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Effects of E-cigarette Vapor on Zebrafish Cell Regeneration and Wound Healing

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I. Abstract:

The use of electronic cigarettes has skyrocketed in recent years, especially among adolescents. According to the FDA's 2018 National Youth Tobacco Survey, there has been a 78% increase in e-cigarette use in high school students from 2017 to 2018 and a 48% increase among middle school students (Center for Tobacco Products, 2020). In regard to wound healing, smoking traditional tobacco cigarettes is known to have negative effects, such as lowering oxygen availability and increasing skin necrosis (Aköz et al., 2002). The full physiological effects of e-cigarettes are not known, and research is being performed to improve our understanding on the effects that these products may have on our bodies. Zebrafish have recently been used as a model organism for studying the effects of cutaneous wound healing (Richardson et al., 2013). They have an adaptive immune system as adults which gives them an advantage for research over other vertebrates for immune system and healing studies (Novoa & Figueras, 2012). This research is focused on studying the effects of electronic cigarette vapor on wound healing by comparing the pigmentation level changes of adult zebrafish after they undergo a caudal fin clip procedure. The level of pigmentation was hypothesized to be reduced in the zebrafish exposed to the e-cigarette chemicals compared to zebrafish who were not exposed to e-cigarette chemicals. The results indicate that electronic cigarette vapor had no significant effects on the pigment pattern in adult Zebrafish. There was no significant difference in pigment area fraction change (ΔPAF) between treatment groups. Future study is needed to determine if higher exposure levels will cause an effect. If zebrafish are immune to the detrimental effects of nicotine and electronic cigarette vapor, further research is needed to determine the mechanisms behind this, as these pathways may hold the answers to improved wound healing treatments and applications.

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II. Introduction: **Cowritten with Jenna Pumneo**

Since its commercial success after its release in 2003, the use of e-cigarettes has dramatically risen, not only for their use in quitting smoking, but also as a new drug that is popular among young individuals. According to the FDA's 2018 National Youth Tobacco Survey, there has been a 78% increase in e-cigarette use in high school students from 2017 to 2018 and a 48% increase among middle school students (Center for Tobacco Products, 2020). E-cigarettes are marketed as being less harmful than traditional tobacco cigarettes, but they are still known to have many negative health effects. In regard to wound healing, smoking traditional tobacco cigarettes is known to have detrimental effects (Aköz et al., 2002). An increased risk of skin necrosis has been observed in patients who smoke throughout the surgical process (Aköz et al., 2002). It is known that nicotine, whether introduced through inhalation or intravenously, leads to peripheral skin vasoconstriction (Aköz et al., 2002). Nicotine exposure on humans has been shown to cause a decrease in cutaneous blood flow (Davies & Ismail, 2016). This leads to negative effects in wound healing, such as the observed skin necrosis. Other negative known effects on wound healing from tobacco cigarettes are from carbon monoxide. The carbon monoxide binds more strongly to hemoglobin than oxygen making it difficult for oxygen to reach the tissue in need of repair (Aköz et al., 2002). The full physiological effects of e-cigarettes are not known, and research continues to improve our understanding regarding the effects that these products may have on our bodies.

Further study is needed on e-cigarette effects on the whole body and on how prolonged exposure may affect wound healing, particularly in regard to pigmentation and scarring. This research is intended for that purpose. Zebrafish have recently been used as a model organism for

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studying the effects of cutaneous wound healing (Richardson et al., 2013). Zebrafish, because of their remarkable ability to regenerate tissue with little scarring, make excellent model organisms for studying wound healing (Poss et al., 2003). They develop an adaptive immune system as adults which gives them an advantage for research over other vertebrates for immune system and healing studies, as their short life cycle means they reach adulthood sooner, and thus develop the necessary immunity sooner (Novoa & Figueras, 2012). In addition, zebrafish are small in size, have relatively rapid life cycle, and breed easily, making them ideal for lab studies (Novoa & Figueras, 2012).

This research is aimed at answering the scientific question of how the chemicals in e-cigarettes may affect wound healing and regeneration of the caudal fin of adult zebrafish after prolonged use of the chemicals in e-cigarettes. We are interested in comparing the level of pigmentation of the caudal fin after the fin clip procedure to the levels before the fin clip. This will allow us to determine if e-cigarette vapor exposure effects the regenerative abilities and the scarring processes of the zebrafish.

The level of pigmentation in the regenerated fins will be compared for three separate test groups of fish. Treatment Group One (TG1) will be exposed to e-cigarette vapor only before the dissection. Treatment Group Two (TG2) will be exposed to e-cigarette vapor both before and after fin dissection. The final Control Group (CG) will not be exposed to any e-cigarette vapor (Control Group). It was hypothesized that the level of pigmentation will be reduced in treatment groups two and one when compared to the control group, as nicotine is known to reduce oxygen to healing wounds, leading to decreased function and potential death of the pigment stem cells utilized in the wound healing process (Page et al., 2016; Rawls & Johnson, 2000, 2001).

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III. Materials Methods: Cowritten with Jenna Pumneo

All methods were approved on July28, 2020 by The University of Akron Institutional Animal Care and Use Committee (IACUC) according to protocol number 20-02-05 BFD.

A. Animals & Treatment Groups

To assess the effects electronic cigarette vapor has on healing and regeneration processes, 48 adult Zebrafish (*Danio rerio*) were obtained from The University of Akron vivarium. Requirements for selection included: healthy male or female with no lesions or physical deformations, non-lethargic, and older than three months.

Zebrafish were randomly split into three groups, each group containing 16 fish. Treatment group two (TG2) was exposed to e-cigarette smoke both before and after caudal fin amputation. Treatment group one (TG1) was exposed to e-cigarette smoke only before caudal fin amputation. The control group (CG) was not exposed to e-cigarette smoke throughout the entire experiment.

B. Aquarium Design

Adult Zebrafish were housed in six tanks (**Figure 1**), intentionally constructed by the lab to isolate fish for continuous individual observation. Each tank was divided into 10 sections with a mesh filter connecting each section. The mesh filter allowed for consistent water movement and quality throughout the entire tank. The front and back sections spanned the entire width of the tank. No fish were housed here, instead, outflow tubes were situated to drain water from the tank into the sump. Between these sections, eight equal partitions housed eight individual fish. Above each partition, an inflow tube connected to an electric motor deposited water from the connected sump. Each treatment group was connected to individual sump systems, meaning two tanks each were connected to one sump. This was to ensure equal exposure levels to both tanks in each

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treatment group. Within the sumps, Marineland C-Series Canister Bio-Filter Balls Filter Media, were utilized to promote healthy bacteria growth. The aquarium was connected to an automatic timer to maintain a 14:10 hour light/dark cycle which imitates the natural molecular clock of Zebrafish. No mechanical filters were used in the setup as they would potentially remove the electronic cigarette vapor from the water.

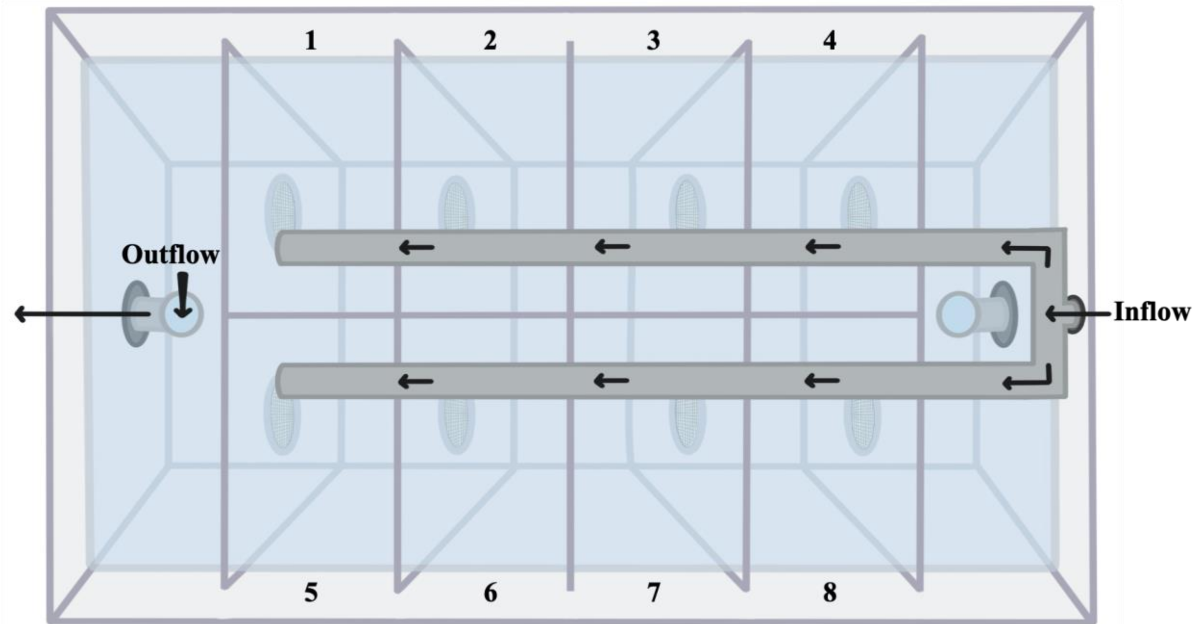

Figure 1. Aquarium design shown from the aerial perspective. Each numbered partition housed one zebrafish. Tubes connecting the inflow and outflow to the sump are not pictured.

C. Husbandry

Adult Zebrafish received standard husbandry and care following the approved University of Akron Institutional Animal Care and Use Committee (IACUC) protocol. Prior to the experiment, the aquarium was filled with dechlorinated water and cycled using 13 Zebrafish, (two to three fish in each tank). Water quality was tested daily until ammonia, nitrite, and nitrate

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concentrations were cycled. Water quality was tested as needed after cycling fish were replaced by experimental fish.

Twenty-four hours prior to first exposure of vapor infused water, the experimental fish were acclimated to the aquarium. Throughout the experiment, water temperature in each sump was maintained between 27 to 29°C by two Eheim Jager Thermostat Aquarium Heater, 250-watt water heaters. The fish were fed Gemma 500 twice daily. Tanks were cleaned every other day to maintain adequate water quality and water movement throughout the system. Partial water changes of approximately 16.6% were performed daily for each sump. 15 L of water was changed from each sump and collected into labeled five-gallon buckets using a syphon. Additional water changes were performed when nonstandard ammonia, nitrite, or nitrate concentrations were detected. Water quality was checked using the API Freshwater Aquarium Master Test Kit, 800 count. Paper and electronic records were kept documenting each tank's daily temperatures, feeding, cleaning, water changes, deaths, and water quality concentrations. Full records can be found in the appendix section.

D. Vapor Chemicals

Vapor was collected using a three-way valve syringe apparatus (**Figure 2**) connected to the Joyetech eGo AIO, second-generation vaping device. At the start of every collection, the device was fully charged, and the mouthpiece was positioned to allow maximum airflow. The liquid holding chamber was filled to the max fill line with 70:30 PG:VG, cinnamon flavored, 18mg/mL nicotine vaping liquid. To imitate standard vaping habits and to prevent overheating of the vape pen, each puff lasted four seconds with 30 seconds between each puff. To further

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prevent the coil from overheating, a device was used for no more than nine puffs within a 15-minute window.

Each four-second-long puff collected 55mL of vapor within the syringe. The vapor was expelled into a flask containing 1000mL of deionized (DI) water. The vapor was infused into the water through vigorous shaking. A total of 18 puffs were infused into every 1000mL of DI water. Several gallons of vapor infused water were collected at a time and diluted to a concentration of nine puffs per L for water changes. Nine puffs per L was hypothesized to be a physiologically relevant concentration for zebrafish (Matta et al., 2007).

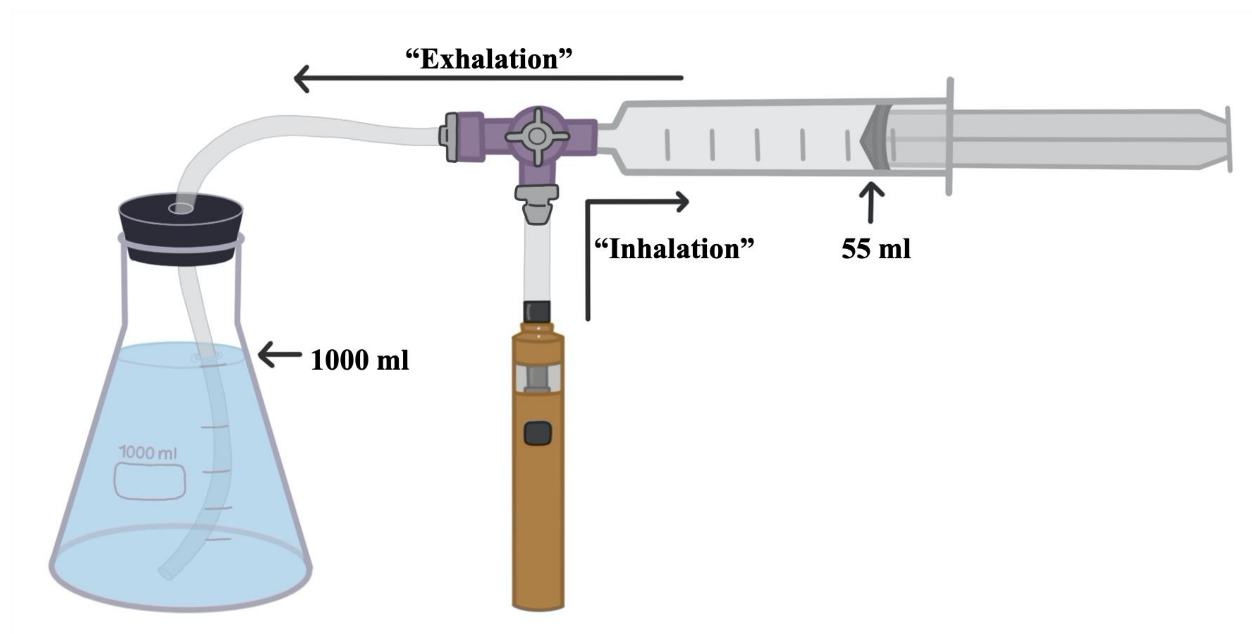

Figure 2. Three-way valve apparatus connecting the vaping device, syringe, and Erlenmeyer flask containing 1000ml DI water.

E. Exposure

Prior to fin amputation, both treatment groups received vapor infused water changes at a concentration of nine puffs per liter for two weeks. Following fin amputation, vapor infused

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water changes were continued for TG2 to stimulate prolonged exposure to e-cigarette vaping. Exposure continued until the end of the designated regeneration period. TG1 underwent a full system water change to reduce remaining vapor chemicals after their exposure period was complete. For the duration of the experiment, CG received water changes with no vapor infusion. Care was taken with each water change to avoid contaminating clean materials with vapor infused water.

F. Procedure

The three groups underwent 50% caudal fin clip two weeks after the initial exposure period. For this procedure, individual fish were transferred from their tanks to water containing 0.01% tricaine methanesulfonate (MS-222). To maintain a pH of seven, the anesthetic agent was buffered with equal parts sodium bicarbonate. Fish were considered successfully anesthetized after observing a loss of equilibrium (change from ventral to dorsal decubitus position) and exhibited no response to a soft stimulus probing the caudal fin. Throughout this process, gill movement was also monitored to ensure the fish were continuing to respire. Zebrafish were then removed from the water and briefly placed into a Petri dish. When fish were not submerged in water, a pipette was used to continuously pass anesthetic water over their gills. This maintained the animals state of sedation and ensured continual respiration.

Caudal fins were then evenly spread out and photographed for the before procedure photograph using a Nikon D3500 camera attached to a Leica MZ12.5 Stereo Microscope. The microscope magnification was set to a constant magnification of 1.25 for all fish and the photographs were taken at 4000 x 6000 pixels. Either the top or bottom caudal fin was amputated using a sterilized scalpel. An even number of top and bottom fin amputations were performed and documented by randomly assigning each zebrafish into a top or bottom fin amputation

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group. After amputation, a two to three drops of 2 % Lidocaine was applied to the cut fins as a precautionary pain reliever. A post procedure photo was captured before recovering the fish.

Zebrafish were recovered individually in an aerated tank filled with heated dechlorinated water. Gills were monitored until equilibrium was restored and normal swimming behavior was observed. The fish were then returned to their separate tanks to be monitored post-operation.

G. Euthanasia

One week post procedure, half of each group was euthanized and photographed. Zebrafish euthanized at this time were randomly selected and used to document pigmentation levels halfway through the recovery period. The remaining fish were euthanized and photographed two weeks post procedure. For each group, individual fish were transferred from their tanks into water containing 0.05% MS-222 buffered with equal parts sodium bicarbonate. Fish were considered successfully euthanized after observing a loss of equilibrium, no response to soft stimuli, no response to strong stimuli, and lack of gill movement. Post regeneration photographs were taken using the same set up as the fin clip procedure. Death was then confirmed by performing a cervical severing with scissors. Fish were then collected and frozen.

H. Pigment Measurements

Pigment measurements were performed using ImageJ software in the following manner: For each fish, images from before and after the procedure, as well as post regeneration period were added into the ImageJ program. The polygon selection tool was used to trace the desired areas. These areas were added to the ROI manager as they were made. The areas measured for each fish were Total Area Before (TAB), Total Area After (TAA), Area Removed (AR), Total Area Post Growth (TAPG), Area Minus Regeneration (AMR), and Area Grown (AG). TAB was

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defined as the entire area of the fin before the procedure. TAA was defined as the area of the fin remaining after the fin clip procedure was performed. AR was defined as the region of fin that was removed during the fin clip procedure. TAPG was defined as the entire area of the fin after the designated regeneration period (one or two weeks depending on treatment group). AMR was defined as the area of the fin remaining after the fin clip procedure was performed. AMR should be the same value as TAA, and thus was used to scale the values to account for differences in fin spreading between photographs. AG was defined as the area of the fin that grew post procedure, for either one or two weeks depending on treatment group.

TAB was measured using the before procedure photo. TAA and AR were measured using the before procedure photo while comparing it to the after-procedure photo as a guide to where the fin clip was made. TAPG, AMR and AG were measured using the post regeneration period photos. The regions of interest for pigment measurements were the AR and AG areas.

Once the areas of interest were defined using the original photos, they needed to be adjusted for measurement. For pigment measurements, the before procedure and post regeneration photos were processed to remove their backgrounds using the subtract background tool. The rolling ball radius was set at 50 pixels and a light background was selected. After the background was removed, the image types were converted from RGB color to RGB stack. The red stack was used for further processing, as this gave the best boundary between the pigmented and nonpigmented areas. The threshold of the red stack was adjusted to define the pigmented areas for analysis. This was done using a lower threshold of zero and an upper threshold of 232. The settings were set to default, dark background, and black and white, so that the pigmented areas were defined as black and the nonpigmented areas were defined as white.

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After the image processing was complete, the ROI manager was used to select the AR from the before procedure photos. The measuring tool was then used to determine the area fraction of the selected regions. The area fraction determines the percentage of your selected area that is white, which was used to define the percentage of the removed fin that did not have pigment. This measurement was also performed for the AG region of the post regeneration photo.

The percentages of pigment before and after regeneration were used to determine the change in pigment area fraction in each fish, as described in the Analysis and Calculations section.

J. Analysis and Calculations

a. Pigment Area Fraction Change Calculations

The pigment in the AR and AG regions were measured using the area fraction. The change in pigment area fraction (ΔPAF) was determined using **Equation 1**. With AFAG being the area fraction of the area grown and AFAR being the area fraction of the are removed.

Equation 1: $\Delta PAF = AFAG - AFAR$

A positive ΔPAF indicates that there was more pigment in the post regeneration period than before the procedure. A negative ΔPAF indicates that there was less pigment in the post regeneration period than before the procedure.

b. Pigment Area Fraction Change Analysis

An ANOVA test was performed to determine if there was a significant difference ($P<0.05$) between treatment groups for the change in pigment area fraction. Tukey post hoc analyses were performed for any difference in treatment that was found to be significant.

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IV. Results

The data indicate that electronic cigarette vapor had no significant effects on the pigment pattern in adult Zebrafish. There was no significant difference in pigment change between treatment groups. All raw data and calculations can be found linked in the appendix section.

Figure 3 shows ΔPAF as a function of the treatment group for the one Week regeneration group. No significant difference in percent area fraction change was observed between the treatment groups (P= 0.933). The mean of CG (4.21 ± 1.80%) was less than the mean of TG1 (4.34 ± 1.68%). The mean of TG2 (3.51 ± 1.68%) was less than that of CG. These results were unexpected, as electronic cigarette liquid exposure was expected to increase pigmentation in regenerated fins.

Figure 4 shows ΔPAF as a function of the treatment group for the two weeks regeneration group. No significant difference in percent area fraction change was observed between the treatment groups (P= 0.579). The mean of CG (6.82 ± 1.61%) was more than the mean of TG1 (5.65 ± 1.61%). The mean of TG2 (4.22 ± 1.86%) was less than that of TG1 and CG. These results were unexpected, as electronic cigarette liquid exposure was expected to increase pigmentation in regenerated fins.

Figure 5 shows ΔPAF as a function of the treatment group with an overlay of both regeneration periods for comparison. **Figure 5** showed that the means of ΔPAF for the two weeks regeneration groups (4.21 ± 1.76%, 4.34 ± 1.65%, and 3.50 ± 1.65%) were larger than the means of the one-week regeneration groups (6.82 ± 1.76%, 5.65 ± 1.65%, and 4.22 ± 1.90%). This difference, however, was not significant (P=0.750).

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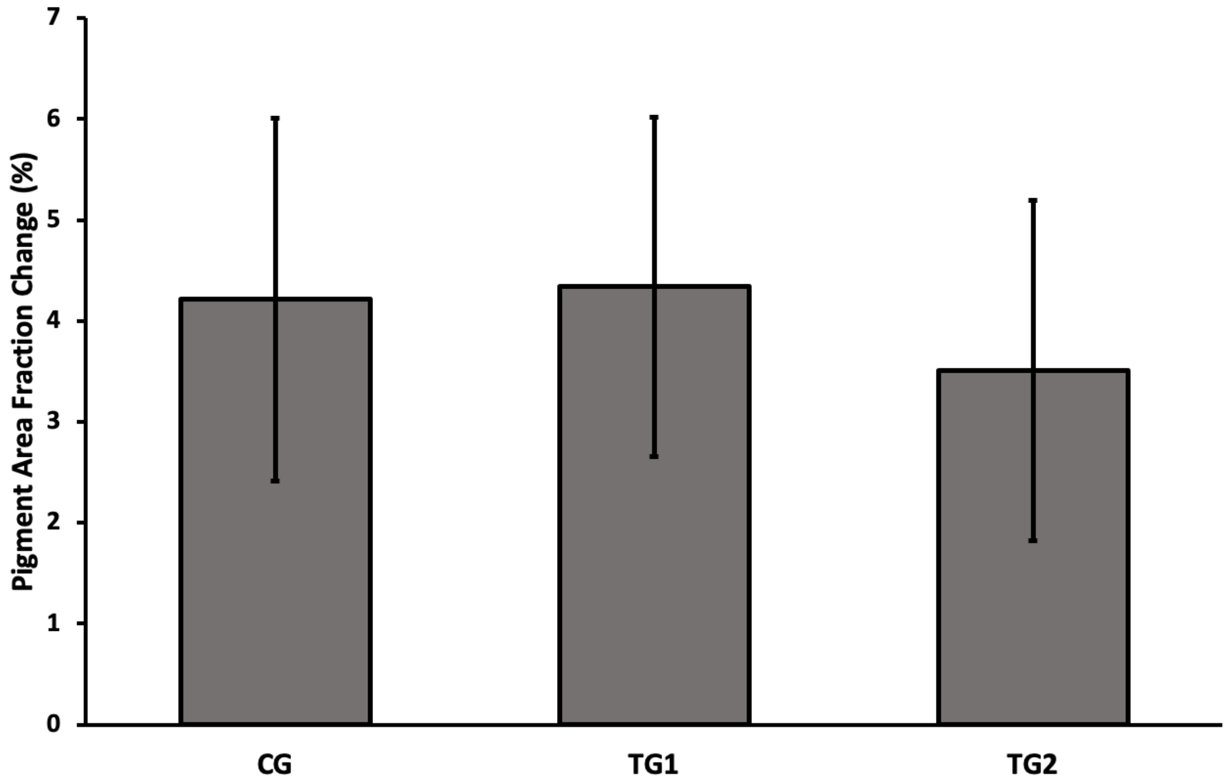

Figure 3: Pigment area fraction change (%) as a function of treatment group for the regeneration period of one week. Data indicate no significant difference in pigment area fraction change between groups (P= 0.9326). Error bars represent ±1 standard error.

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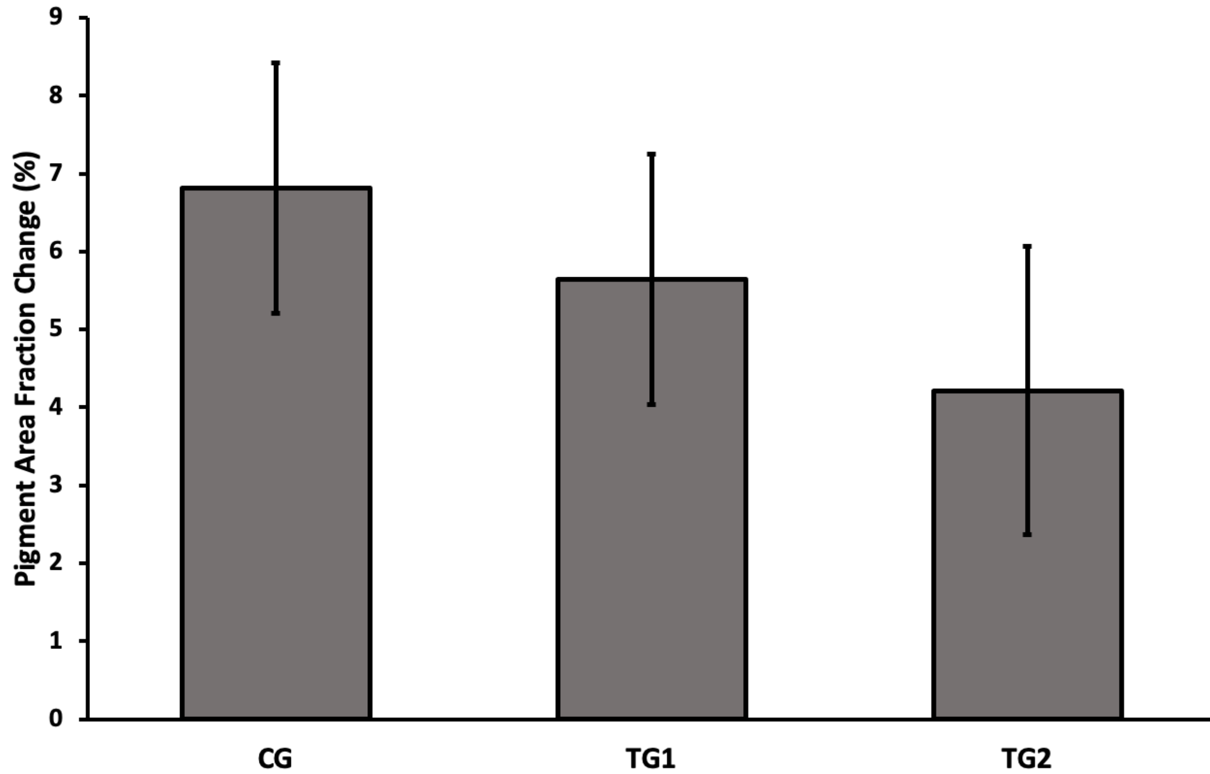

Figure 4: Pigment area fraction change (%) as a function of treatment group for the regeneration period of two weeks. Data indicate no significant difference in pigment area fraction change between groups (P= 0.5786). Error bars represent ±1 standard error.

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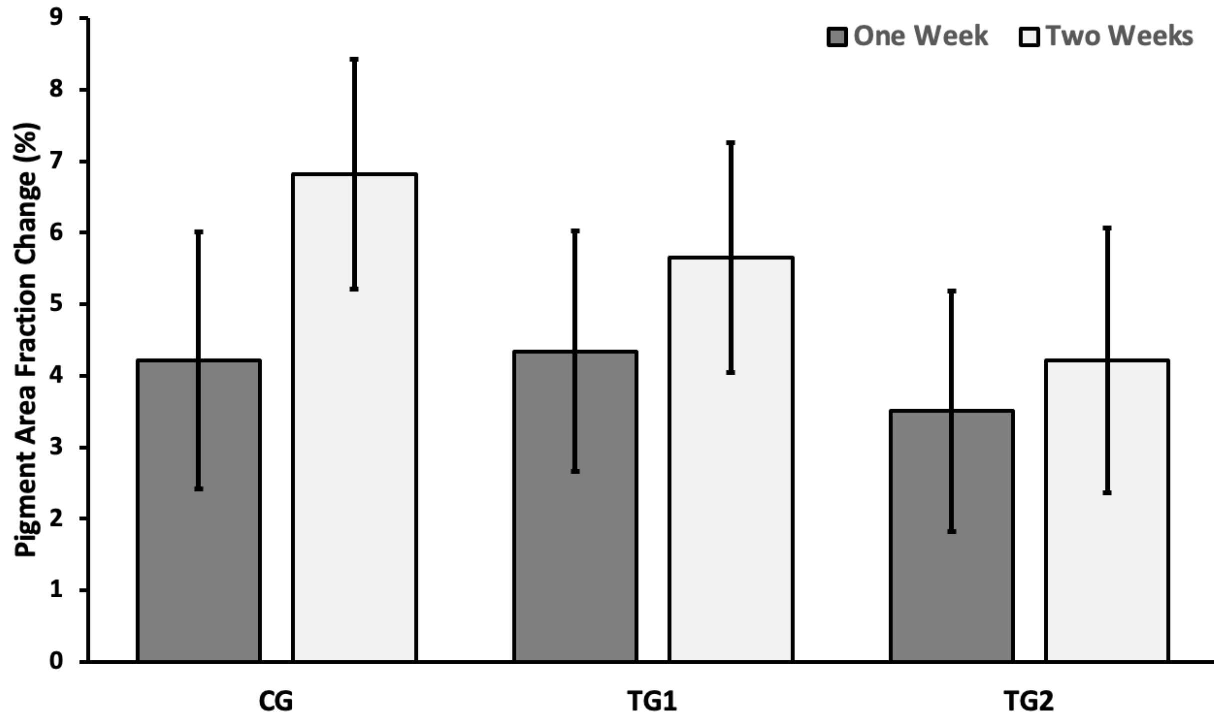

Figure 5: Pigment area fraction change (%) as a function of treatment group for the regeneration period of one and two weeks. Data indicate no significant difference in pigment area fraction change between one and two weeks (P= 0.7489). Error bars represent ±1 standard error.

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V. Discussion

This experiment revealed that electronic cigarette vapor exposure had no significant effects on pigment levels in adult zebrafish. These results were unexpected, as they do not coincide with previous findings that have shown deleterious effects of nicotine exposure, smoking, and electronic cigarette use on wound healing (Aköz et al., 2002; Davies & Ismail, 2016; Page et al., 2016; Rawls & Johnson, 2000).

No significant difference in change of pigment area fraction was found between treatment groups for either the one-week regeneration period or the two-week regeneration period. This was unexpected as use of cigarettes by patients post-surgery has been shown to impair wound epithelialization, decrease hemoglobin's oxygen affinity, and reduce oxygen delivery in humans (Aköz et al., 2002). There have been observations of a reduction in cutaneous blood flow after the use of electronic cigarettes (Page et al., 2016). It is known that a pigment stem cell population will begin to replicate to replace melanocytes upon amputation (Rawls & Johnson, 2000, 2001). The reduced carrying capacity of hemoglobin and reduced oxygen delivery mechanisms were hypothesized to impair stem cell replication and proliferation. Therefore, the pigmentation process was expected to be impaired, leading to a reduction in pigmentation after regeneration in the treatment groups. Evidence of this impairment was not found, as the results were not significant for either time frame. Zebrafish are known for their remarkable ability to regenerate tissue with little scarring (Poss et al., 2003). Mammals tend to heal through repair mechanisms, which leads to an imperfect healing process, often resulting in pigmentation and scaring (Poss et al., 2003). Zebrafish, and other some other low level vertebrates, heal through regeneration mechanisms rather than repair mechanisms, meaning that damaged cells and structures are flawlessly replaced (Poss et al., 2003). The blastema formation plays a crutial role

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in the regeneration process of Zebrafish (Poss et al., 2003). Dedifferentiation in blastemal cells may be occuring in Zebrafish, which would asist in the regeneration process, as it would allow for greater proliferation and migration of cells and materials (Poss et al., 2003). Because of possible dediferentiation and the presence of pigmentation stem cells seen in Zebrafish, they have the ability to quickly regenerate fins of near perfect quality. Dedifferenetiation and stem cell proliferation may not be affected by e-cigarette vapor in the same way that the repair mechanisms of mammals are, which would could explain the lack of significant resultas in this study. This regenenerative ablity may make them impervious to the scarring and pigmentation effects seen in other mammals, but the underlying mechanisms of this regeration need to be further explored for potential theraputic benefits.

The design of this experiment could be improved in several ways. The first way would be to extend the length of exposure to vapor, both before and after the fin clip procedure. This experiment used two weeks of exposure before and after the procedure, but a longer period of time may be necessary for the effects of long-term vapor exposure to take place. Another way to improve the experiment would be to add treatment groups with different vapor, flavor, and nicotine exposure levels. Our experiment only used exposure levels of 9 puffs/L of 70:30 PG:VG, cinnamon flavored, 18 mg/mL nicotine vaping liquid. This was proposed to be a physiologically relevant level of nicotine (Matta et al., 2007). In addition, this level may not have been truly representative of a physiologically relevant level, as the differences between how vapor chemicals cross the human lung epithelium and the epithelium of gills have not been studied.

Another way to better improve the experiment is to regularly test the treatment group water for the concentration of vapor chemicals. Evaporation of water from the heated water tanks

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is inevitable. Subsequently, vapor chemicals could have also evaporated over time leading to a decrease in the level of exposure. Vapor solutions were kept sealed before addition to the tanks, and water changes were done daily to help minimize loss of vapor chemicals, but vapor levels were never verified through chemical testing.

Finally, the animal selection methods could be improved. The selection criterion for animals included that they were healthy, male or female, adult zebrafish with no visible lesions. No selection for fin size or pigment level was made originally. However, there is wide variation in fin size, pigmentation, and shape in zebrafish. In the future, care should be taken to select individuals with similar fin characteristics in order to better control the experiment. Selection could also be improved by increasing the sample size of the experiment. The observed effect size for this experiment is medium (0.25). Thus with a desired power of 0.80 and alpha value of 0.05, the sample size of each group should be 36 for any future studies.

In conclusion, there was no evidence found that electronic cigarette vapor affects wound regeneration in regard to pigmentation in adult zebrafish. Future study is needed to determine if higher exposure levels will cause effects. If zebrafish are immune to the detrimental effects of nicotine and electronic cigarette vapor, further research is needed to determine the mechanisms behind these enhanced regenerative abilities. These pathways may hold the answers to improved wound healing treatments and applications to reduce potential scaring in human and other mammalian repair pathways.

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VI. Appendix

A. Exclusions

As per protocol, any diseased or distressed fish was to be removed from the study and euthanized. Fish 4C was euthanized before data collection due to a persistent, unexplained lesion. Fish 8A was also excluded from the final data analysis, due to the inability to collect accurate pigment data. Fish 8A was believed to have a problem with its swim bladder causing it to be unable to control the height of swimming. As a result, its back fin was damaged from dragging along the bottom of the tank, making it difficult to gauge accurate pigment and regeneration measurements. This compounding variable led to its exclusion in the study.

B. Log Sheets and Measurements

Daily Log Sheet for Fish Tanks:

Pigmentation and Meaturements:

C. Tables

	Mean	Standard Error	P Value
ΔPAF 1 Week			
CG	4.21	1.80	
TG1	4.34	1.68	0.933
TG2	3.51	1.68	
ΔPAF 2 Weeks			

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CG	6.82	1.61	0.579
TG1	5.65	1.61	
TG2	4.22	1.86	

Table 1: Shows the mean, standard error, and P values for ΔPAF for each regeneration time and treatment group. No significant P values are shown for ΔPAF at either one- or two-weeks regeneration time (P=0.933, P=0.579 respectively).

	Mean	Standard Error	P Value
ΔPAF Comparing Both Weeks			
1-CG	4.21	1.76	0.749
1-TG1	4.34	1.65	
1-TG2	3.51	1.65	
2-CG	6.82	1.65	
2-TG1	5.65	1.65	
2-TG2	4.22	1.90	

Table 2: Shows the mean, standard error, and P values for ΔPAF to compare pigment change in each regeneration period. No significant P value was shown, indicating no difference in pigment between one- and two-weeks regeneration time (P=0.749)

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