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Physiological Effects of Nicotine and Lead (II) Acetate on Zebrafish Embryo Development

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Abstract: E-cigarettes have become increasingly popular in the United States, with the Centers for Disease Control and Prevention (2023) reporting that a 47% increase in e-cigarette sales at US retail outlets from 2019 through 2023. Additionally, Vilcassim et al. (2023) estimate that 2.2% to 7% of pregnant women use e-cigarettes, potentially exposing their developing fetus to harmful chemicals. While most existing literature has studied the effect of nicotine alone, this study investigates the interplay of two key e-cigarette components—nicotine and lead (II) acetate—on embryonic development using a zebrafish (Danio rerio) model. Zebrafish embryos were divided into four groups, each raised in a 10 mL solution: control (embryo medium), 5 µM nicotine, 5 µM lead (II) acetate, and a combination of 5 µM nicotine and 5 µM lead (II) acetate. They were observed from 0-5 days post-fertilization (dpf). Heart rate and spinal curvature data were measured at 5 dpf. The embryos were then euthanized, and their craniofacial cartilage was analyzed via Alcian blue staining. Results showed a significant reduction in heart rate in the combined group compared to the control; however, spinal curvature and craniofacial cartilage data were inconclusive. Despite these mixed findings, the observed embryonic deformities underscore the dangers of e-cigarettes and the need for further research on the developmental impacts of their ingredients.

1. Introduction

Smoking tobacco has been a public health concern for decades; however, e-cigarette smoking is a relatively new phenomenon. Despite the growing awareness of the risks, ignorance of the dangers of e-cigarette use persists (Gupta et al. 2020). Although public health efforts have sought to reduce smoking, their efficacy is questionable. Palmer et al. (2022) found that many long-term smokers, along with adolescents with no history of smoking, are turning to e-cigarettes over their combustible tobacco counterparts. This increased prevalence of e-cigarettes invites research to study their physiological effects.

Nicotine, a drug widely known for its addictive properties, has been extensively studied for its detrimental effects on the nervous system. Physiological issues, such as developmental delays and disorders, as well as the dysfunction of several organ systems, have been attributed to chronic exposure to nicotine (Gould et al. 2023). In contrast, lead, a toxic metal also detected in e-cigarette products, is less commonly recognized despite its significant health risks (Lee et al. 2020). For example, Karmach et al. (2022) indicates the possibility of cartilage deformities with exposure to chemicals like lead (II) acetate.

While the effects of nicotine and lead individually have been extensively studied, fewer studies have investigated their combined effects on the physiology of humans, especially adolescents, involving variables such as heart rhythm alterations and the development of deformities of spinal curvature and craniofacial cartilage. This study used zebrafish embryos as a model to answer the central: what are the effects that chronic exposure to nicotine and lead (II) acetate have on physiological development?

Zebrafish (Danio rerio) are a widely used model organism in toxicology research due to their genetic and developmental similarities to humans. Many zebrafish genes associated with neurological diseases are orthologous to those in humans (Lee et al. 2014). Essentially, the genes of both species can be traced to a singular gene in a common ancestor (Glover et al. 2019). This may be the cause of the similarities in development, features, and morphology of the central nervous system between zebrafish and humans, making them a suitable model for the assessment of neurotoxins (Klee et al. 2011). This study hypothesized that chronic exposure to a combination of nicotine and lead (II) acetate would have deleterious effects on zebrafish embryos. These effects were expected to manifest as three variables: decreased heart rate, increased spinal curvature, and increased deformities in the craniofacial cartilage. To test this hypothesis, four groups of 20 embryos each were raised from 0 days post-fertilization (dpf) to 5 dpf in 10mL of the following solutions: embryo medium (control), nicotine, lead (II) acetate, and a mixture of nicotine and lead (II) acetate. On 6 dpf, heartbeat data was collected by placing each zebrafish embryo onto a petri dish using methyl cellulose and tracking its heartbeat for sixty seconds (Chang et al. 2021). Subsequently, the software LAS EZ was used to capture images of the embryos for spinal curvature analysis using the software Image J. The embryos were then euthanized with cold water. An Alcian blue staining procedure was utilized to analyze the craniofacial cartilage and spinal defects in each fish. This concluded one trial and was repeated twice more to ensure robust and accurate results.

2. Materials & Methods

2.1 Zebrafish Housing and Care

Wild-type zebrafish embryos (Danio rerio) were the specific model organisms used in this study. Eighty 0 dpf embryos were raised from 0 dpf to 5 dpf for this study. They were maintained in an embryo water-filled petri dish in the 28-degree incubator upon delivery. At 0 dpf, they were separated into four groups of 20 embryos, each in 10 mL of solution. Group 1 was the control exposed only to embryo medium; Group 2 was exposed to 5 µL of nicotine. Group 3 was exposed to 5 µL lead (II) acetate, and Group 4 was exposed to 5 µL

nicotine and 5 µL lead (II) acetate combined. They were raised until 5 dpf, after which data were collected. Afterward, the zebrafish were euthanized in freezing water.

2.2 Treatment Groups

Embryos were separated into a control and three different treatment dishes. The chemicals used were nicotine and lead (II) acetate. The stock solution for lead (II) acetate was produced with 0.5g of 379.33g/mol lead (II) acetate and 100 mL of embryo water combined to make a 5 µM solution. The initial concentration of nicotine was 24,600 µM, which was then diluted to 5 5 µM. The first treatment was made for group 2, exposed to only nicotine, with 2.025 µL of 5uM nicotine and 8.997 µL of embryo water. The second treatment was made for group 3, exposed only to lead (II) acetate, consisting of 3.79uL of 5uM lead (II) acetate and 8966.2 µL of embryo water. Finally, the third treatment was made for group 4, exposed to both chemicals, with 2.025 µL of 5 µM nicotine, 3.79 µL of 5 µM lead (II) acetate, and 8994.18 µL of embryo water. These concentrations were devised through previous trials, which helped to determine the highest concentrations for each chemical possible while keeping the embryos alive for the experiment duration. The control group's solution was created with 9 mL of embryo water and 1 mL of embryo water from embryo transfer.

2.3 Heartbeat Data Collection

At 6 dpf, methylcellulose was used to calm each embryo to ensure more accurate heart rate data (Liang et. al, 2007). A 1000 µL pipette was used to carefully take one embryo with as little embryo water as possible and transfer it onto a new petri dish. Then, a drop of methyl cellulose was taken and placed on the embryo. This ensured the embryo moved as little as possible. The petri dish was then placed on a dissection microscope and the heart rate was collected (Liang et. al, 2007).

2.4 Spinal Curvature Data Collection

The spinal curvature data was collected along with the heartbeat data. Following the mounting of zebrafish embryos using methylcellulose, the software LAS EZ was launched on a computer connected to the dissection microscope. This was then used to capture an image of the embryo on its side for the future analysis of its angle of development (Kimmer et. al, 1995). These steps were repeated for each embryo per group.

2.5 Cranio-facial Cartilage Data Collection

Following euthanasia, an Alcian staining procedure was performed in which embryos were first transferred to a 15 mL conical tube filled with 4% paraformaldehyde (PFA), a fixative to prepare tissues for microscopic analysis, and the tube was wrapped with aluminum foil (Neuhass et. al, 1996). Afterward, a washing cycle began using a plastic pipette, and the PFA from the tube was removed as much as possible. The removed PFA volume was replaced with a phosphate-buffered saline with a Tween 20 (PBT) solution. Afterward, the PBT solution was again carefully removed. The PBT was then replaced in the tube and then removed. This wash was performed three times in total. After the final wash, the tray was refilled with a 30% hydrogen peroxide solution. The trays were then incubated for one hour, followed by replacing and refilling the 30%

hydrogen peroxide solution and allowing incubation once again.

During the incubation period, an Alcian blue solution was created. The stain was then added to the zebrafish trays and incubated overnight. The next day, the trays were rinsed with acidic ethanol thrice and incubated for 20 minutes. They were then rehydrated in an HCl-EtOH series. Once the embryos were fully hydrated, a microscope slide was prepared by placing a drop of methylcellulose and placing the blue-stained embryo in the drop, and observing underneath the microscope. Then, ventral, dorsal, and side profile pictures were taken for each embryo using the software Logger Pro. The images were used to measure the M-PQ angle, seen as the triangle shape under their jaw, for each embryo (Raterman et. al, 2020). Qualitative data on the visibility of bone density were also collected.

2.6 Statistical Analysis

All three variables were analyzed using ANOVA and Tukey-HSD tests. A bar graph was created from the means of each group to show the distribution of data points for each variable. This allowed for comparison of the different subgroups. All graphs were produced in the software JMP.

2.7 Euthanasia

As the embryos were needed for craniofacial cartilage analysis after euthanasia, the NIH (2009) protocol was modified to euthanize them with cold water rather than bleach. Embryos were placed in cold water and then into the fridge for 20 minutes to ensure the water reached the ideal euthanasia temperature threshold of 2-4 degrees C.

3. Results

3.1 The relationship between the average heart rate of zebrafish embryos among different exposure groups

To determine whether the heart rate of zebrafish embryos was significantly affected by the treatments, the heartbeats were counted under a dissection microscope on day 5 post-fertilization. Fig 1 shows the average heart rate observed across the three trials performed among the embryos in each treatment group. ANOVA and Tukey's post hoc analysis, performed using JMP software, revealed a significant difference between the combined treatment and the control groups ($p = 0.0001$, $F = 8.0584$). There were also significant differences noticed between the combined and nicotine groups and between the combined and lead (II) acetate groups.

3.2 The relationship of the spinal curvature in zebrafish embryos between exposure groups

To determine whether spinal curvature deformity is a developmental risk associated with exposure to these chemicals, we analyzed the feature through the software LAS EZ at 5 dpf. Fig. 2 displays the average spinal curvature data collected across the three trials. ANOVA and Tukey's post hoc tests revealed no statistically significant differences between the combined treatment and individual chemical groups. However, significant differences were observed between the nicotine and control groups, as well as between the combined and control groups ($p = 0.0004$, $F = 6.6392$). Notably, the combined treatment group did not differ significantly from the individual chemical groups.

3.3 The relationship between the M-PQ angle in zebrafish embryos among different exposure groups

An Alcian blue staining procedure was performed on dpf 6 of the first trial to evaluate the craniofacial cartilage deformities in the zebrafish embryos. The primary feature assessed was the angle of the jaw cartilage, seen as a black band in Fig 3. The wider the band is, the higher the level of its deformity is. Fig 3. shows the control embryo as having the narrowest angle of development, indicating it is the least deformed. Fig 4. provides a comprehensive graph summarizing the results. ANOVA and Tukey's post hoc tests revealed a statistically significant difference between the control and combined groups ($p = 0.0019$, $F = 13.1302$). However, there were no statistical differences found between the combined and individual chemical groups.

4. Discussion

The purpose of this study was to assess the physiological changes induced by the main components of e-cigarettes on zebrafish and to thereby translate such patterns to adolescents. Many pieces of literature have investigated the different types of effects of nicotine on humans; however, research on other chemicals has been lacking. This study focused on two significant chemicals in the makeup of e-cigarettes, nicotine and lead (II) acetate, and the effect of their chronic exposure on the development of humans. Using zebrafish embryos, it was hypothesized that chronic exposure to a combination of nicotine and lead (II) acetate would result in an additive effect, which would result in reduced heart rate, increased spinal curvature, and underdeveloped craniofacial cartilage structures.

4.1 Effects on Heart Rate

In testing the heartbeat variable, data across all three trials found that there is a significant difference in the heart rate of the combined group compared to the remaining three groups. There was a more significant reduction of heart rate of the embryos exposed to 5uM of both chemicals ($p < 0.0001$) than from the control, nicotine, or lead (II) acetate groups individually. This suggests that there is an additive effect of nicotine and lead (II) acetate on the cardiovascular health of the organism. Some prior research in mice found exposure to lead (II) acetate to increase cardiovascular risk factors such as higher blood pressure (Ungváry et al. 2002). Additionally, nicotine has been shown to cause heart defects in zebrafish models (Hussen et al. 2023). This could support the notion that these chemicals combined cause an additive effect on the cardiovascular health of the zebrafish model.

4.2 Effects on spinal curvature

Next, in the analysis of the spinal curvature, there was a significant difference between the combined and control groups. However, this difference was not significant when compared to the individual chemicals. With the post-hoc statistical comparison seen in Fig 2, it seems that nicotine affects spinal development the most, with lead (II) acetate not being significantly different from the control, nicotine, or combined groups. Such capacities of nicotine are also found in a study from Svoboda et. al (2002), highlighting the detrimental effects of nicotine on spinal motoneuron development

in embryonic zebrafish. In nicotine-exposed embryos, they found that nicotine delays the differentiation of dorsal motor neurons and dorsal spinal neurons. Consequently, as lead (II) acetate has no significant difference from the control group, there is no synergistic effect when combined. Thus, the hypothesized outcome of comparatively greater defined spinal curvature due to an additive effect of nicotine and lead (II) acetate is not supported.

4.3 Effects on craniofacial cartilage development

Finally, this study examined craniofacial cartilage development using an Alcian Blue staining procedure to analyze the M-PQ angle of the cartilage. As seen in Fig 3, there is a significant difference between the control and combined groups in which the cartilage was more deformed. However, our hypothesis remains unsupported because there is no significant difference among the individual chemical groups. As opposed to the spinal curvature, it seems the lead (II) acetate has a greater detriment to the M-PQ angle of this craniofacial cartilage structure, as the ANOVA test shows it is significantly different from the control and nicotine groups. Yan et. al. (2022) elucidate the skeletal toxicology that commences as a result of exposure to lead (II) acetate. Exposure to this chemical promotes osteoclast formation, which ultimately leads to cartilage defects and bone loss.

4.4 Limitations

Although this study was conducted as accurately as possible, some limitations hindered its progression. We were not able to perform the Alcian blue staining protocol for all three trials due to constraints within the laboratory in which the stains were performed. Thus, the statistical results derived from the M-PQ angle are inconclusive. Additionally, this study relied on a single concentration of each chemical, which may have limited our ability to understand dose-response relationships; higher or lower doses may reveal different additive effects. These limitations were obstacles to our study as they became obstacles to more accurately understanding the effects of nicotine and lead (II) acetate, especially on the variables that were left unsupported.

5. Conclusion

In conclusion, our hypothesis that chronic exposure to a combination of nicotine and lead (II) acetate would have deleterious effects on zebrafish embryos was not supported. Nevertheless, there were developmental differences observed, which indicate e-cigarettes, while perhaps a safer alternative to traditional cigarettes, still produce an outcome of developmental deformities. Lead (II) acetate and nicotine promote decreased physiological development and increased developmental deformities. These results indicate the developmental effects of chronic e-cigarette use on adolescents. This can be especially harmful to pregnant women and their developing fetuses.

In the future, other variables important to development and physiology, such as body length, intersegmental vessels, and dorsal longitudinal anastomotic vessels, may be assessed. In addition, it will be worth assessing the effects of varying concentrations of nicotine and lead (II) acetate on an organism in order to better translate the results to human beings. Finally, other model organisms such as mice can

be used to better understand the effects of these chemicals across species with phylogenetically similar regions.

Supplements

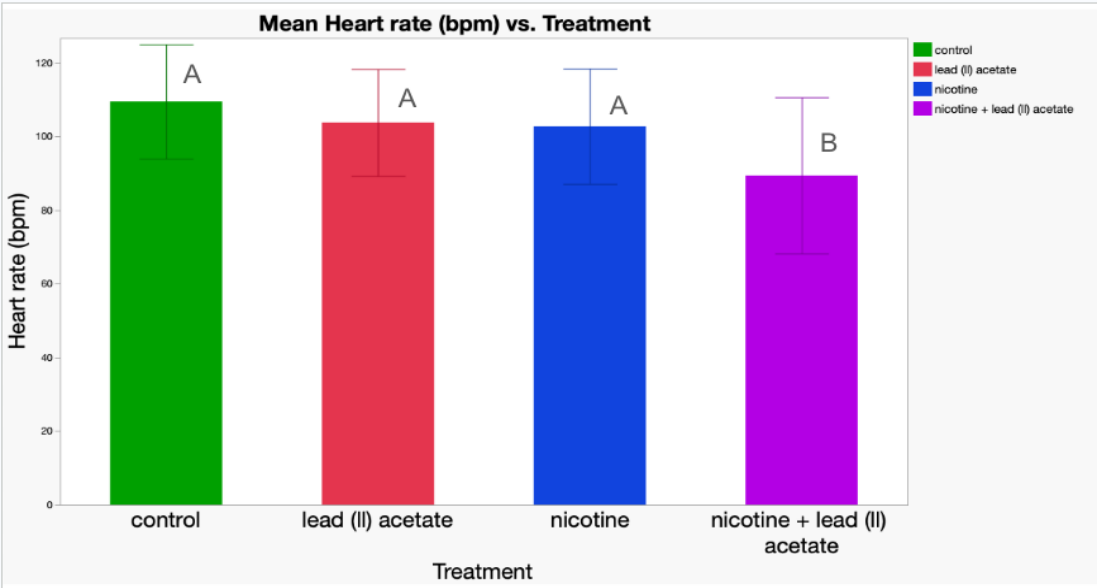

Figure 1. Mean beats per minute (BPM) of the heart of 6dpf wild-type zebrafish embryos after exposure. Letters were derived from the Tukey HSD test, and columns with differing letters have statistical significance. Each error bar was constructed using 1 standard deviation from the mean.

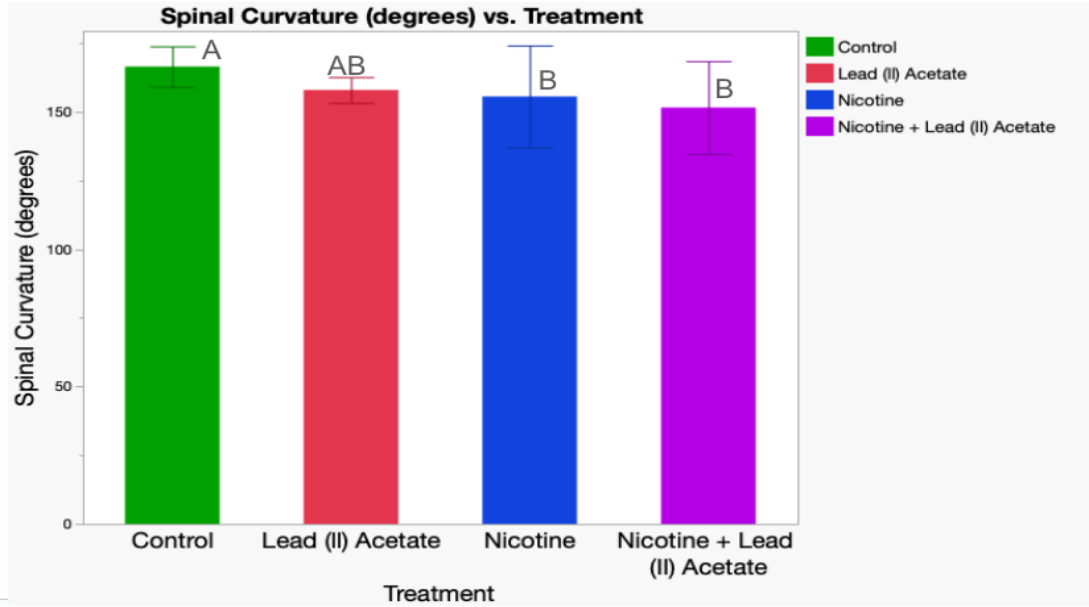

Figure 2. Mean spinal curvature (degrees) of 6-dpf wild-type zebrafish embryos after exposure. Significance was determined using Tukey's HSD test; different letters (A, B, AB) indicate statistically significant differences between groups. Error bars represent ±1 standard deviation from the mean.

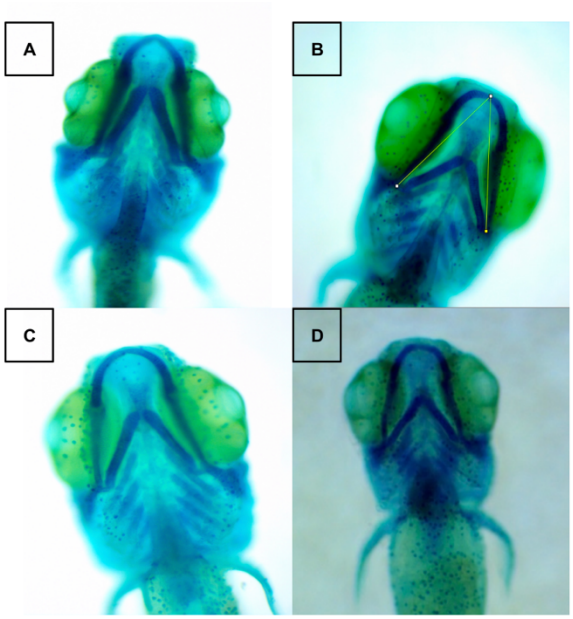

Figure 3. Alcian blue-stained images of zebrafish embryos at 5dpf from the control (A), lead (II) acetate (B), nicotine (C), and combined (D) groups. A wider M-PQ angle was observed in group D compared to group A, indicating underdevelopment of the craniofacial cartilage.

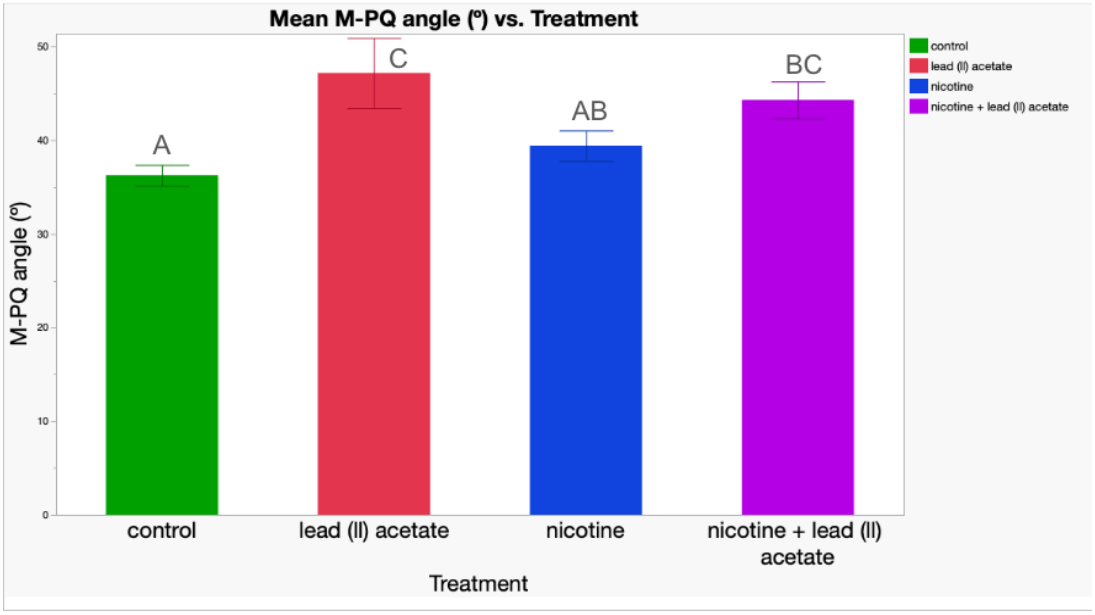

Figure 4. Mean MP-Q angle (degrees) of 6-dpf wild-type zebrafish embryos after exposure. Letters derived from the Tukey HSD test represented significance. Each error bar was constructed using one standard deviation from the mean.

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