T2=0.295 ± 0.014). Likewise, after 72 hours, treatment 1 and treatment 2 had significantly lower absorbances (T1=0.326 ± 0.012; T2=0.205 ± 0.019) than the untreated group (0.963 ± 0.007; $p<0.01$). When analyzing each group's absorbances at each timepoint, two trends were apparent. Firstly, the absorbances for both treatment groups decreased at each timepoint (Figure 9). Secondly, the absorbance for the untreated group increased at each timepoint. Therefore, the rate of proliferation was increasing for the untreated group over 72 hours, whereas it was decreasing for both treatment groups over the same period of time.

Effect of vaping on migration of esophageal keratinocytes

Cellular migration was measured using the wound healing assay to determine if exposure to treated media would impact cellular motility (Figure 10). After 24 hours of treatment, treatment group 2 had the largest average scratch width (658.4 ± 21.5), followed by treatment group 1 (624.2 ± 27.9), indicating decreased migration. The untreated group had the smallest

average scratch width after 24 hours (72.5 ± 53.1). This trend continued after 48 and 72 hours (Figure 11). However, the untreated group also had the smallest average scratch width initially (Table 5). Due to this, comparing the scratch widths between experimental groups is not the most accurate way to evaluate differences in migration.

As a better comparison, the percentage of scratch closure was calculated at each timepoint using this equation:

*% of Scratch Closure = [(Initial Scratch Width - Scratch Width (t=24, 48, or 72h)) / Initial Scratch Width] * 100*

After 24 hours, neither of the treatment groups demonstrated any migration into the scratch (Table 6). Furthermore, this was consistent after 48 and 72 hours (Figure 12). In fact, the scratch width increased in both treatment groups after 72 hours, albeit insignificantly (Percentage of Scratch Closure: T1=-1.3%; T2=-0.9%). On the other hand, the untreated cells had almost completely filled the scratches after 72 hours (Percentage of Scratch Closure: 96.3%). The untreated group quickly migrated into the scratch, with most of the scratch already filled after 24 hours (Percentage of Scratch Closure: 86.8%).

However, these results were only consistent in the first two trials. The third biological replicate demonstrated no significant migration into the scratch for the untreated group (Figure 13), along with both treatment groups. This caused us to perform the wound healing assay a fourth time, to determine if the third trial was outlier data (Table 7). The fourth trial had results consistent with the third, with all three experimental groups demonstrating no significant migration into the scratch (Figure 14; $p=0.46$). Therefore, the effects of vaping on the migration of esophageal keratinocytes cannot be concluded.

Although the results for the effect of vaping on cellular migration was inconclusive, live and dead cell counts were measured after 72 hours to see if the number of live cells present may have impacted those results. Even though the first two and last two trials had inconsistent results

for cellular migration, all four trials demonstrated consistent results for cell counting after 72 hours. Both treatment groups had significantly less live cells than the untreated group (Table 8; $p<0.01$). This was expected, since it is consistent with the results of the cell viability experiment. Furthermore, the untreated group had the highest percentage of cells that were live cells (84.3%). Treatment group 1 had a live cell percentage of 62.5% and treatment group 2 had the lowest percentage (56.1%). This was also consistent with the results of the cell viability experiment.

Discussion

The aim of this research was to determine if human esophageal keratinocytes would exhibit potentially carcinogenic properties after being subjected to vaping acutely. Cancerous cells are known to proliferate more rapidly and frequently than normal cells, exhibit abnormal phenotypes in comparison to normal cells, and metastasize throughout the body in later stages of the disease. This led us to expect to observe changes to the phenotype of the cells and increased rates of proliferation, migration, and viability.

The morphology of the EPC1 cells after vaping treatment was altered. Healthy EPC1 cells are irregular and cuboidal in shape. This allows the epithelial cells to grow and form layers, filling the space between other cells nearby. This is important for the function of the cells, allowing them to provide protection from microbial infection and protect from other mechanical and environmental stressors. However, EPC1 cells treated in vaped media exhibited a smaller, more rounded shape in comparison to healthy EPC1 cells. This result would require an increased number of cells to be present in order to serve the function required. The nature of the treated cells being rounded too may lead to them losing cellular adhesions to adjacent cells, preventing the stratified epithelial barrier from functioning effectively. These results align with prior research conducted on the effects of vaping on bronchial and lung epithelial cell morphology.[28-29]

The human bronchial epithelial cell line (BEAS-2B cells) used for research had a cobblestone appearance when healthy.[28] After exposure to vaping for only 24 hours, the bronchial epithelial cells became rounded, having a smoother surface and lost their cobblestone appearance. The morphology also changed for lung epithelial cells (A549 cells), demonstrating increased vacuolization and alterations to their cellular membranes.[29] Furthermore, this effect was apparent when exposing the lung cells to TC smoke, albeit less pronounced. Vaping e-cigarettes appears to impact the morphology of human epithelial cells, even when exposed only over a short period of time (<72 hours).

As mentioned earlier, cancerous cells are known to proliferate more rapidly and frequently than healthy cells. Therefore, we expected EPC1 cells in vaped media to also proliferate more rapidly, demonstrating cancerous behavior. However, our results differed from expectations, reflected by the results of the MTT assay. It is a colorimetric assay used to measure the formation of formazan by viable cells with active metabolism. The experimental group with the highest absorbance has the greatest metabolic activity, indicating a higher proliferation rate than the other experimental groups. In this study, both treatment groups had a significantly lower rate of proliferation than the untreated group ($p<0.01$). This is consistent with other studies investigating the effects of vaping on nasal and bronchial cell proliferation.[30-31] Furthermore, these studies also compared the rate of proliferation in the presence and absence of nicotine. Even in nicotine-free aerosols, the cells had a decreased rate of proliferation, suggesting that exposure to the other chemicals present in vape juice also negatively impacts the health of human epithelial cells.

Another characteristic of cancers is metastasis, or the ability to spread from one area of the body to another. This usually happens by part of the tumor traveling through the bloodstream

or lymphatic system.[32] To break off the primary tumor these cells need to lose their adhesion to adjacent cells and increase cellular motility. This allows the increased migration of cancerous cells required for metastasis. Therefore, we expected to observe similar properties in the EPC1 cells grown in vape-treated media. Overall, our results were inconclusive, due to inconsistency in the untreated group. However, across all four trials, neither vaping treatment group demonstrated any migration at all. Therefore, the results of the wound healing assay suggest that vaping does not increase cellular motility, since both treatment groups did not migrate into the scratches at all. Once again, these results are somewhat consistent with studies focusing on the effects of vaping on oral fibroblast migration,[33-34] which concluded that vaping decreases the migration of the cells. This study cannot presently conclude that vaping decreased migration in esophageal keratinocytes, but vaping certainly did not increase migration since there was no cellular migration at all after 72 hours. However, future research should reinvestigate this due to the inconsistency of the untreated group. One possible explanation for this inconsistency was due to contamination being present before starting the third biological replicates. After decontaminating the incubator, the untreated cells did not migrate into the wound as they did in the first two trials.

Cancer cells are described as immortal,[35] suggesting that cellular viability would increase if the vape treatment is causing healthy EPC1 cells to become carcinogenic. Although there was not a significant difference in the dead cell counts between the untreated group and either of the treatment groups ($p=0.56$), the percentage of live cells was much greater in the untreated group. This suggests that exposure of EPC1 cells to vape juice aerosol decreased the viability of the cells. One explanation for these results is that the untreated group was grown in ideal conditions for cell growth and proliferation, whereas the treated groups were exposed to the toxic chemicals present in the aerosol from the vape juice. The toxicity of vape juice aerosol damages their

cellular membranes and may induce cell death. This conclusion is supported by research on bronchial and lung epithelial cells.[28-29] Studies found that exposure to vape juice aerosol caused an increase in lactate dehydrogenase (LDH) release, which occurs when the plasma membrane of a cell is damaged. In fact, vaping led to more than a 50% increase in LDH release in bronchial epithelial cells.[28] Furthermore, LDH release is an indicator of cells undergoing apoptosis or cell death.[36] Therefore, it was unsurprising that these studies also reported decreased cell viability after exposure to vape juice aerosol.[28-29]

Current research evaluating the effects of vaping on oral squamous cell carcinomas (OSCC) suggests that vaping increases cell proliferation and migration.[37] The results of our study do not suggest carcinogenic properties in the treatment groups. Although the morphology of the cells was altered, the decreased proliferation rate and viability of the treated groups are not suggestive of carcinogenic changes in the cells. In hindsight, it is unsurprising that the proliferation rate and viability of the treated groups decreased compared to the untreated group. Our hypotheses were based on the results of EPC1 cells demonstrating carcinogenic characteristics, but the results would not reflect the expected results unless a significant number of cells became carcinogenic quickly. Another explanation for the decreased rate of proliferation and viability of the cells was due to overexposure to vaping, with the treated media becoming toxic to the EPC1 cells. This is supported by the increased percentage of total cells counted as dead cells, along with the proliferation rate of the treated groups decreasing at each timepoint (24, 48, 72hr).

Although exposing esophageal keratinocytes to vaping did not result in the cells demonstrating carcinogenic properties, the results of decreased viability and proliferation rate are still concerning for people who vape. The function of these keratinocytes is to protect the

esophagus from damage by providing a barrier from food and reflux of the contents of the stomach. As mentioned earlier, the keratinocytes became smaller and rounded after exposure to vaping. This would require more cells to provide an effective barrier for the esophagus in comparison to the untreated cells which are irregular and cuboidal in shape, allowing the cells to be packed tight and prevent gaps from being present in the epithelial layer. Furthermore, the decreased viability and proliferation rate of the keratinocytes suggests that it would be more difficult for the esophagus to recover from any damage that occurs to the esophageal epithelial cells, which could develop into long-term health problems.

One limitation to this research was the duration of the experiments. Firstly, each result was evaluated daily for three days. The established proliferation rate of esophageal epithelial cells is approximately one division every 2.4 days.[38] This means that we could expect the cells to divide once or twice during each experimental replicate. Since cancers are caused by DNA mutagenesis, this gives limited opportunities for mistakes to be made during DNA replication and cell division. Furthermore, only one cell needs to mutate and become carcinogenic to proliferate and develop into a future tumor. Even if this occurred in this study, the impact of it would likely be so minimal it would go undetected due to the short experimental design. It likely would not impact the results significantly in only 72 hours. To expand on the research of this study, each experimental replicate should last 1 to 2 weeks. Since the duration of the experiment increases, the media should be replaced every 72 hours for each experimental group. This would allow the cells to divide several times across the experiment, providing insight on how newly divided cells grow, proliferate, and potentially adapt to vape-treated media. This also provides more opportunities for mistakes to be made during DNA replication and cell division, increasing the likelihood of cells to become carcinogenic.

Another limitation to the research conducted is the duration of the vaping treatment. The total duration of the vaping treatment for treatment 1 was 3 minutes and 20 seconds. Treatment 2 was only 5 minutes in total. People do not vape this frequently over that period of time. However, they are exposed to the chemicals in the vape juice aerosol over many months and years. Since the puff and interpuff intervals used for this study do not represent habitual vaping, the potential of vaping leading to cancer cannot be dismissed even though the results provide evidence that suggests that it does not.

For this study, we used a 5sec puff/5sec interpuff interval. Prior research on puff and interpuff intervals varies with their durations, with common puff durations ranging from 2 seconds to 6 seconds.[39] There has been less research focusing on interpuff durations, resulting in a larger range (5 sec - 58 sec).[40] We opted for 5 second puff and 5 second interpuff intervals for a few reasons. Firstly, it can take up to two seconds for the coil of the e-cigarette to reach the temperature required to produce aerosol consistently. Secondly, at puff durations of longer than 5 seconds, the e-cigarette can overheat.[40] The 5 second puff/5 second interpuff interval was simple and long enough to expose the media to the vape juice aerosol, while ensuring the e-cigarette would not overheat and cause damage to the user or laboratory equipment.

Future research should expand on the experiments performed to determine if the trends observed after acute exposure to vaping are similar to the long-term impacts of vaping. This could be done by running experiments across several weeks instead of several days. This would provide insight on how vaping may impact a passage of cells over several cell divisions, as opposed to one passage of cells and the new cells made after one cell division. Furthermore, future research can experiment with different puff and interpuff intervals to find intervals more

representative of how people vape. This would provide insight on if the results of this study highlights trends that are apparent over a longer duration of exposure to vaping.

Tables and Figures

Figure 2. E-cigarette used to create treatment 1 and treatment 2 media for each experiment performed. It is an eGo-T vape pen with a CE4 Clearomizer tank containing 1.6mL of vape juice.

Figure 3. Vape juice used in e-cigarette to create treatment 1 and treatment 2 media. It is from the brand Saltbae and is the Sweet Caramel Tobacco flavor, containing 50mg/mL of nicotine.

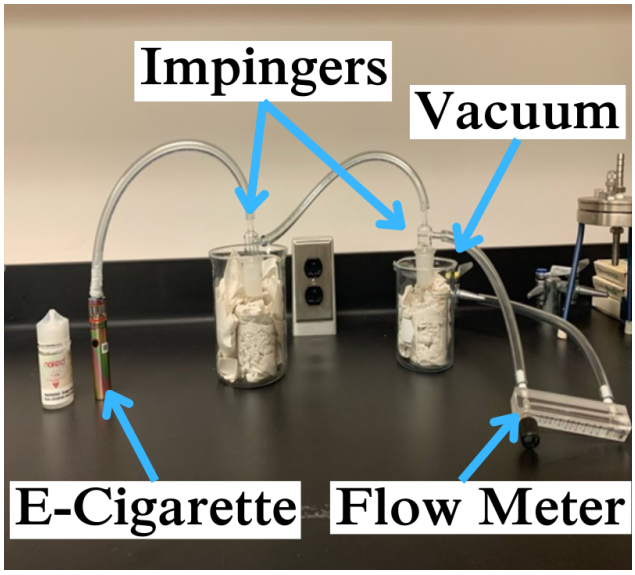

Figure 4. Laboratory set up used to vape into EPC1 media. The impinger closest to the e-cigarette contained 20mL of EPC1 media, which became treatment 1 or 2 media after vaping 5 second puffs and 5 second interpuffs for 20 or 30 rounds, respectively. The second impinger functioned to prevent overflow of vapor or media into the flow meter or laboratory vacuum. The vacuum was turned on and the flow meter was adjusted to ensure vape juice aerosol was pulled into the EPC1 media, without having the media overflow into the second impinger.

Table 1. Treatment group protocols used for each experiment performed.

	Treatment 1	**Treatment 2**	**Untreated**
Puff Interval	5 seconds	5 seconds	n/a
Interpuff Interval	5 seconds	5 seconds	n/a
Rounds	20	30	n/a

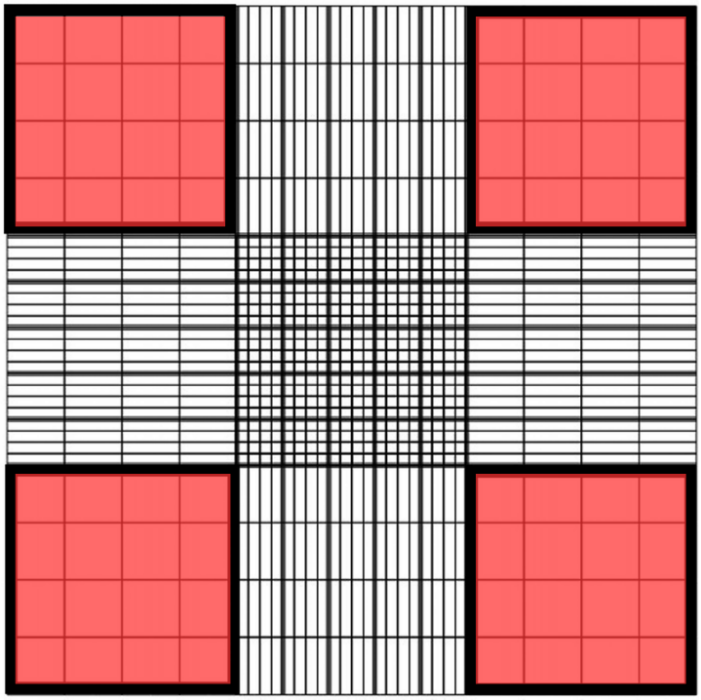

Figure 5. Diagram of a hemocytometer grid. The four quadrants highlighted in red were the squares used for counting live, unstained cells as well as dead, stained cells using the Trypan blue dye exclusion method.

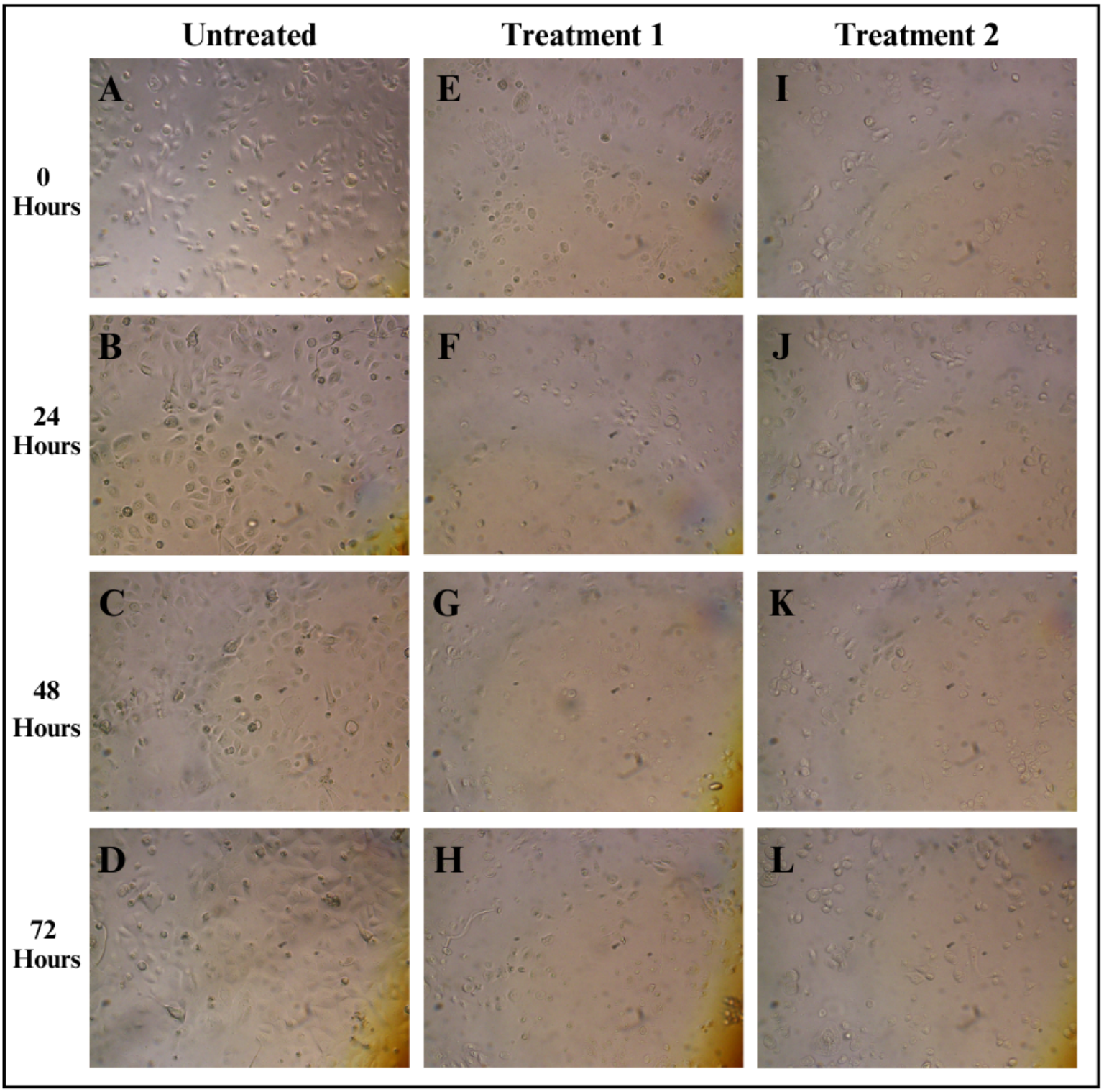

Figure 6. Phenotypic images of esophageal keratinocytes cultured in untreated, treatment 1, and treatment 2 media. The cellular phenotype of both treatment groups changed, becoming more rounded and smaller than the irregular cuboidal shape of the untreated cells. The confluency of both treatment groups was less than the untreated group. There were no observed differences in morphology when comparing treatment 1 and treatment 2 cells (n=3, total magnification=100X).

Table 2. Average number of live cells (± SEM) counted using the Trypan blue dye exclusion method. Both treatment groups had less live cells than the untreated group at each timepoint. This difference was only statistically significant at 72 hours ($p<0.01$; n=3).

	24 Hours	48 Hours	72 Hours
Treatment 1	6,943 ± 1,387	11,113 ± 3,674	12,500 ± 2,408
Treatment 2	8,333 ± 4,167	12,500 ± 2,408	12,503 ± 4,167
Untreated	16,667 ± 4,167	58,333 ± 30,262	66,667 ± 9,081

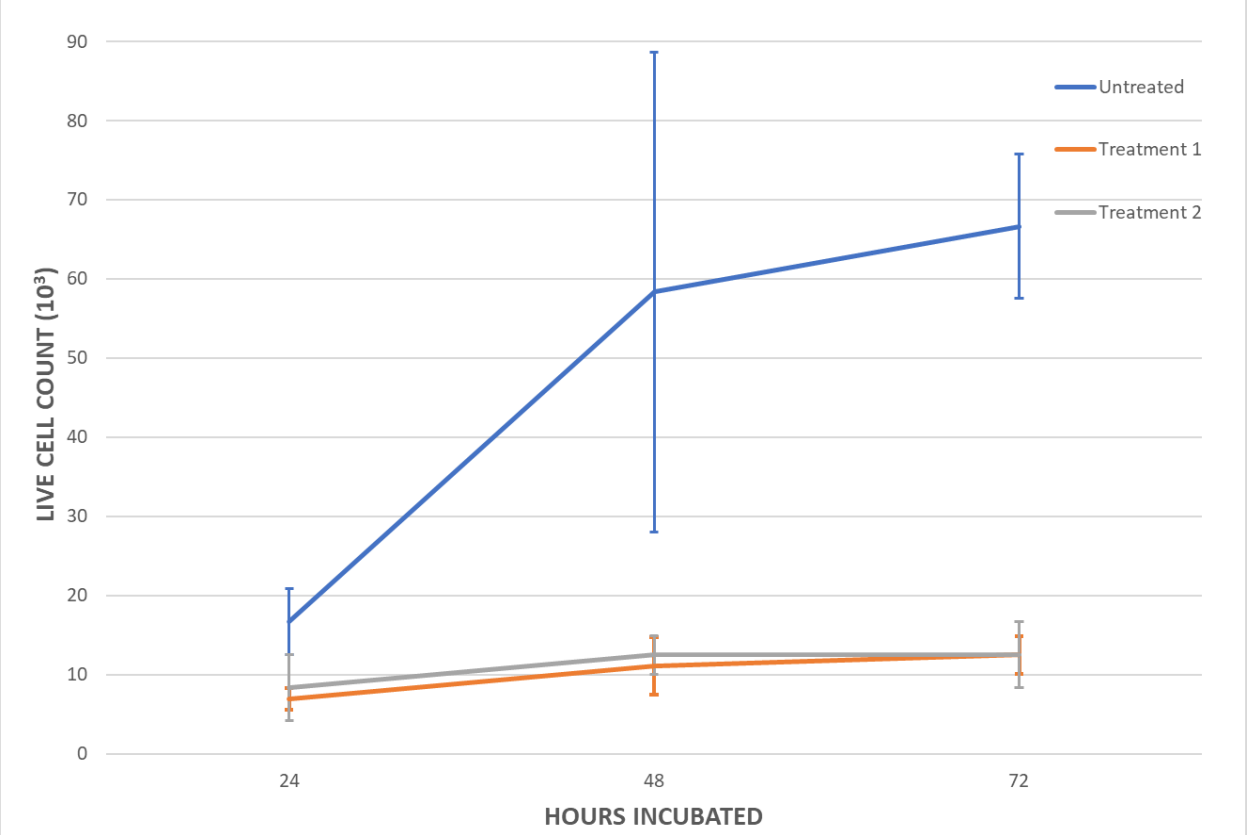

Figure 7. Average number of live cells counted ($x10^3$) using the Trypan blue dye exclusion method. Vape juice aerosol exposure to EPC1 cells resulted in a decreased live cell count (treatment 1 and 2). Error bars represent the standard error of the mean (n=3).

Table 3. Average number of dead cells (± SEM) counted using the Trypan blue dye exclusion method. Both treatment groups had less live cells than the untreated group at each timepoint. (n=3).

	24 Hours	48 Hours	72 Hours
Treatment 1	0 ± 0	2,780 ± 1,390	4,170 ± 2,405
Treatment 2	1,390 ± 1,390	1,390 ± 1,390	12,500 ± 2,408
Untreated	0 ± 0	0 ± 0	8,333 ± 8,333

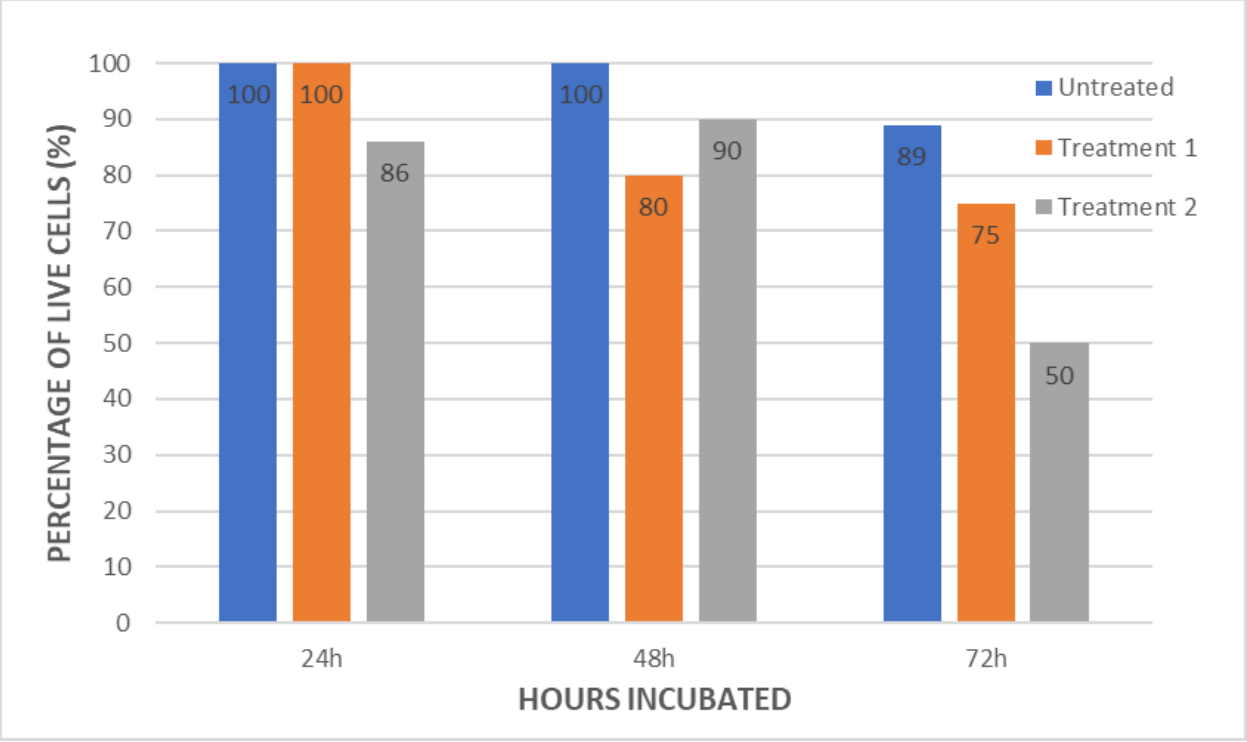

Figure 8. Percentage of live EPC1 cells after exposure to vape treatment. After 72 hours, the untreated cells had the highest percentage of live cells (89%), followed by treatment 1 (75%) and treatment 2 cells (50%). Both treated groups had a lower percentage of live cells than the untreated group at 48 hours. At 24 hours, both the untreated and treatment 1 groups had 100% live cells, while treatment 2 had only 86% live cells (n=3).

Table 4. Average absorbance measured (± SEM) of EPC1 cells using the MTT assay to compare the proliferation rate of cells after exposure to vaping. Absorbance was read at 562 nm. Both treatment groups had a significantly decreased rate of proliferation in comparison to the untreated group ($p<0.01$). Cells with increased exposure to vape juice aerosol (treatment 2) had a decreased rate of proliferation in comparison to cells with less exposure to vape juice aerosol (treatment 1; $p<0.01$; $n=3$).

	24 Hours	48 Hours	72 Hours
Treatment 1	0.511 ± 0.013	0.403 ± 0.006	0.326 ± 0.012
Treatment 2	0.338 ± 0.011	0.295 ± 0.014	0.205 ± 0.019
Untreated	0.761 ± 0.010	0.856 ± 0.017	0.963 ± 0.007

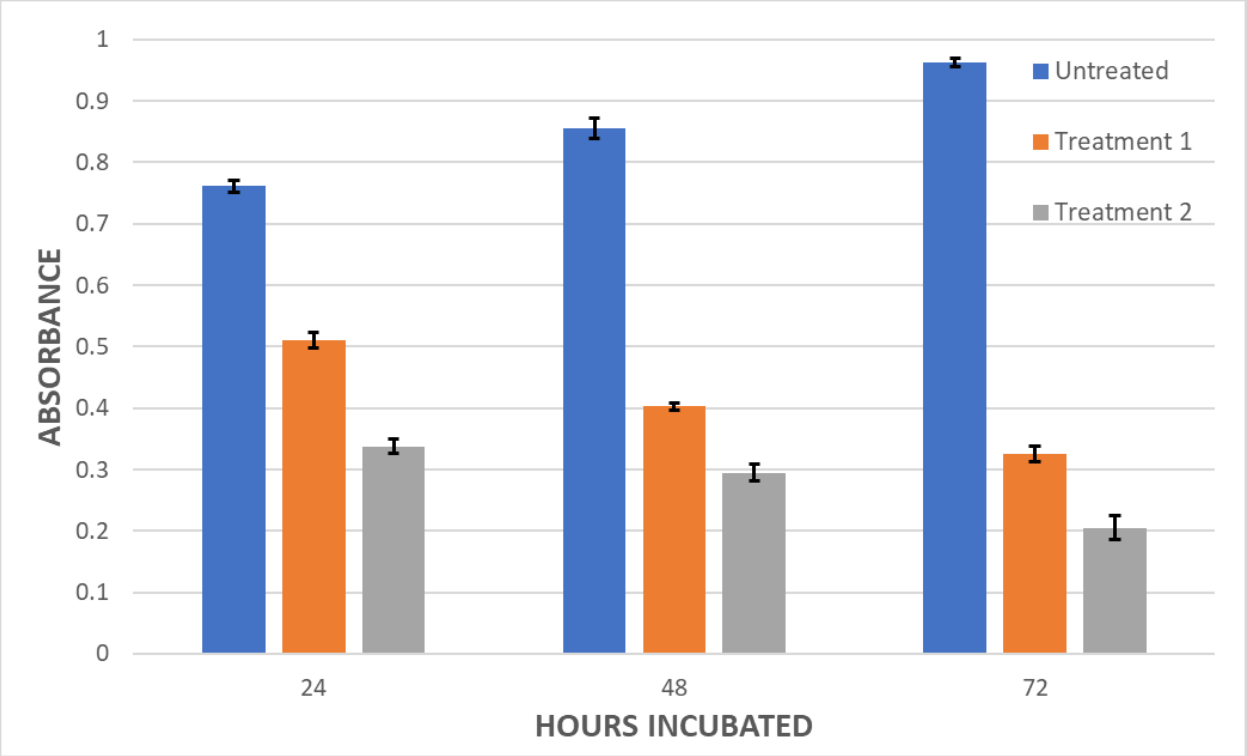

Figure 9. Average absorbance measured to compare the proliferation rate of cells after exposure to vaping. The proliferation rate of the untreated group increased at each timepoint. The proliferation rate decreased at each timepoint in both groups exposed to vaping. Absorbance was read at 562 nm. The error bars represent the standard error of the mean ($n=3$).

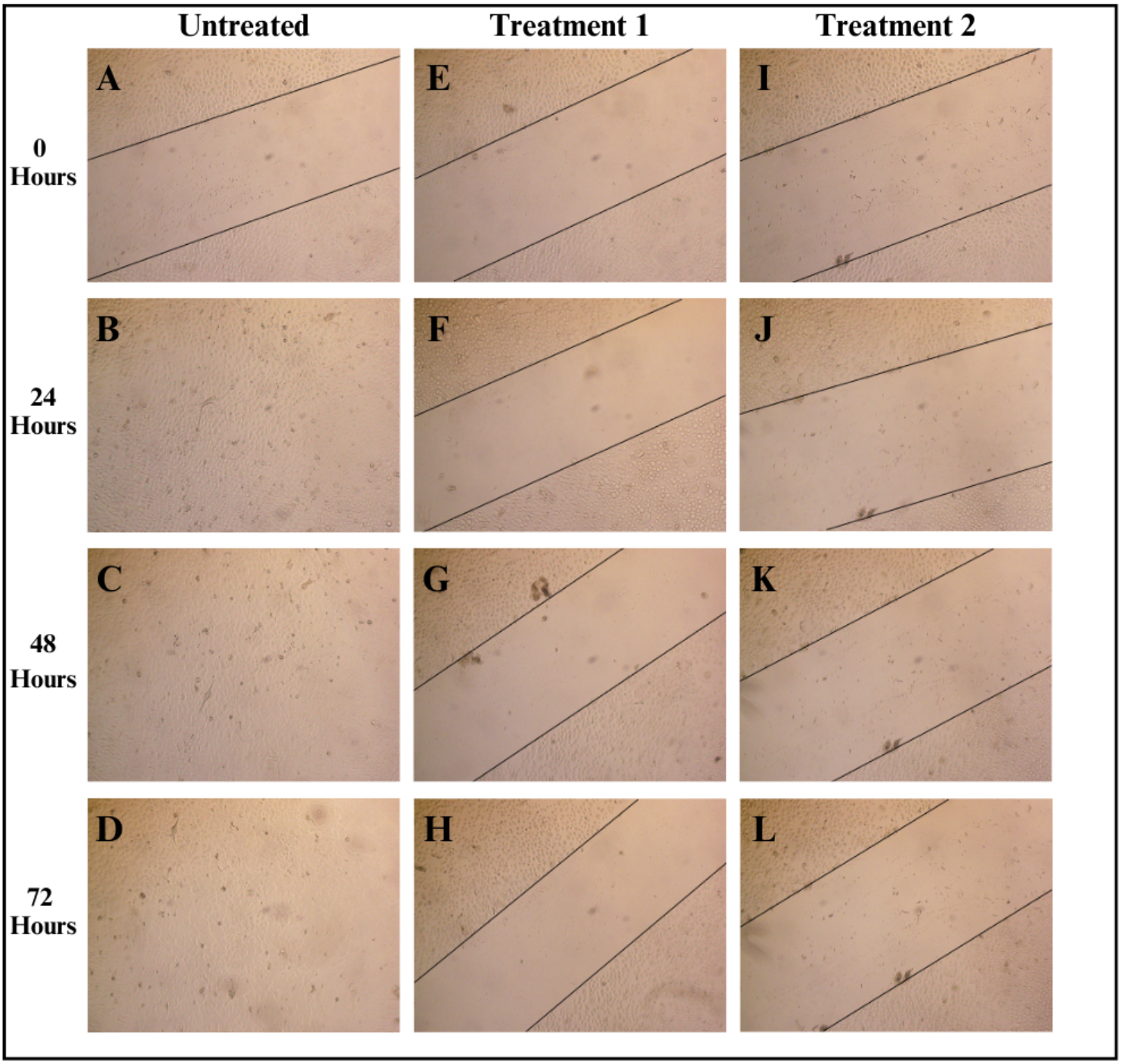

Figure 10. Migration pattern of EPC1 cells into the wound observed in biological replicates 1 and 2. The untreated group quickly migrated into the wound, reaching almost 100% confluency after 72 hours. Both treatment groups did not migrate into the wound at all (n=2, total magnification=50X).

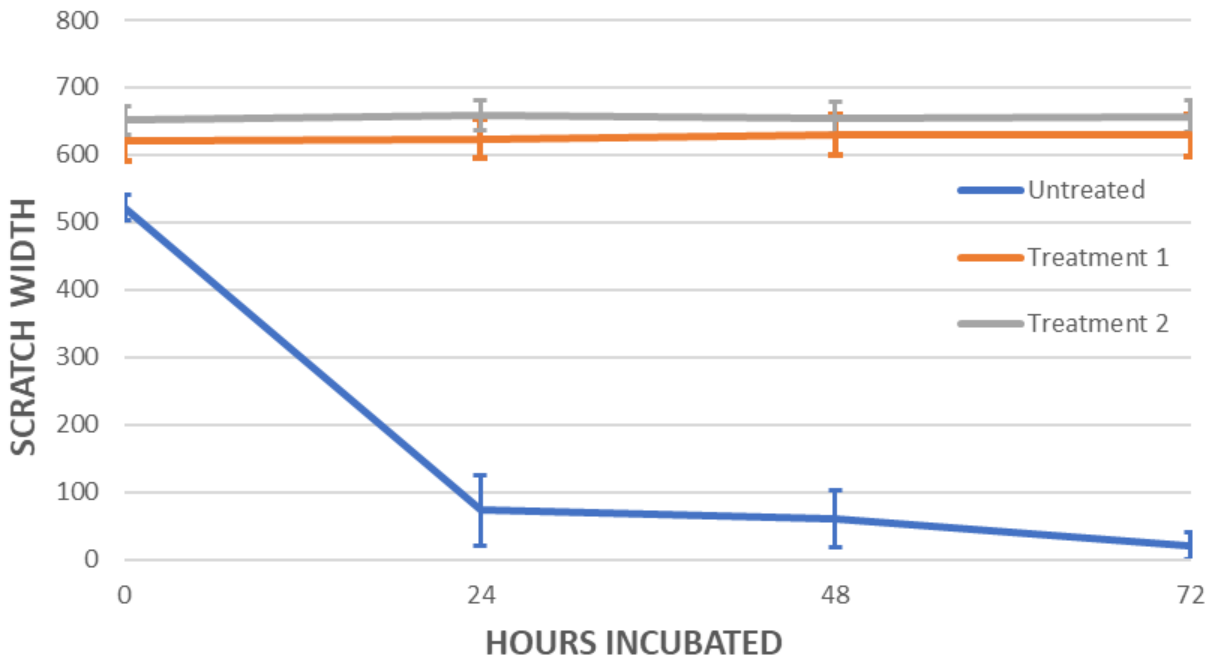

Figure 11. Average scratch width measured in a wound healing assay (biological replicates 1 and 2). The scratch width decreased at each timepoint for the untreated group. The treated groups demonstrated no significant migration into the wound ($p>0.05$). Error bars represent the standard error of the mean (n=2).

Table 5. Average scratch width (from first two biological replicates) measured for wound healing assay (± SEM). EPC1 cells exposed to vape juice aerosol demonstrated decreased migration into the scratch. Scratch width was measured using ImageJ ($p<0.01$; n=2).

	0 Hours	**24 Hours**	**48 Hours**	**72 Hours**
Treatment 1	621.1 ± 29.3	624.2 ± 27.9	630.3 ± 29.5	629.1 ± 30.7
Treatment 2	651.7 ± 21.4	658.4 ± 21.5	654.8 ± 23.8	657.5 ± 22.8
Untreated	521.8 ± 19.6	72.5 ± 53.1	59.3 ± 42.3	20.3 ± 20.3

Table 6. Average percentage of scratch closure from the wound healing assay (biological replicates 1 and 2). EPC1 cells exposed to vape juice aerosol had decreased migration in comparison to untreated cells ($p<0.01$; $n=2$).

	0 Hours	24 Hours	48 Hours	72 Hours
Treatment 1	0%	-0.58%	-1.49%	-1.26%
Treatment 2	0%	-1.04%	-0.42%	-0.85%
Untreated	0%	86.8%	89.3%	96.3%

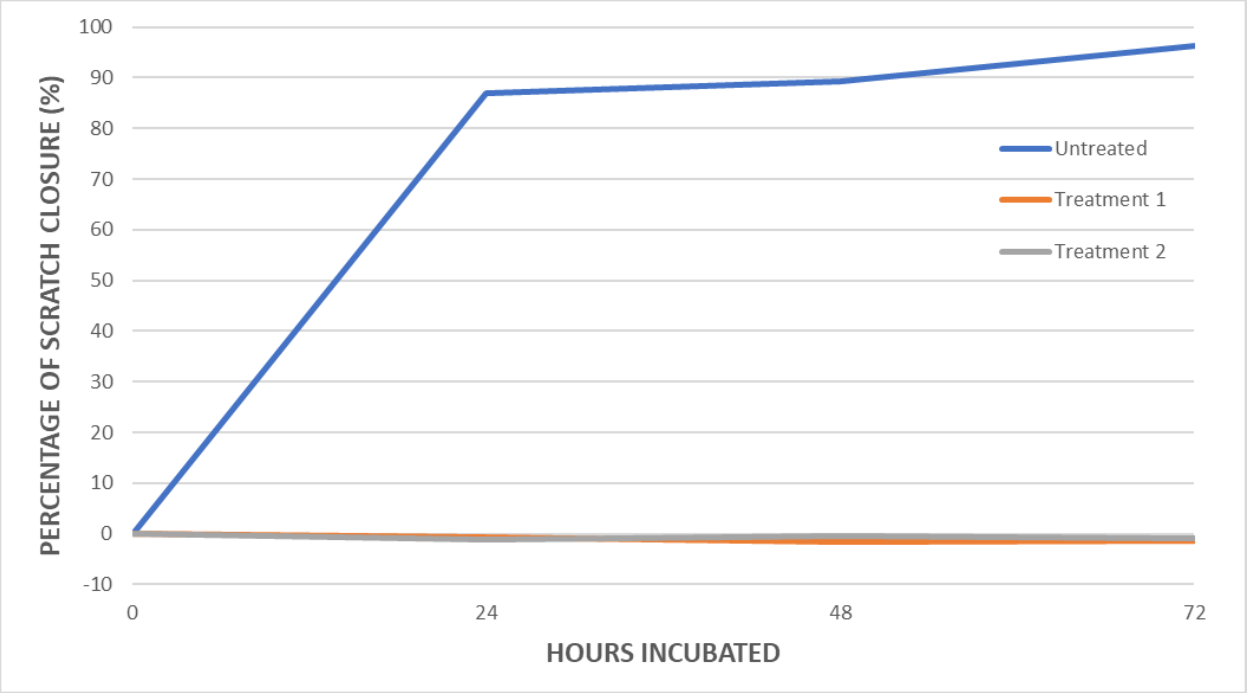

Figure 12. Average percentage of scratch closure (biological replicates 1 and 2) from the wound healing assay. The untreated cells almost completely filled the wound after 72 hours. Neither of the treated groups migrated into the scratch at all ($n=2$).

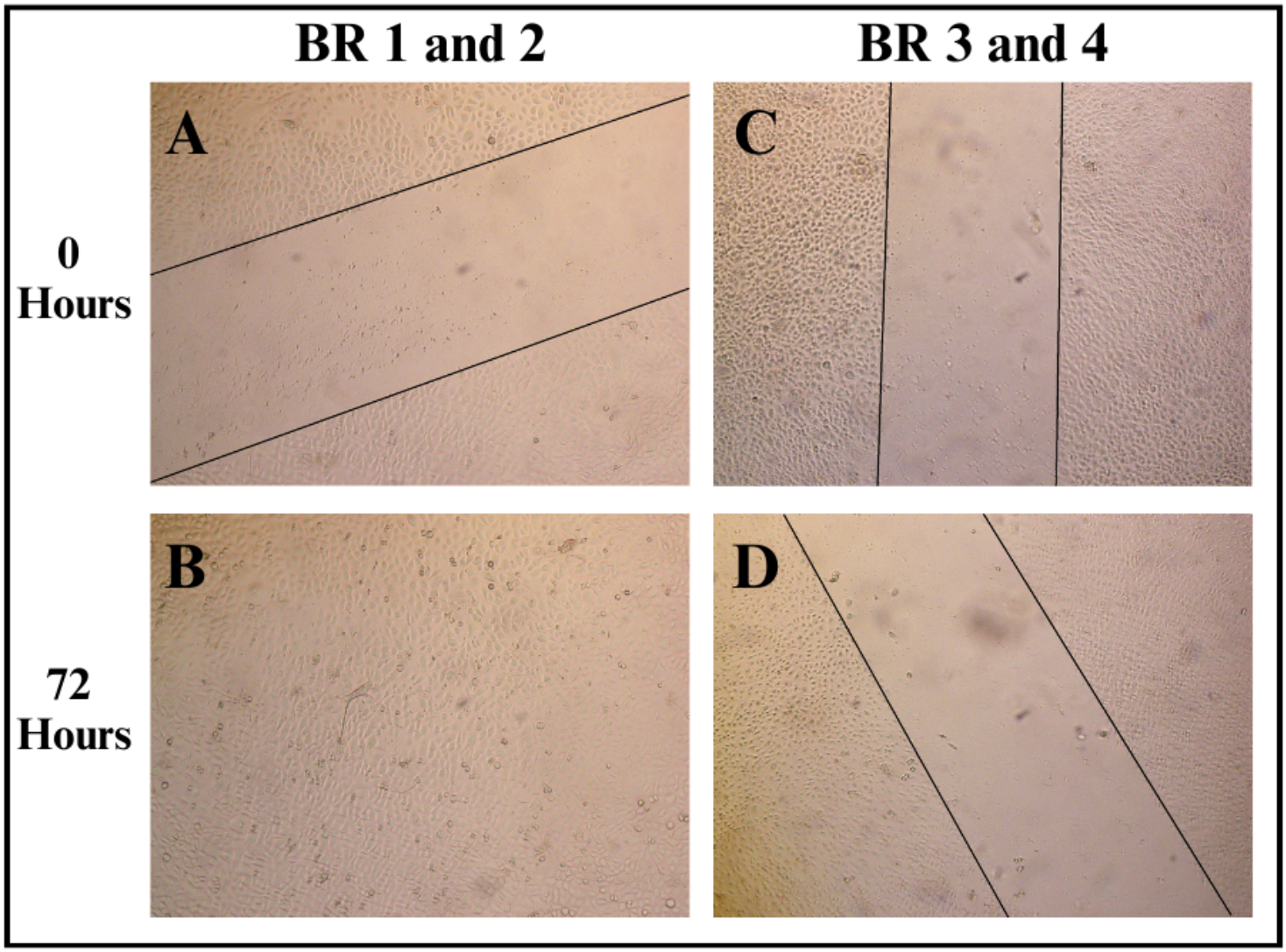

Figure 13. Difference in migration of the untreated groups by biological replicate (BR). Biological replicates 1 and 2 demonstrated significant migration into the wound in the untreated group, whereas biological replicates 3 and 4 demonstrated no migration into the wound at all in the untreated group (n=2, total magnification=50X).

Table 7. Average scratch width (from third and fourth biological replicates) measured for wound healing assay (± SEM). These data differed significantly from the first two biological replicates (Table 5). None of the cells migrated into the scratches, regardless of exposure to vaping. Scratch width was measured using ImageJ (n=2).

	0 Hours	24 Hours	48 Hours	72 Hours
Treatment 1	642.8 ± 23.9	636.5 ± 21.2	634.4 ± 22.9	656.5 ± 18.9
Treatment 2	644.3 ± 25.4	634.8 ± 29.1	645.7 ± 28.7	661.2 ± 19.4
Untreated	598.1 ± 38.0	532.4 ± 36.5	619.4 ± 28.3	600.4 ± 29.7

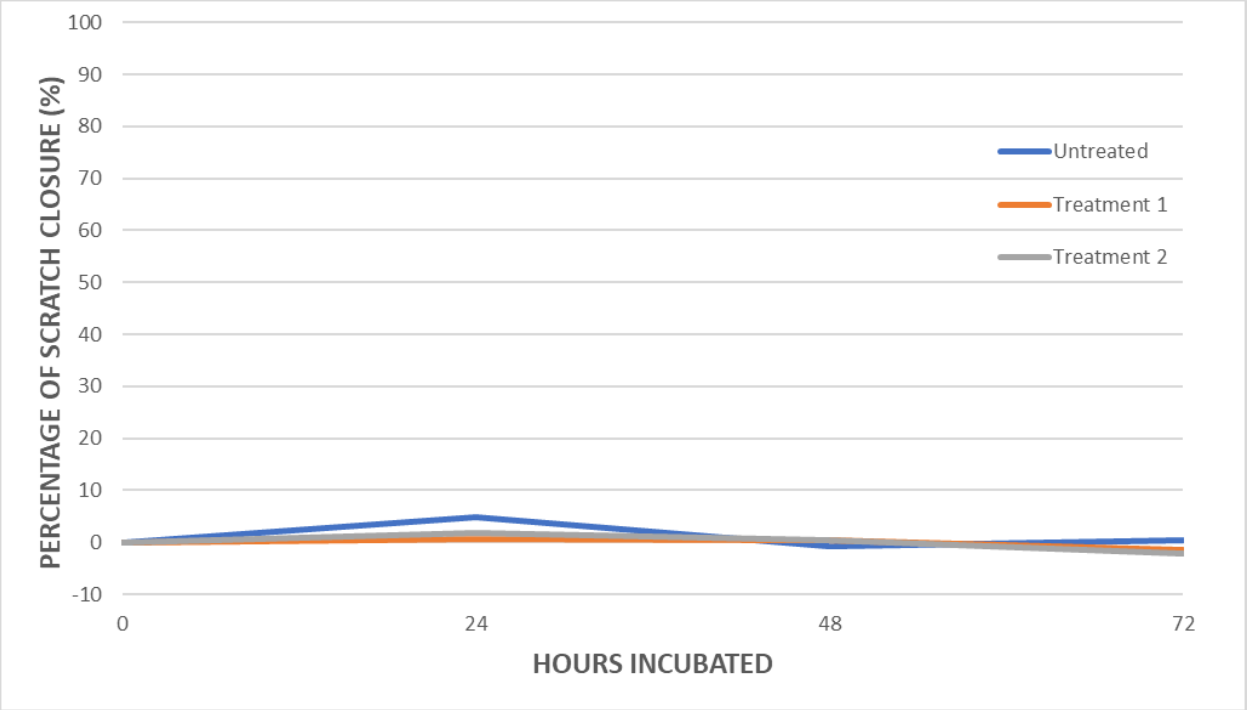

Figure 14. Average percentage of scratch closure (biological replicates 3 and 4) from the wound healing assay. There was no difference in migration between experimental groups over 72 hours (n=2).

Table 8. Total number of live cells counted after 72 hours of the wound healing assay. EPC1 cells exposed to vape juice aerosol had a decreased number of live cells. Furthermore, both treatment groups had a decreased percentage of live cells (T1=62.5%, T2=56.1%) in comparison to the untreated cells (84.3%; n=4).

	Biological Replicate 1	**Biological Replicate 2**	**Biological Replicate 3**	**Biological Replicate 4**
Treatment 1	8,333	8,333	83,333	25,000
Treatment 2	4,167	16,667	79,167	33,333
Untreated	354,167	541,667	379,167	225,000

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Literature Cited

1. Bajwa SA, Toro F, Kasi A. *Physiology, Esophagus.* In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023. Available from: https://www.ncbi.nlm.nih.gov/books/NBK519011/.
2. Curtis BJ, Radek KA. Cholinergic regulation of keratinocyte innate immunity and permeability barrier integrity: new perspectives in epidermal immunity and disease. *J Invest Dermatol*. 2012;132(1):28-42. doi:10.1038/jid.2011.264.
3. Key Statistics for Esophageal Cancer. American Cancer Society. Published January 12, 2023. Accessed November 9, 2023. https://www.cancer.org/cancer/types/esophagus-cancer/about/key-statistics.html#:~:text=Esophageal%20cancer%20is%20more%20common,that%20can%20affect%20these%20chances.
4. Olle DA. Esophageal cancer. *Salem Press Encyclopedia of Health*. January 2023. Accessed November 9, 2023. https://research.ebsco.com/linkprocessor/plink?id=d44d8bdb-ad4d-3338-8f25-dfe873e006fc.
5. What Is Cancer of the Esophagus? American Cancer Society. Published March 20, 2020. Accessed November 9, 2023. https://www.cancer.org/cancer/types/esophagus-cancer/about/what-is-cancer-of-the-esophagus.html.
6. Esophageal Cancer Risk Factors. American Cancer Society. Published June 9, 2020. Accessed November 9, 2023. https://www.cancer.org/cancer/types/esophagus-cancer/causes-risks-prevention/risk-factors.html.
7. Chang P, Friedenberg F. Obesity and GERD. *Gastroenterol Clin North Am*. 2014;43(1):161-173. doi:10.1016/j.gtc.2013.11.009.
8. Treating Esophageal Cancer by Stage. American Cancer Society. Published June 2, 2022. Accessed November 9, 2023. https://www.cancer.org/cancer/types/esophagus-cancer/treating/by-stage.html.

9. Then EO, Lopez M, Saleem S, Gayam V, Sunkara T, Culliford A, Gaduputi V. Esophageal Cancer: An Updated Surveillance Epidemiology and End Results Database Analysis. *World J Oncol*. 2020;11(2):55-64. doi:10.14740/wjon1254.
10. SEER Cancer Stat Facts: Common Cancer Sites. National Cancer Institute. Accessed November 9, 2023. https://seer.cancer.gov/statfacts/html/common.html.
11. Survival Rates for Esophageal Cancer. American Cancer Society. Published March 1, 2023. Accessed November 9, 2023. https://www.cancer.org/cancer/types/esophagus-cancer/detection-diagnosis-staging/survival-rates.html.
12. Prono L. Electronic Cigarettes. *Salem Press Encyclopedia*. May 2022. Accessed November 9, 2023. https://research.ebsco.com/linkprocessor/plink?id=09c07b1d-be46-3d4b-9f7d-9863a065cd71.
13. About Electronic Cigarettes (E-Cigarettes). Centers for Disease Control and Prevention. Published May 4, 2023. Accessed November 9, 2023. https://www.cdc.gov/tobacco/basic_information/e-cigarettes/about-e-cigarettes.html.
14. New Vintage 2021 Population Estimates Available for the Nation, States and Puerto Rico. United States Census Bureau. Published December 21, 2021. Accessed November 9, 2023. https://www.census.gov/newsroom/press-releases/2021/2021-population-estimates.html#:~:text=The%20voting%2Dage%20resident%20population,of%20the%20population%20in%202021.
15. Park-Lee E, Ren C, Cooper M, Cornelius M, Jamal A, Cullen KA. Tobacco Product Use Among Middle and High School Students - United States, 2022. *MMWR Morb Mortal Wkly Rep.* 2022;71(45):1429-1435. Published 2022 Nov 11. doi:10.15585/mmwr.mm7145a1.
16. Seiler-Ramadas R, Sandner I, Haider S, Grabovac I, Dorner TE. Health effects of electronic cigarette (e-cigarette) use on organ systems and its implications for public health. *Wien Klin Wochenschr*. 2021;133(19-20):1020-1027. doi:10.1007/s00508-020-01711-z.

17. Hartmann-Boyce J, McRobbie H, Bullen C, Begh R, Stead LF, Hajek P. Electronic cigarettes for smoking cessation. *Cochrane Database Syst Rev*. 2016;9(9):CD010216. Published 2016 Sep 14. doi:10.1002/14651858.CD010216.pub3.
18. QuickStats: Cigarette Smoking Status Among Current Adult E-cigarette Users, by Age Group — National Health Interview Survey, United States, 2015. *MMWR Morb Mortal Wkly Rep.* 2016;65:1177. DOI: http://dx.doi.org/10.15585/mmwr.mm6542a7.
19. Travis N, Knoll M, Cadham CJ, Cook S, Warner KE, Fleischer NL, Douglas CE, Sánchez-Romero LM, Mistry R, Meza R, Hirschtick JL, Levy DT. Health Effects of Electronic Cigarettes: An Umbrella Review and Methodological Considerations. *Int J Environ Res Public Health*. 2022;19(15):9054. Published 2022 Jul 25. doi:10.3390/ijerph19159054.
20. McNeil A, Brose LS, Calder R, Hitchman SC, Hajek P, McRobbie H. 2015. *E-cigarettes: an evidence update. A report commissioned by Public Health England.* https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/733022/Ecigarettes_an_evidence_update_A_report_commissioned_by_Public_Health_England_FINAL.pdf
21. Kaisar MA, Prasad S, Liles T, Cucullo L. A decade of e-cigarettes: Limited research & unresolved safety concerns. *Toxicology*. 2016;365:67-75. doi:10.1016/j.tox.2016.07.020.
22. Coke LA. Vaping and Use of E-Cigarette Products in Adolescents: A New Cardiopulmonary Crisis. *J Cardiovasc Nurs.* 2020;35(3):225-228. doi:10.1097/JCN.0000000000000679.
23. Ayton P, Weiss-Cohen L. Smoking versus vaping: how (not) to communicate their relative harms. *Journal of Risk Research*. 2021;24:2, 198-214, DOI: 10.1080/13669877.2020.1749117.
24. Wiley ER, Seabrook JA. Nicotine and Nicotine-Free Vaping Behavior among a Sample of Canadian High School Students: A Cross-Sectional Study. *Children (Basel)*. 2023;10(2):368. Published 2023 Feb 13. doi:10.3390/children10020368.
25. Zuckermann AME, Williams GC, Battista K, Jiang Y, de Groh M, Leatherdale ST. Prevalence and correlates of youth poly-substance use in the COMPASS study. *Addict Behav*. 2020;107:106400. doi:10.1016/j.addbeh.2020.106400.

26. Gillman IG, Pennington ASC, Humphries KE, Oldham MJ. Determining the impact of flavored e-liquids on aldehyde production during Vaping. *Regul Toxicol Pharmacol.* 2020;112:104588. doi:10.1016/j.yrtph.2020.104588.
27. Tobacco. National Cancer Institute. Published January 23, 2017. Accessed November 9, 2023. https://www.cancer.gov/about-cancer/causes-prevention/risk/tobacco.
28. Pinkston R, Zaman H, Hossain E, Penn AL, Noël A. Cell-specific toxicity of short-term JUUL aerosol exposure to human bronchial epithelial cells and murine macrophages exposed at the air-liquid interface. *Respir Res*. 2020;21(1):269. Published 2020 Oct 17. doi:10.1186/s12931-020-01539-1.
29. Cervellati F, Muresan XM, Sticozzi C, et al. Comparative effects between electronic and cigarette smoke in human keratinocytes and epithelial lung cells. *Toxicol In Vitro*. 2014;28(5):999-1005. doi:10.1016/j.tiv.2014.04.012.
30. Rouabhia M, Piché M, Corriveau MN, Chakir J. Effect of e-cigarettes on nasal epithelial cell growth, Ki67 expression, and pro-inflammatory cytokine secretion. *Am J Otolaryngol*. 2020;41(6):102686. doi:10.1016/j.amjoto.2020.102686.
31. Gonzalez-Suarez I, Marescotti D, Martin F, Scotti E, Guedj E, Acali S, Dulize R, Baumer K, Peric D, Frentzel S, Ivanov NV, Hoeng J, Peitsch MC. In Vitro Systems Toxicology Assessment of Nonflavored e-Cigarette Liquids in Primary Lung Epithelial Cells. Applied *In Vitro Toxicology* 2017 3:1, 41-55. doi:10.1089/aivt.2016.0040.
32. Calibasi-Kocal G, Basbinar Y. Introductory Chapter: Cancer Metastasis. *IntechOpen*. 2018. doi:10.5772/intechopen.81914.
33. Alanazi H, Park HJ, Chakir J, Semlali A, Rouabhia M. Comparative study of the effects of cigarette smoke and electronic cigarettes on human gingival fibroblast proliferation, migration and apoptosis. *Food Chem Toxicol*. 2018;118:390-398. doi:10.1016/j.fct.2018.05.049.
34. Shaikh ZN, Alqahtani A, Almela T, Franklin K, Tayebi L, Moharamzadeh K. Effects of electronic cigarette liquid on monolayer and 3D tissue-engineered models of human gingival mucosa. *J Adv Periodontol Implant Dent*. 2019;11(2):54-62. Published 2019 Dec 18. doi:10.15171/japid.2019.010.

35. Duesberg P, McCormack A. Immortality of cancers: a consequence of inherent karyotypic variations and selections for autonomy. *Cell Cycle*. 2013;12(5):783-802. doi:10.4161/cc.23720.
36. Kumar P, Nagarajan A, Uchil PD. Analysis of Cell Viability by the Lactate Dehydrogenase Assay. *Cold Spring Harb Protoc*. 2018;2018(6):10.1101/pdb.prot095497. Published 2018 Jun 1. doi:10.1101/pdb.prot095497.
37. de Lima JM, Macedo CCS, Barbosa GV, Castellano LRC, Hier MP, Alaoui-Jamali MA, da Silva SD. E-liquid alters oral epithelial cell function to promote epithelial to mesenchymal transition and invasiveness in preclinical oral squamous cell carcinoma. *Sci Rep*. 2023;13(1):3330. Published 2023 Feb 27. doi:10.1038/s41598-023-30016-0.
38. Piedrafita G, Kostiou V, Wabik A, Colom B, Fernandez-Antoran D, Herms A, Murai K, Hall BA, Jones PH. A single-progenitor model as the unifying paradigm of epidermal and esophageal epithelial maintenance in mice. *Nat Commun.* 2020;11(1):1429. Published 2020 Mar 18. doi:10.1038/s41467-020-15258-0.
39. Beauval N, Verrièle M, Garat A, Fronval I, Dusautoir R, Anthérieu, S, Garçon G, Lo-Guidice JM, Allorge D, Locoge N. Influence of puffing conditions on the carbonyl composition of e-cigarette aerosols. *Int J Hyg Environ Health*. 2019;222(1):136-146. doi:10.1016/j.ijheh.2018.08.015.
40. Gupta A,Tayyarah R, Gillman G, Garner C, Stevens R. Machine Vaping of Electronic Cigarettes - A Comparison of Puffing Regimes#. *Contributions to Tobacco & Nicotine Research*. 2021;30(3) 127-136. doi:10.2478/cttr-2021-0009.

Images

Drzezo. Normal esophageal anatomy and physiology. Abdominal Key. Published August 1, 2016. Accessed November 9, 2023. https://abdominalkey.com/normal-esophageal-anatomy-and-physiology/