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Article

The effect of nicotine and lead on neuron morphology, function, and α-Synuclein levels in a *C. elegans* model

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SUMMARY

An electronic cigarette (e-cigarette) is a device that simulates cigarette-tobacco smoking by atomizing a liquid into an inhalable vapor. It is often billed as a smoking cessation device that is safer than cigarette smoking, however, these devices are feared to work as a gateway to cigarette smoking through nicotine addiction. In addition to e-cigarette injury and death, there are also neurological implications to using an e-cigarette. Our research aimed to determine the effect of chemicals commonly found in e-cigarette aerosol, specifically nicotine and lead, on dopaminergic neuron morphology and α-synuclein levels using the model organism *Caenorhabditis elegans*. Our hypothesis stated that should exposure to these chemicals have an effect, then we would observe negative neurological implications such as changes in dopamine neuron morphology and possible neurodegeneration. We used transgenic *C. elegans* to assess the effects of nicotine and lead on α-synuclein levels and neuron morphology. *C. elegans* were treated with different doses of lead, nicotine, or a mixture of to simulate the neurological effects of e-cigarette use. We determined dopamine and acetylcholine-dependent behavior using a 1-nonanol assay and an aldicarb assay, respectively. We observed a significant dopaminergic deterioration upon the treatment of lead and nicotine. At higher concentration of each treatment (lead, nicotine, and the combination), we observed *C. elegans* (NL5901 and BZ555) strains were smaller with an increase in protein aggregation. Our findings indicate that using e-cigarettes may induce morphological changes that are not favorable for normal brain function and could lead to a decrease in individuals' e-cigarette use.

INTRODUCTION

"Vaping," or the use of electronic cigarettes (e-cigarettes), consists of atomizing a liquid (which contains many compounds) into an inhalable vapor. These e-cigarette devices vaporize liquid, rather than burning substances, to generate an aerosol instead of smoke, have been thought to be safer than traditional smoking. Thus, they are often intended for smoking cessation, a safer vehicle for nicotine (akin to a nicotine patch) that still provides flavor and pleasure without the known dangerous consequences of consistent cigarette use (1). In reality, vaping is feared to work in reverse as a gateway to cigarette smoking through nicotine addiction (1).

Given its intended purpose as a smoking cessation tool, e-cigarette solution is known and registered to contain nicotine (2). However, some brands of pods, including JUUL, contain mixtures known as nicotine salts, which are nicotine combined with an acid. JUUL pods contain nicotine benzoate, a combination of nicotine and benzoic acid. These salts are much easier to inhale and provide much more available nicotine, increasing the intake per unit (2). One JUUL pod contains as much nicotine as one pack of standard cigarettes (2). By increasing the amount of nicotine intake per unit, the exposure and potential effects could be amplified.

The use of e-cigarettes may also put the user at risk of heavy metal exposure. Due to the electronic heating process, as well as the inexpensive construction of vape devices, users of e-cigarettes often accidentally inhale heavy metals that have leached from manufactured components and have then been aerosolized (3). In one instance, researchers detected significantly higher concentrations of lead, copper, tin, and nickel in aerosolized e-cigarette vapor than in cigarette smoke (3).

Metal exposure increases the risk of the development of Parkinson's disease (PD) (4). PD is a neurological disease stemming from the loss of dopaminergic neurons in the substantia nigra, a structure of the midbrain that helps to regulate movement and other processes (4). PD also results in the development of Lewy-Bodies, protein clusters that overwhelm a cell's functions and leads neuronal death resulting in a reduced capacity in that part of the brain. The disease is characterized by a variety of symptoms, including motor symptoms, such as tremors, bradykinesia (slowness of movement), rigidity, and a stooped posture, and non-motor symptoms, such as Individuals with PD often, dementia, anxiety, and depression (4). Most cases of PD are idiopathic, meaning that the exact causes of the cause are unknown, though some have known genetic etiologies (5). Notably, there is a decrease in the rate of incidence of PD among cigarette users, and studies have shown that nicotine has some positive effect on the survival of dopaminergic neurons in a *Drosophila melanogaster* model (6).

A critical protein in the progression of PD, and a potential factor in AD (as it interacts with tau protein and beta-amyloid)

is the α-synuclein protein. This protein is most abundant in the brain, mainly in the hippocampus, hinged near the axon terminal with the synaptic vesicles (mainly responsible for the chemical release between neurons). The α-synuclein protein is encoded by *SNCA*. Aggregations of α-synuclein (the formations of which are preceded by accumulations of the protein) lead to the formation of Lewy-Bodies (8).

The α-synuclein protein has been shown to be the leading cause of the death of dopamine-producing neurons (9). Dysregulation in the dopamine system can cause many neurological complications, including PD and depression (10). Specifically, the National Institutes of Health (NIH) found a significant cross-sectional association between e-cigarette users who also tend to fall into depression (11). A rare mutation of *SNCA* is a known cause of PD and over-expression of *SNCA* is toxic (12).

A proven model of PD, *Caenorhabditis elegans* are transparent nematodes that live in temperate soil environments. The worms, like humans, possess developed nervous systems, making them ideal for neurological research (13). A specific *C. elegans* strain, NL5901, can express the human α-synuclein protein, which can then be visualized by fusing the protein to a yellow fluorescent protein (14). Fluorescence microscopy of NL5901 can reveal the extent of accumulation of the protein in a sample. Another strain, BZ555, has a green fluorescence protein that can be observed in the soma of its dopaminergic neuron, enabling visualization of dopaminergic expression (15).

We examined the neurodegenerative effects of exposure to e-cigarette chemicals, namely nicotine and lead, utilizing various neurotoxicological assays. Our hypothesis stated that should exposures to these chemicals have an effect, then we would see an increase in repulsion time in the 1-nonanol assay (indicating decreased dopaminergic function), an increase in the percentage of paralyzed worms in the aldicarb assay (indicating decreased acytylcholinergic function), a decrease in fluorescence of BZ555 worms (indicating decreased dopaminergic function), and an increase in fluorescence of NL5901 worms (indicating increased α-synuclein aggregation, characteristic of PD). We observed a significant dopaminergic deterioration upon the treatment of lead and nicotine. At higher concentration of each treatment (lead, nicotine, and the combination), we observed *C. elegans* (NL5901 and BZ555) strains were smaller with an increase in protein aggregation. Overall, our results from these assays present potential evidence for neurodegeneration as a result of exposure to e-cigarette chemicals.

RESULTS

We treated *C. elegans* with increasing amounts of nicotine and lead to determine the effect exposure has on neurons. The methods were split into phases, including the seeding of *E. coli* OP50 as a food source for the nematodes, the culture of *C. elegans* (NL5901 and BZ555 strains were chosen for their ideal fluorescence expression traits), age synchronization using sodium hypochlorite treatment (in order to ensure all results observed were due to the manipulation of chemical treatment as opposed to age variability), treatment with different doses of lead acetate and nicotine based on previous literature (100, 250, 500 and 1000 μM), and the fluorescence imaging of the nematodes.

Past research has shown that repulsion time after exposure to 1-nonanol correlates with dopamine levels (16). In order to evaluate dopamine-dependent motor behavior (as an indirect measure of dopamine levels), 1-nonanol-based repulsive behavior was quantified. Worms with optimum levels of dopamine exhibit repulsive behavior when exposed to 1-nonanol, whereas worms with lower levels of dopamine require prolonged time to show the repulsive behavior; increases in dopamine correspondingly decrease 1-nonanol repulsion time (17,18). Previous studies in worms have validated the assay by exposing the worms to DAT inhibitor, bupropion HCl, and simultaneously conducting the assay in worms overexpressing cat-2 (which encodes tyrosine hydroxylase) and cat-2 mutants (18). Additionally, RNAi of cat-2 has also been shown to enhance repulsion time in worms (19). Furthermore, cat-2 mutants have also shown increased repulsion time in response to octanol with reversal in the presence of exogenous dopamine (20).Treatment with nicotine at a lower dose (100 μM) led to a slight, though insignificant ($p > 0.05$) decrease in repulsion time, signifying an increase in dopamine levels, whereas the inverse happened at higher doses, with a slight, though insignificant increase in repulsion time **Figure 1A**. Treatment with lead led to a similar reduction in dopamine levels as doses increased as seen from the slight increase in repulsion time (**Figure 1B**). As per a single factor ANOVA test, the individual nicotine and lead 1-nonanol results were not statistically significant. Treatment with the lead and nicotine mixture led to a reduction in dopamine levels as the dose increased from 100 μM to 250 μM, as seen through the significantly ($p < 0.05$, $p < 0.01$ respectively) increased repulsion time (**Figure 2**).

Aldicarb is an acetylcholinesterase inhibitor and exposure leads to accumulation of acetylcholine, resulting in flexion of muscles. The percentage of worms paralyzed at a time point is proportional to acetylcholine levels. The aldicarb assay

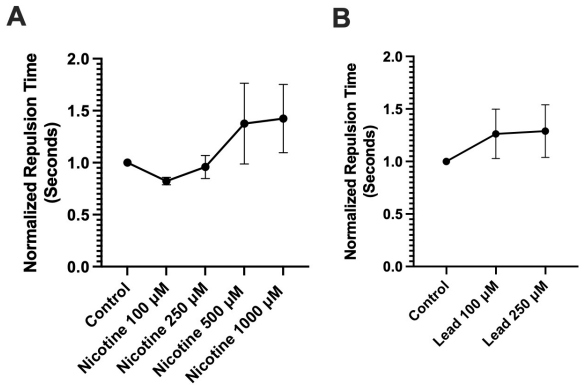

Figure 1. Nicotine and lead affected dopamine-dependent behavior though the 1-nonanol assay. Normalized repulsion time as per the 1-nonanol assay in response to a vehicle control (water) and different doses of **(A)** nicotine, and **(B)** lead in *C. elegans* strain NL5901. Error bars represent standard error. As per a one-way ANOVA test, none of the variable groups are significant versus control ($p > 0.05$).

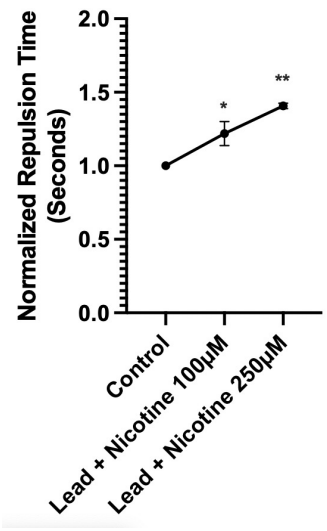

Figure 2. The nicotine and lead combination affected dopamine-dependent behavior through the 1-Nonanol Assay. Normalized repulsion time as per the 1-nonanol assay in response to a vehicle control (water) and different doses of lead and nicotine in *C. elegans* strain NL5901. Error bars represent standard error. As per a single factor ANOVA test, the data for the lead and nicotine 250 µM group is highly statistically significant (**p = 0.0058). The data for the lead and nicotine 100 µM group is also statistically significant (*p = 0.0331).

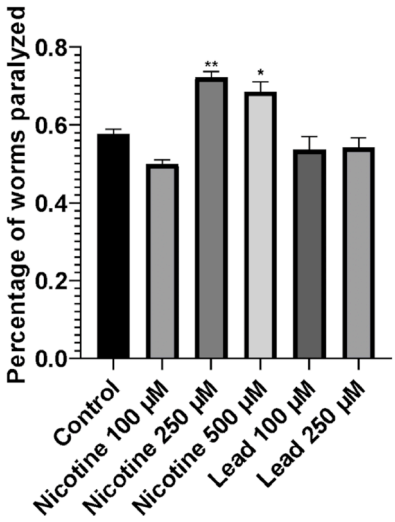

Figure 3. Nicotine and lead affected acetylcholine-dependent behavior though the aldicarb assay. Percentage of worms paralyzed as per the aldicarb assay in response to a vehicle control (water) and different doses of lead (L), nicotine (N), and lead and nicotine (LN) in *C. elegans* strain NL5901. Error bars represent standard error. As per a single factor ANOVA test, the data for the nicotine 250 µM group is statistically significant (**p = 0.0028) and the data for the nicotine 500 µM is also statistically significant (*p = 0.0212). The rest of the data is not statistically significant (p > 0.05).

has been successfully utilized to study functional alterations resulting from perturbed synaptic transmission (21). Previous studies have indicated its efficacy in determining the effect on cholinergic transmission in phytomolecules and established pharmacological AcHE inhibitors such as donepezil (17,22). Therefore, this assay measured the level of acetylcholine-induced paralysis within the nematodes. Treatment with nicotine led to a decrease in the percentage of worms paralyzed as the dose increased to 100 µM and from 250 µM to 500 µM, which is suggestive of an increase in acetylcholine-dependent neurotransmission (**Figure 3**). However, a significant ($p < 0.05$) reduction in acetylcholine-led neurotransmission occurred as doses increased from 100 µM to 250 µM, as evident through the increase in the percentage of worms paralyzed; a further significant reduction in neurotransmission occurred between nicotine and lead. The treatments with individual lead and nicotine concentrations lead to no significant change in acetylcholine-led neurotransmission (**Figure 3**). Unfortunately, due to the time and lab scheduling constraints, the Aldicarb assay was not performed on the lead and nicotine mixture at concentrations of 100 and 250 µM.

Treatment with nicotine led to a reduction in dopamine levels as doses increased (**Figure 4A**), as evident by the decreased observed fluorescence intensity. There was no observable difference in fluorescence intensity between the worms treated with 100 µM and 250 µM of lead (**Figure 4B**). Worms with 250 and 1000 µM mixtures of nicotine showed to a significantly ($p < 0.05$) lower level of α-synuclein, as

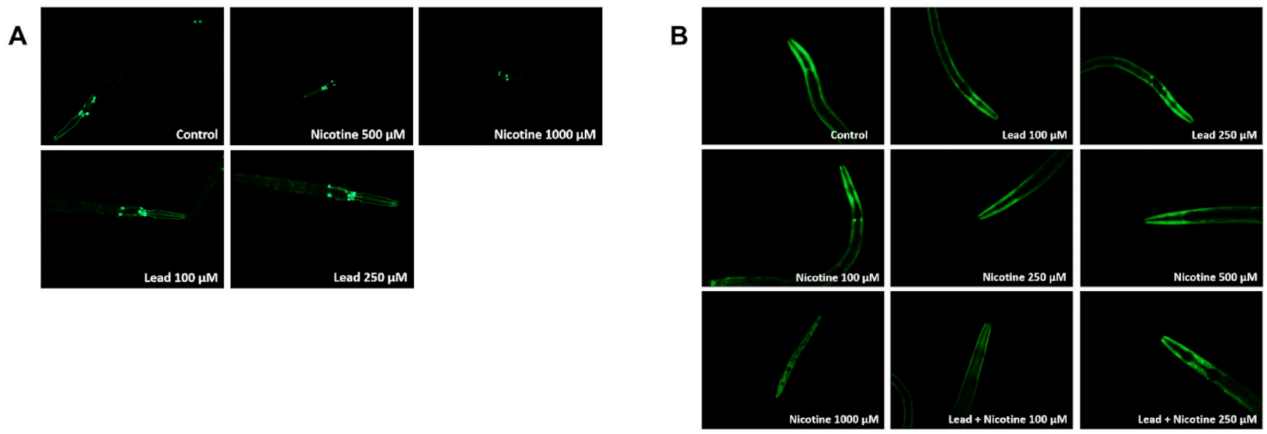

Figure 4. Nicotine and lead affected the dopaminergic neurons and α-synuclein expression. (A) Dopaminergic neuron fluorescence in *C. elegans* strain BZ555 and **(B)** α-synuclein fluorescence in *C. elegans* strain NL5901.

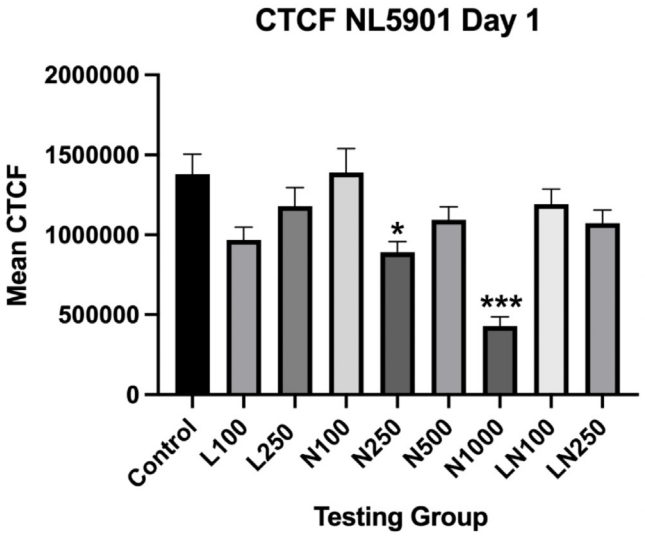

Figure 5. Nicotine and lead affected the CTCF for the NL5901 strain on Day 1. Mean CTCF response to a vehicle control (water) and different doses of lead (L), nicotine (N), and lead and nicotine (LN) in *C. elegans* strain NL5901 on day 1 of experimentation. The numbers following the letter in each group indicate concentrations of substance (in μM). Error bars represent standard error (average error across all groups 4.90%). As per a single factor ANOVA test, the data for the nicotine 250 μM group is statistically significant (*p = .02202) and the data for the nicotine 1000 μM is also statistically significant (***p < 0.0001). The rest of the data is not statistically significant (p > 0.05).

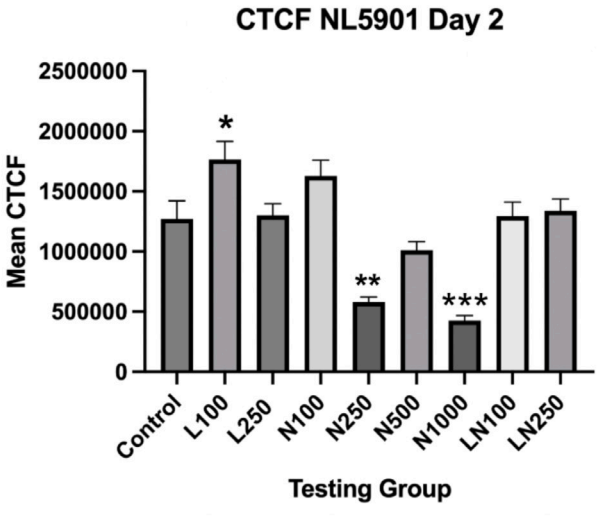

Figure 6. Nicotine and lead affected the CTCF for the NL5901 strain on Day 2. Mean CTCF response to a vehicle control (water) and different doses of lead (L), nicotine (N), and lead and nicotine (LN) in *C. elegans* strain NL5901 on day 2 of experimentation. The numbers following the letter in each group indicate concentrations of substance (in μM). Error bars represent standard error (average error across all groups 7.39%). As per a single factor ANOVA test, the data for the lead 100 μM group is statistically significant (*p = .03679), the data for the nicotine 250 μM is statistically significant (**p = .00094), and the data for the nicotine 500 μM (***p < 0.0001). The rest of the data is not statistically significant (p > 0.05).

seen from the decrease in observed fluorescence intensity; on day 1, there was no other significant ($p < 0.05$) impact on fluorescence (**Figure 5**).

24 hours after the first measurement, on day 2, worms with 250 and 1000 μM mixtures of nicotine continued to show significantly ($p < 0.05$) lower level of α-synuclein, as seen from the decrease in observed fluorescence intensity; in addition, the group treated with 100 μM of lead showed a significant ($p < 0.05$) increase in α-synuclein aggregation (**Figure 6**). The rest of the groups maintained insignificant ($p > 0.05$) changes in α-synuclein aggregation versus control.

DISCUSSION

Our research aimed to determine the effect of e-cigarette chemicals and chemical combinations, specifically nicotine and lead, on dopaminergic neuron morphology and α-synuclein levels. *C. elegans* were treated with different doses of lead, nicotine, or both. The results suggest that chemicals present in e-cigarette aerosols could have adverse neurological effects at high concentrations. We hypothesized that if exposures to these chemicals have an effect, then we would see an increase in repulsion time in the 1-nonanol assay, an increase in the percentage of paralyzed worms in the aldicarb assay, a decrease in fluorescence of BZ555 worms, and an increase in fluorescence of NL5901 worms. NL5901 worms can express the human α-synuclein protein and reveal the extent of accumulation of the protein in a sample. The BZ555 strain enables the visualization of dopaminergic expression (15). Our hypothesis was partially supported as some higher doses of nicotine did cause an increase in the rate of acetylcholine-induced paralysis; nicotine and lead combined led to increased repulsion time; and nicotine alone caused lower dopaminergic neuron fluorescence intensity. Though the study power was limited, and some exposure/assay combinations lacked statistical significance, the results suggest a wide array of potential neurological implications, each of which could warrant further study.

The aldicarb assay, which measures acetylcholine-induced paralysis, exhibited increased rates of paralysis effects with higher doses of nicotine (at 250 and 500 μM concentrations). There was an insignificant effect on acetylcholine-induced paralysis from both lead concentrations, and the 100 μM nicotine concentration yielded a lower rate of paralysis but was insignificant. More trials should be run to confirm whether it had an effect. The nicotine doses differed from the vehicle control with higher concentrations demonstrating a negative impact on acetylcholine receptors, of which nicotine serves as an agonist (20). The difference between short- and long-term exposure to nicotine should be noted here—the worms were exposed for longer than 2 or 3 days, relative to their measured 50% survival rate of 10 days, and so the long-term effects of nicotine on the neurons were observed (23). Although certain levels of consumed nicotine can reduce the risk of PD in that it provides a type of neuroprotection, it is unknown whether higher concentrations of nicotine can, in turn, increase the risk of PD (24). In addition, higher rates of paralysis signify lower levels of acetylcholine, which have been linked to PD (25).

In the 1-nonanol assay, which examines dopamine-dependent motor behavior, none of the individual-substance trials (just lead or just nicotine) returned any results with a normalized mean repulsion time that was significant against

the control. However, the lead and nicotine combined concentrations did return significantly elevated normalized repulsion times.

The results from NL5901 worms indicate that, at most doses, there is no effect of nicotine or lead on α-synuclein aggregation; this was the same across both days of testing. At higher concentrations (250 and 1000 µM), the aggregation of α-synuclein was consistently lower, indicating that increased nicotine consumption could reduce α-synuclein aggregation. Interestingly, however, the aggregation of α-synuclein at (500 µM) was not significant on either day of testing; this is a result that must be further investigated. Overall, findings indicate that α-synuclein aggregation may not be one of the negative side effects of increased nicotine and lead exposure.

The results from BZ555 worms indicate lower dopaminergic activity at 500 and 1000 µM of nicotine, as worms at those doses displayed a significantly decreased fluorescence in the neurons surrounding the worm's head. Furthermore, qualitatively, there was a noticeable difference in size between the vehicle control and the 500 and 1000 µM exposed worms. We observed that as the dosage increased, the size of the worms decreased. It is known that repeated nicotine exposure can impair or permanently damage neural circuits in adolescents; this aligns with our findings in the worms (26). The findings from these data can be rationalized with findings from other research surrounding neurological implications of nicotine and lead, as well as α-synuclein. One interesting finding from these data was the clear increase in repulsion time after the nicotine and lead combination exposure, despite insignificant single-substance exposures, as well as the increase in α-synuclein. Chronic lead exposure is a significant risk factor for the development of PD (27). Akinyemi *et al.,* found that lead exposure-induced dopaminergic dysfunction in *C. elegans*, which corroborates with the finding of higher doses of lead causing a reduction in dopaminergic activity (28). Overall, further trials need to be done to determine the effect of the combined nicotine and lead exposure on the brain.

The unique results of the combination exposure are part of a larger issue at hand – there is not a complete picture of what substances and concentrations an e-cigarette user is exposed to when using an electronic cigarette (29). For example, the increased exposure to nicotine through salts, or ingredients of the salts in combination with lead, nicotine, and other chemicals (like heavy metals) is unknown. As seen here, a combination of exposures can have different effects than individual ingredients, which must be studied further to gain a truly accurate picture of e-cigarette neurotoxicological effects.

In addition, the nicotine α-synuclein expression present an interesting case. Nicotine has been shown to slow oligomerization of α-synuclein in a yeast model, which aligns with the expression presented here (30). Further, traditional smoking has been shown to have an inverse correlation with PD. At the same time, our BZ555 dopaminergic expression indicates that nicotine could hamper dopaminergic function in ways consistent with PD (10).

While some interesting findings were made, the primary caveat to our study was the lack of replicates due to the limited amount of time allotted to conduct this experiment. Stronger conclusions could be reached by increasing the number of trials throughout the experiment to increase the results' credibility, accuracy, and precision, thereby avoiding systematic error.

This experiment yielded results, such the changes in dopaminergic morphology and α-synuclein expression, that support that e-cigarettes can have a notable neurological impact. Due to the amount of nicotine and other heavy metals inhaled being larger than what was tested, dire neurological effects may occur. Additionally, when adolescents are included with adults in consideration of whether e-cigarettes, nicotine, lead, etc., can affect neurological function, the effects are magnified. Due to the developmental process occurring during adolescence, adolescent neurological function can easily be inhibited or damaged with e-cigarettes. Although it is known that addiction to the same will most likely lead to neurological impairment or a mental health disorder, it is yet to be determined whether the sole use of e-cigarettes (compared to e-cigarettes and another addictive substances such as alcohol or drugs) can spark this change. Overall, our experiments present numerous avenues for future study, with the eventual goal of finding the true extent of e-cigarettes' effects on the brain, to safeguard human health.

MATERIALS AND METHODS

Preparation of NGM Plates

The composition of nematode growth medium (NGM) included NaCl (3.0 g), agar (18.0 g), peptone (2.5 g), and distilled water (975 mL). After autoclaving for 45 minutes, the following were added: 1.0 M KH_2PO4 buffer with 6.0 pH (25 mL), 1.0 M MgSO4 (1.0 mL), 1.0 M CaCl2 (1.0 mL), and cholesterol (5.0 mg/mL) in ethanol (1.0 mL). The plates were left at room temperature for 2 days before use to allow for detection of contaminants, and for excess moisture to evaporate (31).

Composition of M9 Buffer

The M9 buffer was composed of KH_2PO_4 (0.022 M), Na2HPO4 (0.042 M), NaCl (0.086 M), and distilled water (1000 mL). After autoclaving, 1.0 M $MgSO_4$ (1.0 mL) was added. The MEM was composed of the M9 buffer (100 mL), 20% dextrose (1 mL), 1.0 M NH_4Cl (1.0 mL), uracil at 4.0 mg/mL (400 µL), and bacterial culture (1 colony or 10 - 20 µL liquid culture).

Preparation of Minimal Essential Media (MEM)

The MEM, which contained the bacterial culture (*E. coli* OP50), was incubated overnight at 37°C while shaking at 200 (RPM). 500 µL of MEM was added to the NGM plates (10 cm) and was spread with a sterilized glass spreader in laminar air flow. The plates were swirled after 2 hours and the culture was evenly distributed on the plates. After the plates were dry, they were incubated at 37°C overnight.

Culture of *C. elegans*

Caenorhabditis elegans strains, BZ555 (egls1[dat-1p::GFP]), NL5901 (pkIs2386 [unc-54p::alphasynuclein::YFP + unc-119(+)], and *E. coli* OP50, were procured from Caenorhabditis Genetics Centre, (University of Minnesota, Minnesota). *C. elegans* strains were grown on the NGM and cultured at 22°C. A synchronized population of worms was obtained by sodium hypochlorite treatment. Embryos were incubated overnight at 22°C to obtain L1-staged worms.

Age Synchronization

Worms were washed using an M9 buffer. Gravid worms settled through gravity and the supernatant containing L1 to L3 and eggs were saved for further subculturing. The final volume of worm suspension was increased to 700 µL and 200 µL of 6% sodium hypochlorite and 100 µL of 5M NaOH was added. The worm bodies were vortexed until dissolved. The samples were centrifuged at 5000 RPM for 2 minutes. The supernatant was discarded, and the pellet was washed with 1 mL M9 buffer while the sample was vortexed vigorously. Centrifugation was repeated 4 times at 5000 RPM for 2 minutes followed by vortexing each time. The solution was transferred in 3.5 cm Petri dishes and the suspension was stored at 15 °C overnight. The suspension was checked for L1 worms the next day.

Treatment with Lead Acetate and Nicotine

100 mM solution of lead acetate and nicotine were prepared in distilled water. Worms were treated for 48 hrs at 22°C with different doses of nicotine (100, 250, 500 and 1000 µM). Nicotine, being a controlled substance, was handled only by members of the lab who were above the age of 21. Worms were exposed to 100 and 250 µM of lead in a similar way; treatments with lead concentrations above 250 µM were not included as the lead began to precipitate in the petri dish. Treatment of worms was conducted in liquid culture as described previously. Higher doses of lead acetate (and lead acetate-nicotine mixture) were prone to precipitation of salts, and so were avoided.

Aldicarb assay for acetylcholine dependent behavior

Determination of acetylcholine-based function was conducted as described (32). Briefly, treated worms were washed three times and were transferred to NGM-aldicarb plates (0.5 mM aldicarb). Aldicarb is an acetylcholinesterase inhibitor and exposure leads to accumulation of acetylcholine, resulting in flexion of muscles. The percentage of worms paralyzed at a time point is proportional to acetylcholine levels. The number of paralyzed worms was counted every 30 min. Any worms lost or injured were not considered as part of the study. The percentage of worms paralyzed was calculated when approximately 50% of worms were paralyzed in the vehicle control group.

1-nonanol Assay

Treated worms were briefly washed three times with the M9 buffer. Worms were placed on NGM plates. The poking lash (dipped in 1-nonanol, Acros Organics, AC157471000) was placed close to the head region of the worms; care was taken not to touch the worms. While conducting the experiment, an effort was applied to not over-immerse the poking lash in 1-nonanol, as excess 1-nonanol on the agar plate comes in contact with the worms while also desensitizing them after prolonged exposure. . Any worm prodded accidentally by the lash was disregarded in order to rule out interference from the mechanosensory stimulus. Time taken for the worms to show repulsive behavior was counted using a stopwatch. The stopwatch was started as the poking lash was placed close to the head region and stopped when the worm reversed and turned its head ≥45°.

Fluorescence Imaging

Worms were washed three times using M9 buffer (centrifugation at 2000 RPM for 2 minutes). Worms were then anesthetized using 5 mM Sodium Azide before being mounted on slides and observed using a FITC filter (Olympus). Semiquantitative analysis for α-synuclein expression was conducted through Image J (NIH) using the formula for CTCF (Corrected Total Cell Fluorescence).

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